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Physical and chemical properties of the metallofluorochromic indicator Calcein

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Physical and chemical properties of the metallofluorochromic indicator Calcein

by

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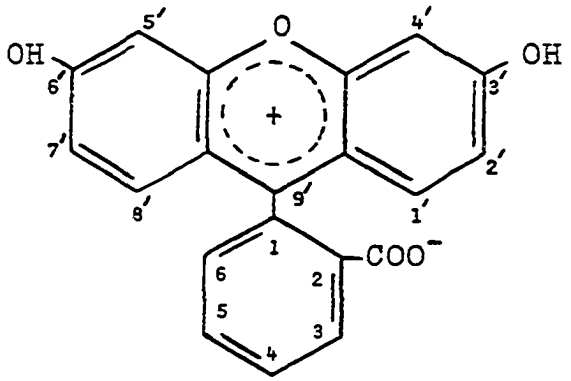
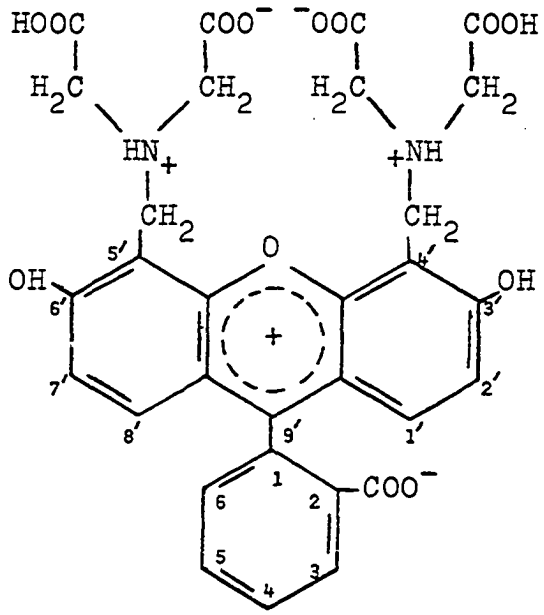
I. INTRODUCTION

Calcein was first synthesized by Diehl and Ellingboe (18) in 1956 and recommended as an indicator to mark the end-point in the titration of calcium in the presence of magnesium with sodium ethylenediaminetetraacetate (EDTA). Calcein is prepared by the Mannich condensation of fluorescein, formaldehyde, and iminodiacetic acid. Two methyleneiminodiacetic acid groups are introduced into the fluorescein molecule. Each group is equivalent to one-half of the ethylenediaminetetraacetic acid molecule. The structures and the numbering of the carbon atoms of fluorescein and Calcein are:



Fluorescein



Calcein

The combination of the fluorescent property of fluorescein and the chelating property of EDTA made Calcein the first of the so-called *metallofluorochromic indicators* and furnished the basis for a variety of applications to chemical analysis. Calcein itself exhibits a yellow-green fluorescence. This fluorescence depends on the acidity of the solution, beginning, as pH is increased, at pH about 4, reaching a broad maximum centered at pH 8, and then decreasing and finally disappearing completely at pH 12. Certain metals, notably calcium, strontium, barium, cadmium, and mercury, form fluorescent compounds with Calcein, the fluorescence of which persists at high pH and is the basis

of analytical methods for these elements. Other metals, notably copper, nickel and cobalt, quench the fluorescence at all values of pH. Aluminum is unusual in that it forms a fluorescent compound with Calcein at low pH. The principal use of Calcein has been in the determination of calcium, particularly in biological materials, both as an indicator in the EDTA titration and in the direct fluorometric determination. A variety of methods making use of Calcein for the determination of other metals have been devised and within the last decade (1967-1977) a novel use as a marker for bone growth has been discovered and applied in various animal bodies.

A sizeable literature on Calcein has now accumulated. By the time Diehl published his 1964 monograph (20), some fifty-five papers had been published on Calcein. Additional papers, including a review article by Diehl (19) in 1967, have appeared and the total literature at the present time (1977) is approaching two hundred publications.

Since the first publication, work on Calcein has gone on at Iowa State University on the fundamental chemistry of Calcein, the rate at which the work has been accomplished having been determined by the characteristics, the exigencies, and the slowness with which research is accomplished by graduate students working toward higher degrees in American universities. Some otherwise unpublished work

of the 1956 to 1963 period, on the purity and properties of Calcein, is reported (24) in the 1964 monograph of Diehl. The work by graduate students is found in the following: Hefley, M.S. thesis (35), 1965; Hefley, Ph.D. dissertation (36), 1967; Huitink, M.S. thesis (41), 1965; Huitink, Ph.D. dissertation (42), 1967; Huitink and Diehl (43), 1974; Huitink, Poe and Diehl (44), 1974; Birze, Ph.D. dissertation (6), 1970; Freytag, M.S. thesis (29); 1971; Hoyle and Diehl (40), 1972 (work not submitted as a thesis for a degree); Martin, M.S. thesis (58), 1974; Markuszewski, Ph.D. dissertation (57), 1976; and the present Ph.D. dissertation.

The work of Hefley was a major contribution to the chemistry of Calcein. The troubles encountered by numerous workers using Calcein sold commercially were traced to impurities; a procedure for preparing Calcein of definite composition was developed; the structure of Calcein was established by NMR spectrometry to be fluorescein-4',5'-bis(methyleneiminodiacetic acid); the acid dissociation constants, the solubility as a function of pH, and the absorption in the ultraviolet and the fluorescence as functions of pH were determined; the nature of the reaction of Calcein with calcium was established. The work of Huitink dealt with the related compounds *Calcein Blue* and *Methyl Calcein Blue*, materials derived from umbelliferone.

The work of Birze was a diversion into the chemistry of the isomeric phthaleins and calceins derived from orcinol. The work of Hoyle dealt with a form of Calcein called *Stato-calcein*, a reagent which is stable in water solution. The M.S. thesis of Martin was devoted to perfecting a method for the preparation of a pure Calcein in good yield and to developing methods for establishing purity. The work of Markuszewski was another major contribution to the chemistry of both fluorescein and Calcein; the structures of the yellow, red and colorless forms of fluorescein were established unequivocally; various physical and chemical behaviors of fluorescein were clarified; these findings were extended to Calcein; and the nature of the reactions of Calcein with certain metals was investigated. The present dissertation is then, in a real sense, a culmination of a series of investigations. In particular, in this dissertation, there is offered a confirmation of the Hefley structure of Calcein by an independent method, a sound and satisfactory procedure for the direct fluorometric determination of calcium, and a study of the electrochemistry of Calcein.

A. Naming and Structure of Calcein

In the initial paper on Calcein, Diehl and Ellingboe (18) stated that they had not obtained a pure substance but one sufficiently pure to serve as an indicator. This announcement, of course, was a challenge to other chemists and as might be expected those who thought they had prepared a purer material assigned their product a new name. Thus, the material of Wilkins and Hibbs (87) became *Calcein W*; that of Körbl, Vydra and Pribil (53) first became *Fluorescein Complexone* and later, because the term *Complexone* is a registered trade mark, *Fluorexone*; that of Wallach and co-workers (82,83) became *Compound A*. Each of these groups of workers employed the same reagents and the Mannich condensation, the variations being simply in choice of solvent, ratios of the reactants, and acidity. The product in each case was the same. The proliferation of names was understandable and excusable on the grounds of human vanity; what has been less fortunate, however, is that two structures, both wrong, were assigned to the material, the assignment being made in one case on the basis of no evidence at all and in the other on very insubstantial evidence.

Thus, the fluorexone of Körbl, Vydra and Pribil has become fluorescein-2',7'-bis(methyleneiminodiacetic acid);

no experimental evidence was offered and the assignment was evidently a mere guess on the part of a reader.[1] Compound A was stated by Wallach, Surgenor, Soderberg and Delano to be the unsymmetrical compound flüorescein-2'-4'-bis-(methyleneiminodiacetic acid), or expressed more technically and impressively by them as 3',6'-dihydroxy-2',4'-bis-[N,N'-di-(carboxymethyl)-aminomethyl]fluoran. The assignment was made on the basis of an absorption band at 800 to 860 cm^{-1} in the infrared spectrum. Every infrared spectrum of Calcein among numerous ones taken at Iowa State University is extremely complex with numerous, almost continuous absorption bands in the region 800 to 860 cm^{-1}, bands which

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[1]The first statement in print that the two iminodiacetic acid groups occupy the 2'- and 7'- positions in the Calcein molecule appears in Chemical Abstracts, Vol. 56, January-June 1962 Subject Index, pages 441 and 933, in which Calcein (and Fluorexone) is given the name *Fluorescein-2',7'-bis[[bis-(carboxymethyl)amino]methyl]-*. The Czech chemists, Körbl and his co-workers, stated on several occasions (53,52,74,54,81, 51) that the structure had not been established. They may, however, have been responsible for misleading the indexer of Chemical Abstracts, for a structural formula showing the groups in the 2'- and 7'- positions appears in an English review paper (52), on the very page in which it is stated that the structure is unknown! This may have been an error by the printer who set the structural formula in type inasmuch as other similar formulas appear in the same paper; in other publications, Körbl and his co-workers draw bonds from the methyleneiminodiacetic acid groups to the centers of the xanthene rings indicating the exact attachment to be unknown.

are barely distinguishable from the background; the work of Wallach, Surgenor, Soderberg and Delano cannot be repeated and their structure was shown by Hefley to be incorrect.

These mistakes in the structure of Calcein are propagated in Chemical Abstracts. To make matters worse, Chemical Abstracts, in the course of their appalling revision of their naming of organic compounds, introduced still further names for Calcein, names unintelligible to all but dedicated computer devotees or chemists blessed with extraordinary persistence. Thus, Chemical Abstracts, Index Guide of January-June 1972, gives

Calcein: Glycine,N,N'-[(3',6'-dihydroxyspiro-[isobenzofuran-1(3H),9'-[9H]xanthene]-2',7'diyl)bis(methylene)bis-N-(carboxymethyl)- [1461-15-0]

Fluorexone: Glycine,N,N'-[(3',6'-dihydroxy-3-oxospiro[isobenzofuran-1(3H),9'-[9H]xanthene]-2',7'-diyl)bis-methylene)]bis[N-carboxymethyl]-[1461-16-0]

Probably the best procedure if it becomes necessary to use Chemical Abstracts after 1972 is to remember to look under Glycine, N,N' followed by the essentially meaningless nomenclature just given.

Still two other designations for Calcein have been introduced, these by workers using Calcein as a marker for bone growth, *DCAF* (Suzuki and Mathews (73), 1966) and *CAL* (Kariyama, Akai and Nishijima (47), 1969). The origin of *CAL* is obvious; the designation *DCAF* is taken from certain letters in the impressive name (for the incorrect structure) given by Wallach and co-workers.

Calcein, Fluorescein Complexone, Fluorexone, Compound A, Calcein W, DCAF, CAL, the *Glycine-N,N'-gibberish* of Chemical Abstracts — it is all *Calcein,* and chemically, the structure is fluorescein-4',5' bis(methyleneiminodiacetic acid).

B. The Titrimetric Determination of Calcium with EDTA Using Calcein as Indicator

In the initial paper on Calcein, Diehl and Ellingboe (18), 1956, the EDTA titration of calcium using Calcein as indicator was adapted to the determination of calcium in limestone, gypsum, and magnesium and sodium salts. Many applications to the determination of calcium in other materials have now been made: to the determination of calcium in bone, teeth and phosphate rock (Yalman,

Bruegemann, Baker and Garn (90), 1959); calcium in lithium salts (Olsen, Diehl, Collins and Ellestad (64), 1961); calcium in ferromanganese slags (Morris (60), 1961); calcium in ores and silicate rock (Wohlmann (89), 1962); calcium in milk (Ntaillianas and Whitney (62), 1964; Sedlacek and Dusek (69), 1966); calcium in caustic soda (Van der Reyden and Van Lingen (78), 1962); calcium in titanium dioxide (Malevannyi (56), 1964); calcium in enamel, dentine and bond (Robinson and Weatherell (68), 1968); calcium in sodium chloride and potassium chloride crystals (Frölich and Siemroth (30), 1973).

That the method yields results even more accurate and precise than the conventional gravimetric methods in the determination of calcium in limestone and dolomite was demonstrated by Diehl and Miller (22).

The fluorometric end-point is best observed in the dark with the titration vessel illuminated by ultraviolet radiation. Several convenient titration boxes have been described (Hermann (37), 1958; Wilkins (86), 1959; Bett and Fraser (5), 1959; Diehl (21), 1964); the titration vessel is best observed through a yellow glass (Diehl (21), 1964). Automatic apparatus has been specifically designed for the titration (Howerton and Wasilewski (39), 1961; Borle and Briggs (7), 1968; Alexander (1), 1971).

The principal application of Calcein has been to the determination of calcium in blood serum. In the bibliography

of the 1964 monograph of Diehl (20) are listed fifteen papers on the subject and more have appeared since.

C. The Direct, Fluorometric Determination of Calcium in Serum with Calcein

The direct fluorometric determination of calcium with Calcein, although it has met with varied success, is extensively used; a bibliography of the principal papers is given in the next few paragraphs. As a result of the work of Hefley (35,36) and that described in Chapter V of the present dissertation, the sources of the troubles in this determination have now been uncovered and surmounted and the determination has been placed on a satisfactory footing.

During the very early work of Diehl and co-workers at Iowa State University, it proved impossible to obtain reproducible results or even calibration curves reproducible from lot to lot of Calcein. Diehl and co-workers did not publish their findings, hoping to first uncover the source of the trouble. Other workers, however, did describe direct fluorometric methods: for calcium, strontium and barium (Körbl, Vydra and Pribil (53), 1958); for various metals (Wallach and Steck (83), 1963); and for calcium in serum (Kepner and Hercules (48), 1963); as was predicted, numerous other workers experienced difficulty repeating these procedures and they were vocal and pointed in their off-the-record

comments. Because of these criticisms, work at Iowa State University was intensified. This work led to the findings of Diehl and Hefley. Hefley traced the principal difficulty to impurities of heavy metals in the Calcein. The metals were principally iron, mercury, zinc and aluminum. They were derived for the most part from the fluorescein used, the metals having been employed as catalysts in the condensation of phthalic anhydride and resorcinol used to manufacture fluorescein. More metals are derived from the various reagents used in the preparation of Calcein and, Calcein being a first class chelating agent for heavy metals, all are carried through into the final product. A second major difficulty arises from the nature of the reaction of calcium with Calcein; this is explained in Chapter V of the present dissertation.

In the meantime, workers have made do with the Calcein available commercially (see, for example, the 1968 paper of Uemura (77) dealing with the determination of calcium in muscle tissue) and have adapted the direct fluorometric determination of calcium in serum to automatic analyzers (Hill (38), 1965; Klein, Kaufman and Isaacs (49), 1967; Mabry and Wyles (55), 1967; De Witt and Parsons (17), 1970; Bandrowski and Benson (2), 1972).

D. Applications of Calcein to the Determination of Various Cations and Anions

Reaction with Calcein appears to divide the metals into two classes. First, those metals which form a fluorescent compound with Calcein at a pH at which Calcein by itself does not fluoresce; such metals are calcium, strontium, barium, aluminum, zinc, cadmium and mercury. Second, those metals which form a nonfluorescent compound with Calcein in the pH range 6 to 9 in which Calcein by itself does fluoresce; among these metals are copper(II), cobalt, manganese, nickel and palladium. Analytical methods involving Calcein make use of both the fluorescent metal derivatives and of the quenching of the fluorescence of Calcein.

In addition to the direct fluorometric method for calcium discussed above, the fluorometric method using Calcein has been devised for cadmium (Hefley and Jaselskis (34), 1974). It would appear that direct fluorometric methods should also be possible for both aluminum and mercury, the reaction of aluminum with Calcein being unusual in that the fluorescence is produced in acid solution. The reaction of aluminum with Calcein was examined in great detail by Markuszewski (57). He found that aluminum reacts with Calcein to form a compound containing three atoms of aluminum and three molecules of Calcein. As the pH of the solution is raised, a hydroxy-aluminum-Calcein compound is formed and at

pH above 9 the aluminum-Calcein compound is completely decomposed. Markuszewski made no attempt to apply his findings to the actual determination of aluminum.

Markuszewski (57) also looked into the nature of the reaction of mercury(II) and Calcein. The combining ratio proved to be one atom of the metal to one molecule of Calcein, thus differing markedly from the compounds of calcium and aluminum with Calcein. Markuszewski found also that the non-fluorescent copper(II) compound contained two atoms of the metal to one molecule of Calcein.

Among the numerous fluorometric methods which have been described are many which are based on the quenching of the fluorescence of a reagent by the addition of a metal ion. Quenching of the fluorescence of Calcein by copper(II) has been used to mark the end-point in the EDTA titrations of metals, an excess of EDTA being added to the solution of the metal being determined and the excess EDTA back-titrated with a standard solution of copper(II). At the end-point, the fluorescence of the Calcein disappears. This method was used by Wilkins for the titrimetric determination of iron (85,88), cobalt(II) (85,88), cobalt(III) (84), chromium (84, 88), copper (84) and titanium (88). The methods are particularly useful in that good end-points are obtained even in solutions of high concentrations of the metal ions in which the intense colors of the metal-EDTA compounds obscure

end-points with visual indicators. Essentially the same procedure was used for the determination of the metals in brass and bronze but a standard solution of triethylenetriamine was used in place of EDTA (Wilkins and Hibbs (87), 1959).

Calcein has been reported to be especially good as an indicator in the titration of bromide, iodide, cyanide and thiocyanate with silver nitrate (Vydra, Markova and Pribil (81), 1961; Tandon and Mehrotra (75), 1964). The indicator action is apparently a combination of the disappearance of fluorescence and adsorption of Calcein on the silver halide. An indirect method for sulfate, based on the back-titration with EDTA of the excess of standard barium chloride added, has also been described (Effenberger (27), 1959).

E. Calcein as a Marker of Bone Growth

When injected into animals, Calcein is laid down in growing bone as the fluorescent calcium derivative. The first paper on the subject was by Suzuki and Mathews (73) in 1966. Five additional papers have now been published: Kariyama, Akai and Nishijima (47), 1969; Olerud and Lorenzi (63), 1970; Sullivan (72), 1972; Solheim (71), 1974; Rasmussen (66), 1975. Work in the area has also been done at Iowa State University (14), with the chicken; by injecting Calcein into fertilized eggs it becomes possible to follow the deposition of calcium in the growing embryo.

F. The Work of the Present Dissertation

The first two chapters of the present dissertation are devoted to reporting extensions and improvements of the investigations reported in my master of science thesis (58), a thesis which dealt principally with improvements in the synthesis of Calcein and with the development of a method of determining the purity of Calcein. A question remained about the concentration of commercial formaldehyde and the stability of formaldehyde; this has been settled by developing and using a method for the quantitative determination of formaldehyde. The two-end-point method of determining the purity of Calcein reported in the master of science thesis has been re-examined and certain improvements made.

It has been known for some time that high concentrations of sodium cause Calcein to fluoresce and that the high alkalinity needed in the determination of calcium is best achieved by the addition of potassium hydroxide rather than sodium hydroxide. In the present work, an attempt was made to further reduce the background fluorescence by using as the titrant dipotassium ethylenediaminetetraacetate rather than the corresponding sodium salt.

The direct fluorometric determination of calcium using Calcein has been improved by the use of a reagent consisting of a one-to-one molar mixture of Calcein and calcium. A

linear calibration curve of high sensitivity results. This work is described in Chapter V.

An investigation into the electrolytic reduction of Calcein is reported in Chapter VI.

The principal work of the thesis is a study of the carbon-13 NMR spectrum of fluorescein, Calcein and related compounds. An independent proof of the structure of Calcein is offered and a foundation is laid for the future studies of the structure of xanthene compounds.

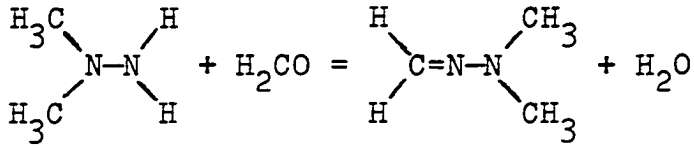
II. NOTE ON THE SYNTHESIS OF CALCEIN. CONCENTRATION AND STABILITY ON STORAGE OF FORMALDEHYDE

In the thesis (58) which I submitted for the degree master of science, I described an improved synthesis for the preparation of Calcein. Calcein is made by the Mannich condensation of fluorescein, iminodiacetic acid and formaldehyde. In the thesis, I made a statement that it was best to use formaldehyde from a freshly-opened bottle. Although this statement was based on certain observations made during the course of numerous preparations of Calcein, no direct check was made during the course of that work, or during that of the earlier workers, Hefley and Huitink, on Calcein and Calcein Blue, on the quality of commercial formaldehyde or on the stability on storage of solutions of formaldehyde. In this chapter I report on a method for the quantitative determination of formaldehyde and measurements which settle these questions.

Formaldehyde is marketed as a 37 to 40 per cent solution in water.

Of several methods for the determination of formaldehyde found in the literature, one method (70) appeared particularly appropriate to this study. The method is based on the reaction of formaldehyde with excess unsymmetrical dimethylhydrazine to form a hydrazone, the excess dimethylhydrazine then being back-titrated with standard hydrochloric acid

in methanol. The principal reaction is



A. Materials and Equipment

Unsymmetrical dimethylhydrazine was obtained from Aldrich; the purity was stated to be 99 per cent and no further purification was attempted. A stock solution, 0.2 M, in ethylene glycol was prepared.

The formaldehyde studied was the "Analyzed Reagent", 37-40 per cent formaldehyde of the J. T. Baker Chemical Company.

Potentiometric titrations were made using a Beckman Zeromatic SS-3 pH meter equipped with a saturated calomel electrode and a glass electrode. Response was quite good with saturated potassium chloride as filler solution for the saturated calomel electrode. No attempt was made to find a nonaqueous filler solution although the necessary titrations were made in nonaqueous solutions.

Hydrochloric acid, 0.1 M, was prepared by dilution of concentrated hydrochloric acid with acetone-free, absolute methanol. The hydrochloric acid was standardized by titrating weighed samples of Fisher, tris(hydroxymethyl)-aminomethane (THAM), which had been ground, sieved, and

vacuum dried. THAM has been shown not to be a primary standard (50) but the purity of this particular lot had been analyzed and found to be 99.9 per cent pure. The indicator used was a mixture of bromcresol green and sodium alizarin sulfonate.

A polyethylene dropping bottle was used for dispensing samples of formaldehyde. The nozzle of the bottle was provided with a two-way valve which allowed a very careful dispensing of the sample. Thus, there was no overflow and minimal exposure to the atmosphere. The bottle was used essentially as a weight buret.

B. Experimental Work

An aliquot, 20-25 ml, of the stock solution of unsymmetrical dimethylhydrazine, 0.2 M, was stirred for thirty minutes to simulate the conditions of the sample and then titrated potentiometrically with a previously standardized solution of hydrochloric acid in methanol. Acetone-free methanol was used as solvent.

The formaldehyde was placed in the dropping bottle and the bottle plus formaldehyde weighed. Then 8-10 drops were dispensed into a beaker and the beaker sealed with parafilm. The bottle plus formaldehyde was weighed again. The difference in weight, about 0.32-0.37 g, corresponded to approximately 0.002 mole of formaldehyde.

A 25-ml aliquot of standardized unsymmetrical dimethylhydrazine was pipetted into the beaker containing the formaldehyde. The beaker was sealed with parafilm. The reaction was allowed to proceed for 30 minutes with stirring. To the mixture was then added 50 ml of acetone-free methanol. The mixture was then titrated with standard hydrochloric acid in methanol. Typical titration curves for the standardization of the solution of unsymmetrical dimethylhydrazine and for the determination of the excess dimethylhydrazine after reaction with formaldehyde are shown in Figures 1 and 2.

C. Results and Discussion

Low results for formaldehyde were obtained when the time of reaction was shorter than the 30 minutes specified above. Some warming of the solution by the mechanical stirring may have occurred, otherwise the reaction was performed at room temperature.

Because the purpose of the experiment was to compare the concentration of formaldehyde immediately after opening the bottle and then again after an interval of exposure to the atmosphere, no attempt was made to study the effects of time and temperature on the analytical method except to standardize conditions and analyze each sample in exactly the same manner.

Figure 1. Titration of unsymmetrical dimethylhydrazine with hydrochloric acid

Concentration of hydrochloric acid: 0.1079 M

Volume of unsymmetrical dimethylhydrazine taken: 25.00 ml

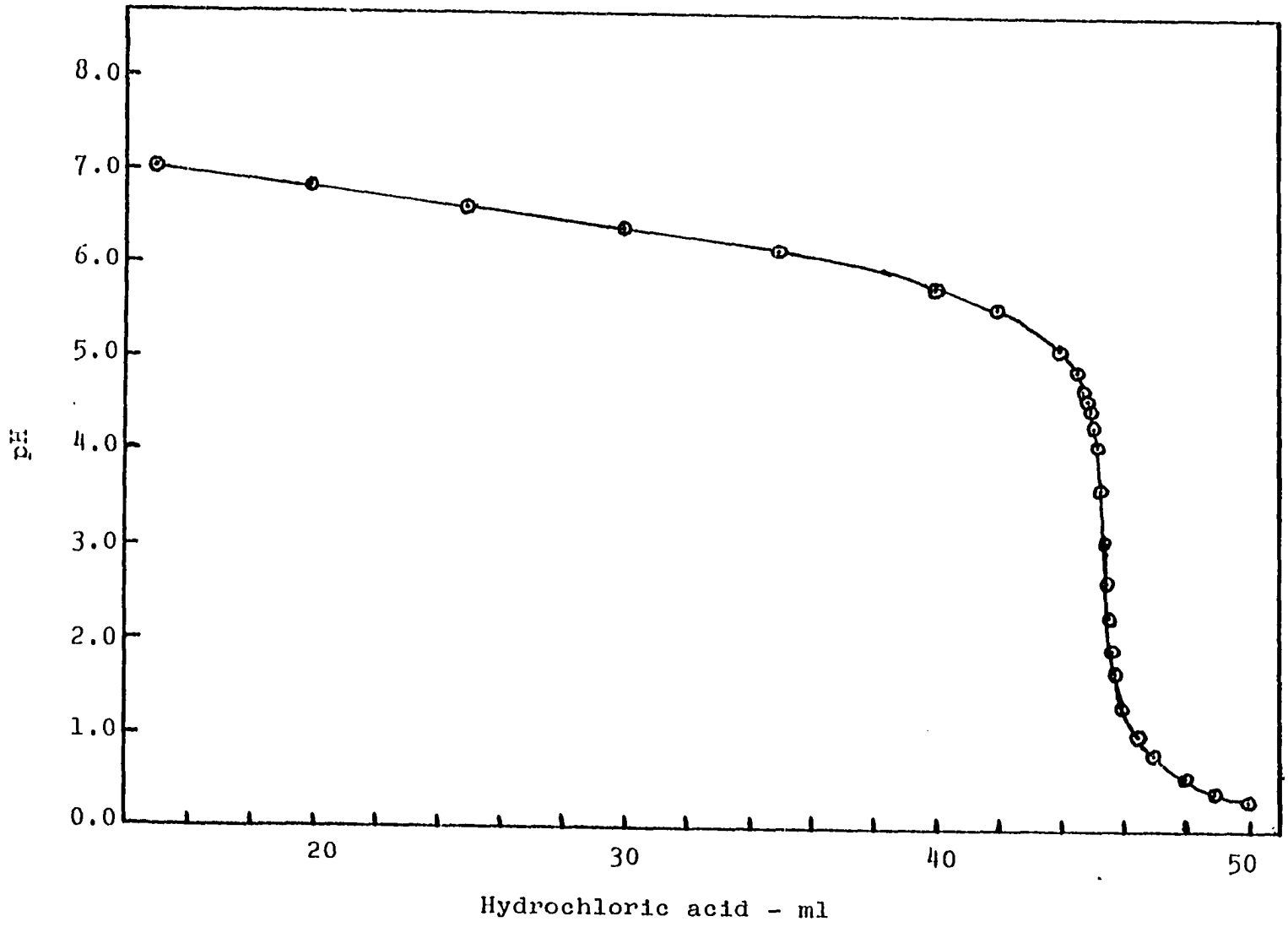

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
pH
20
30
40
50
Hydrochloric acid - ml

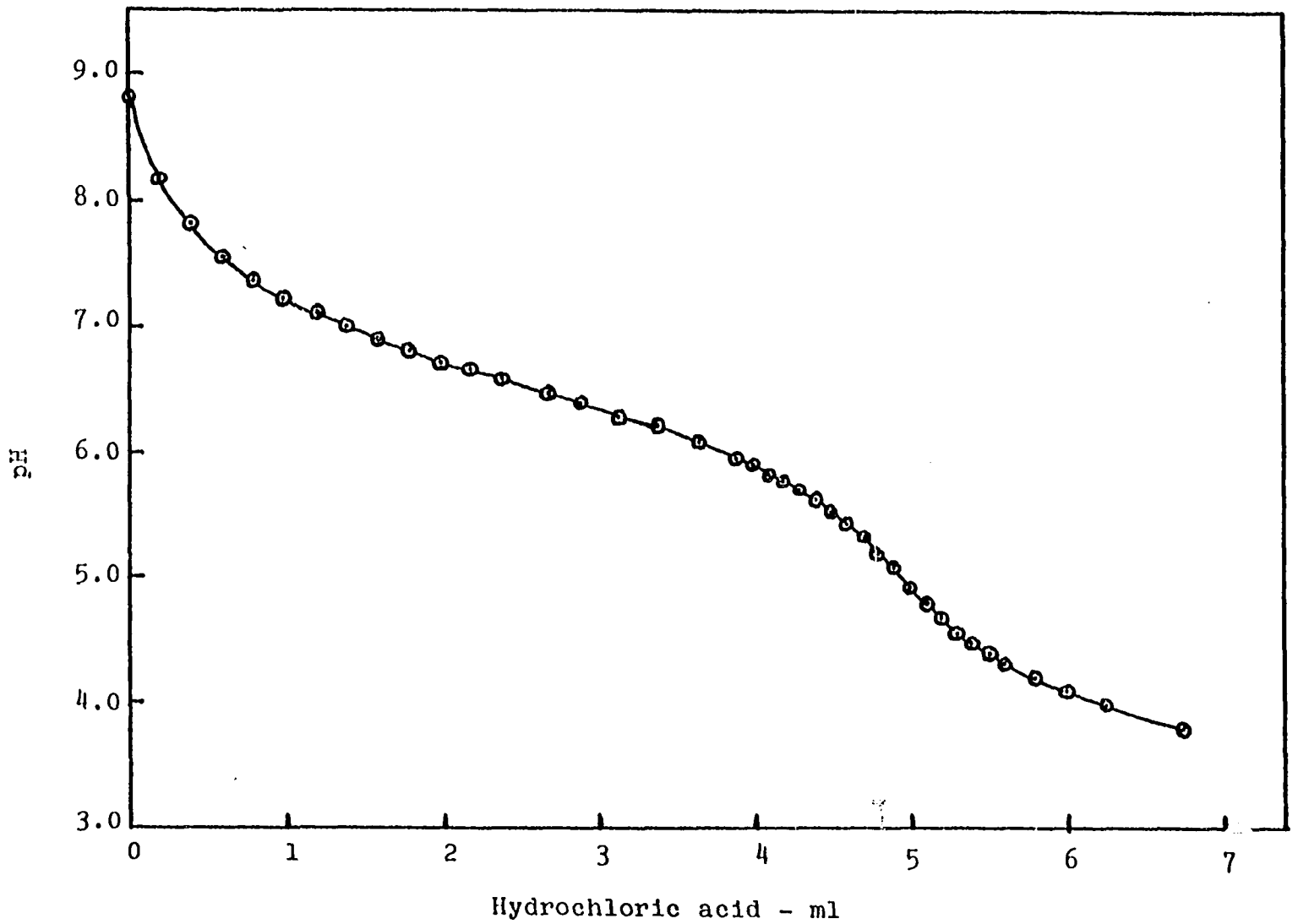
Figure 2. Titration of excess unsymmetrical dimethylhydrazine with hydrochloric acid after reaction with formaldehyde

Concentration of unsymmetrical dimethylhydrazine: 0.1961 M

Volume of unsymmetrical dimethylhydrazine added: 25.00 ml

Concentration of hydrochloric acid: 0.1079 M

Weight of formaldehyde taken: 0.3492 g


9.0
8.0
7.0
6.0
5.0
4.0
3.0
pH
0
1
2
3
4
5
6
7
Hydrochloric acid - ml

The content of formaldehyde found in a freshly-opened bottle was: 37.6, 37.5, and 37.5 per cent. That in the bottle left uncapped for 22 hours was: 37.8, 37.8, and 37.8 per cent.

Apparently, solutions of formaldehyde do not undergo change on standing exposed to air. The statement made in my master of science thesis as to the necessity of using formaldehyde from a freshly-opened bottle is withdrawn.

III. THE PURITY OF CALCEIN AS DETERMINED BY THE TWO-END-POINT METHOD

Previous workers have used the equivalent weight of Calcein, determined by potentiometric titration with standard alkali, as a criterion of purity. Hefley (36) added a known amount of standard potassium hydroxide to a weighed sample of the Calcein and then back-titrated the excess potentiometrically with standard hydrochloric acid. Markuszewski (57) took advantage of the solubility of Calcein in 50 per cent ethanol and titrated directly, potentiometrically, with standard alkali. The calculations of both workers were based on the end-point at which four moles of base are added for each mole of Calcein present. Any fluorescein present in the Calcein is also titrated, two replaceable hydrogen atoms being titrated simultaneously with the four hydrogen atoms of Calcein. The equivalent weights of fluorescein and Calcein, 166.2 and 160.2, respectively, are so close that the method does not give a measure of the amount of fluorescein present.

In the present work, the interference of fluorescein is eliminated by utilizing the property of Calcein of combining with calcium with the expulsion of two hydrogen ions. The sample of Calcein is first titrated to the end-point corresponding to four moles of base added per mole of Calcein and to two moles of base per mole of fluorescein. A 10- to

12-fold excess of calcium as calcium chloride is then added and the titration continued to a second end-point. The volume of alkali added between the two end-points is a measure of the Calcein present. Fluorescein does not interfere because it does not form a slightly-dissociated compound with calcium as does Calcein. This procedure was first described in my master of science thesis (58).

In more recent determinations of the equivalent weight of Calcein by this two-end-point procedure, a significant drift in the pH near the second end-point was observed. This effect was investigated in detail. Three titrations were performed. In the first titration, no attempt was made to compensate for the observed drift. In the second titration, the drift in the pH was allowed to proceed until a steady state was reached. In the third titration, the Calcein was titrated to the first end-point, an excess of standard potassium hydroxide was added, an excess of calcium was then added, and the excess potassium hydroxide was then back-titrated with standard hydrochloric acid. This third procedure proved a decided improvement over the earlier procedure in that the drift in the pH at the equivalence point was negligible.

A. Observations on the Earlier Method

1. Titration 1

A sample of Calcein weighing 0.4145 g was titrated potentiometrically with standard sodium hydroxide, 0.1078 M, using the two-end-point procedure of the earlier method referred to above. The first end-point occurred at 22.65 ml of sodium hydroxide added. No drift was observed during the titration to the first end-point. After passing the first end-point, a 12-fold molar excess of calcium as calcium chloride was added to the solution. The pH dropped for several minutes. The titration was continued before the drift reached a steady state and was completed without any attempt made to compensate for the drift observed between the first and second end-points. The drift in the pH was not observed after passing the second end-point. This end-point occurred at 33.65 ml of sodium hydroxide added. The titration curve obtained is presented in Figure 3.

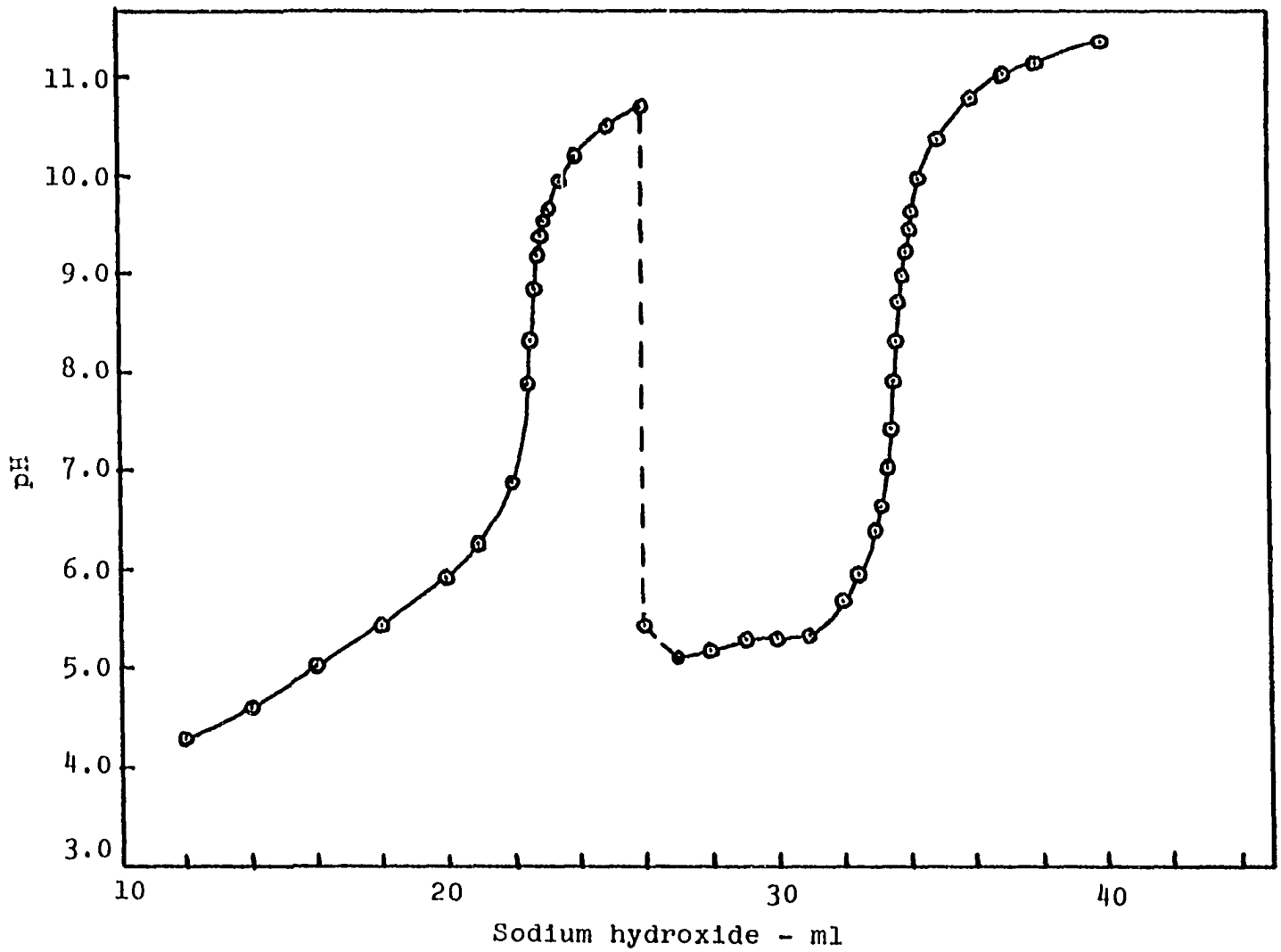
An equivalent weight was calculated for both of the observed end-points. The first end-point corresponded to four moles of base added per mole of Calcein present and to two moles of base added per mole of fluorescein present. The equivalent weight was 169.8 g per equivalent.

The calculation of the equivalent weight at the second end-point is based on the amount of base added between the first and second end-points. This amount corresponds to

Figure 3. Potentiometric titration of Calcein with sodium hydroxide. Twelve-fold molar excess of calcium added after passing the first end-point

Weight of Calcein taken: 0.4145 g

Concentration of sodium hydroxide: 0.1078 M


11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
pH
10
20
30
40
Sodium hydroxide - ml

the removal of the fifth and sixth replaceable hydrogen atoms present in the Calcein molecule. This calculated equivalent weight must then be doubled to obtain the equivalent weight of Calcein. This method was described in my master of science thesis (58). The equivalent weight calculated in this manner was 174.8 g per equivalent. The theoretical equivalent weight of Calcein is 160.2 g per equivalent.

2. Titration 2

A sample of Calcein weighing 0.6153 g was titrated potentiometrically with standard sodium hydroxide, 0.1079 M, using the two-end-point method as described above. No drift was observed during the titration to the first end-point. The first end-point occurred at 33.65 ml of sodium hydroxide added. After passing the first end-point, 1.7 g of calcium chloride dihydrate in 20 ml of deionized water was added to the solution. The pH dropped for twenty minutes and then reached a steady state.

The titration was continued with standard sodium hydroxide. A drift in the pH was observed after each increment of sodium hydroxide added. The amount of sodium hydroxide added, the initial pH, the pH after drift, and the amount of time between readings is listed in Table 1. In almost all cases the drift never completely stopped. Some effort was made to wait until the drift had slowed to a negligible rate. The entire titration curve is shown in Figure 4.

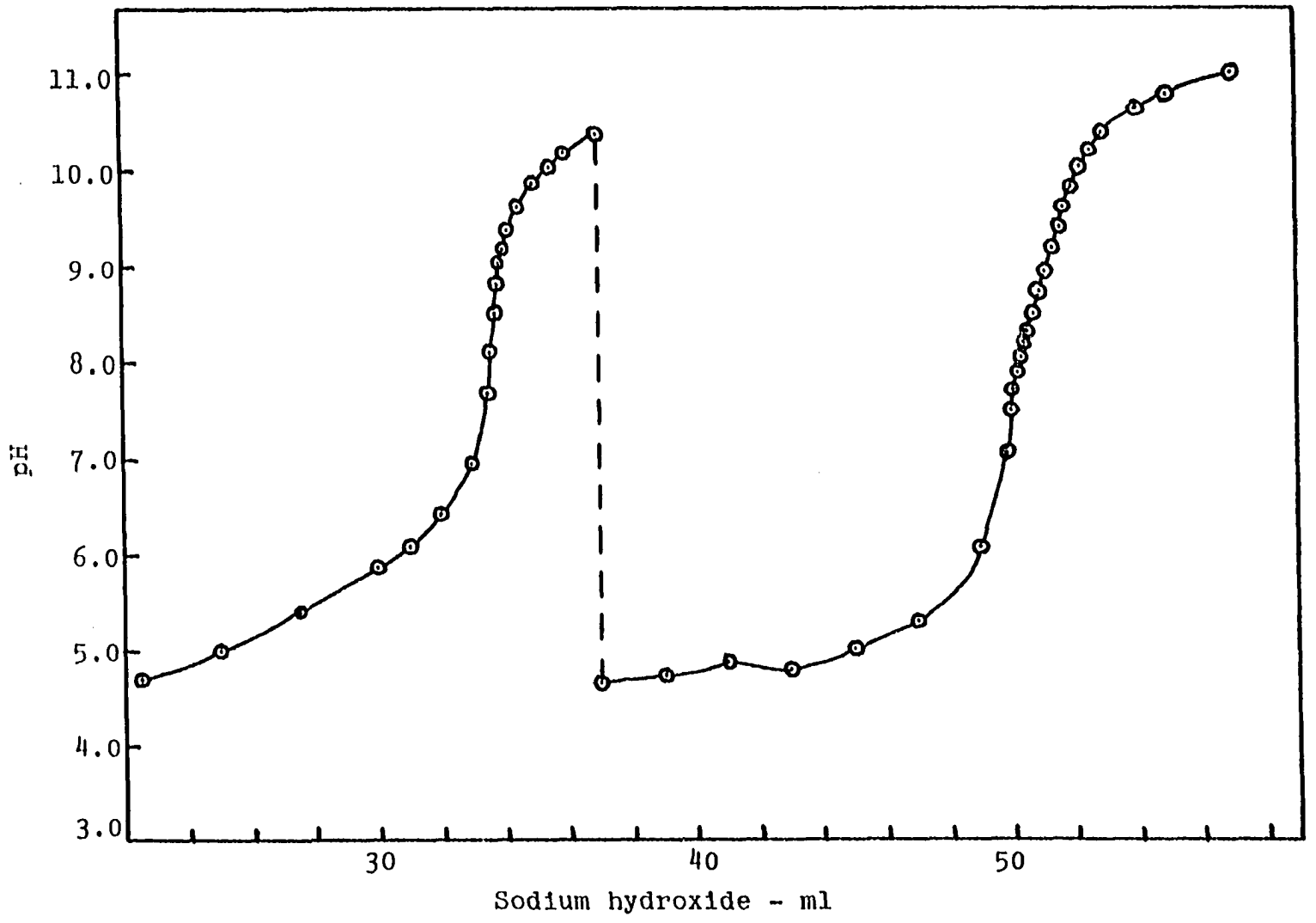
Table 1. Drift in pH near the second end-point in the titration of Calcein with sodium hydroxide in the presence of excess calcium

ml	Initial pH	Drift Time (min)	Final pH
37.00	10.40	20	4.67
39.00	---	5	4.75
41.00	---	4	4.89
43.00	---	10	4.82
45.00	---	13	5.03
47.00	---	13	5.32
49.00	6.22	13	6.12
49.81	7.32	13	7.07
50.00	7.67	13	7.49
50.10	7.85	15	7.72
50.20	8.08	18	7.92
50.30	8.25	25	8.09
50.40	8.32	20	8.22
50.50	8.51	44	8.32
50.60	8.53	25	8.41
50.70	8.62	22	8.53
50.80	8.72	20	8.64
50.90	8.82	20	8.74
51.00	8.92	20	8.85
51.10	9.00	10	8.97
51.20	9.13	10	9.10
51.30	9.24	10	9.22
51.40	9.33	10	9.32

Figure 4. Potentiometric titration of Calcein with sodium hydroxide. Twelve-fold molar excess of calcium added after passing the first end-point

Weight of Calcein taken: 0.6153 g

Concentration of sodium hydroxide: 0.1079 M


11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
pH
30
40
50
Sodium hydroxide - ml

The total elapsed time for the titration was 7.75 hours. Upon completion of the titration the electrodes were rinsed with deionized water, with 1 M hydrochloric acid, and again with deionized water. The electrodes were then placed in standard pH 6.86 buffer for several minutes before taking a reading. The observed pH was 6.89. It was apparent that the pH meter was operating properly and stable.

An equivalent weight was calculated for both end-points as was done in the first titration described above. The equivalent weight at the first end-point was 169.5 g per equivalent. The plot of the pH *versus* the amount of sodium hydroxide added, Figure 4, is flat through the region of the second equivalence point and does not have the expected general shape of a titration curve. This made location of the end-point difficult. The second end-point was estimated at 50.60 ml of sodium hydroxide added. The equivalent weight was 168.2 g per equivalent.

3. Titration 3

A solution of carbonate-free potassium hydroxide, approximately 0.1 M, was prepared in the following manner. Twice the amount of potassium hydroxide pellets necessary to make 2 liters of 0.1 M potassium hydroxide was weighed. Deionized water was added to the pellets, swirled, and the solution immediately decanted. This removed any carbonate from the surface of the pellets. The portion of the pellets

remaining was dissolved and diluted to 2 liters with deionized water which had been boiled and cooled to remove the carbon dioxide. The solution was protected from the atmosphere by a test tube containing some of the potassium hydroxide and fitted with a Bunsen valve. The solution was standardized against primary standard potassium acid phthalate.

A solution of calcium chloride was prepared from primary standard calcium carbonate. A sample of calcium carbonate weighing 8.7385 g was dissolved by adding 56 ml of concentrated hydrochloric acid to the material in a 600-ml beaker. The solution was then baked to dryness and the solid residue which remained was dissolved with deionized water. The solution was transferred to a 200 ml volumetric flask and diluted to volume. The concentration of calcium was 0.437 M.

A sample of Calcein weighing 0.1026 g was suspended in 75 ml of deionized water. Nitrogen gas was passed through a U-tube containing ascarite and then gently bubbled through the suspension of Calcein for 15 minutes prior to the titration in order to remove any carbon dioxide present.

After 15 minutes, the nitrogen gas was passed over the solution while the Calcein was titrated potentiometrically with the carbonate-free potassium hydroxide, 0.0910 M, prepared earlier. No drift was observed during the titration to the first end-point which occurred at 6.61 ml of base added.

After a total of 8.00 ml of base had been added, a 10-ml aliquot of standard, carbonate-free, potassium hydroxide, 0.0910 M, was pipetted into the solution. Then a 5-ml aliquot of 0.437 M calcium chloride was added. The pH rose and reached a steady state almost immediately. Nitrogen gas was again bubbled through the solution for 15 minutes.

The excess potassium hydroxide was back-titrated potentiometrically with 0.0960 M hydrochloric acid in an atmosphere of nitrogen. Only a very slight amount of drift in the pH was observed as the second end-point was approached. The end-point occurred at 7.34 ml of hydrochloric acid added. The entire titration curve is presented in Figure 5.

A 5-ml aliquot of 0.437 M calcium chloride was titrated with 0.0910 M potassium hydroxide to the phenolphthalein end-point. The end-point occurred at 0.22 ml of potassium hydroxide added. This value was taken into account in the calculation of the equivalent weight.

The equivalent weight was calculated for each of the observed end-points. The equivalent weight calculated for the first end-point was 170.6 g per equivalent. The calculation of the equivalent weight for the second end-point was done in the same manner as the previous titrations except that the excess potassium hydroxide had to be taken into account. The equivalent weight was 164.5 g per equivalent.

The drift in pH at the second end-point experienced in titrations 1 and 2 reported above is probably caused by the

Figure 5. a. Potentiometric titration of Calcein with potassium hydroxide

Weight of Calcein taken: 0.1026 g
Concentration of potassium hydroxide: 0.0910 M

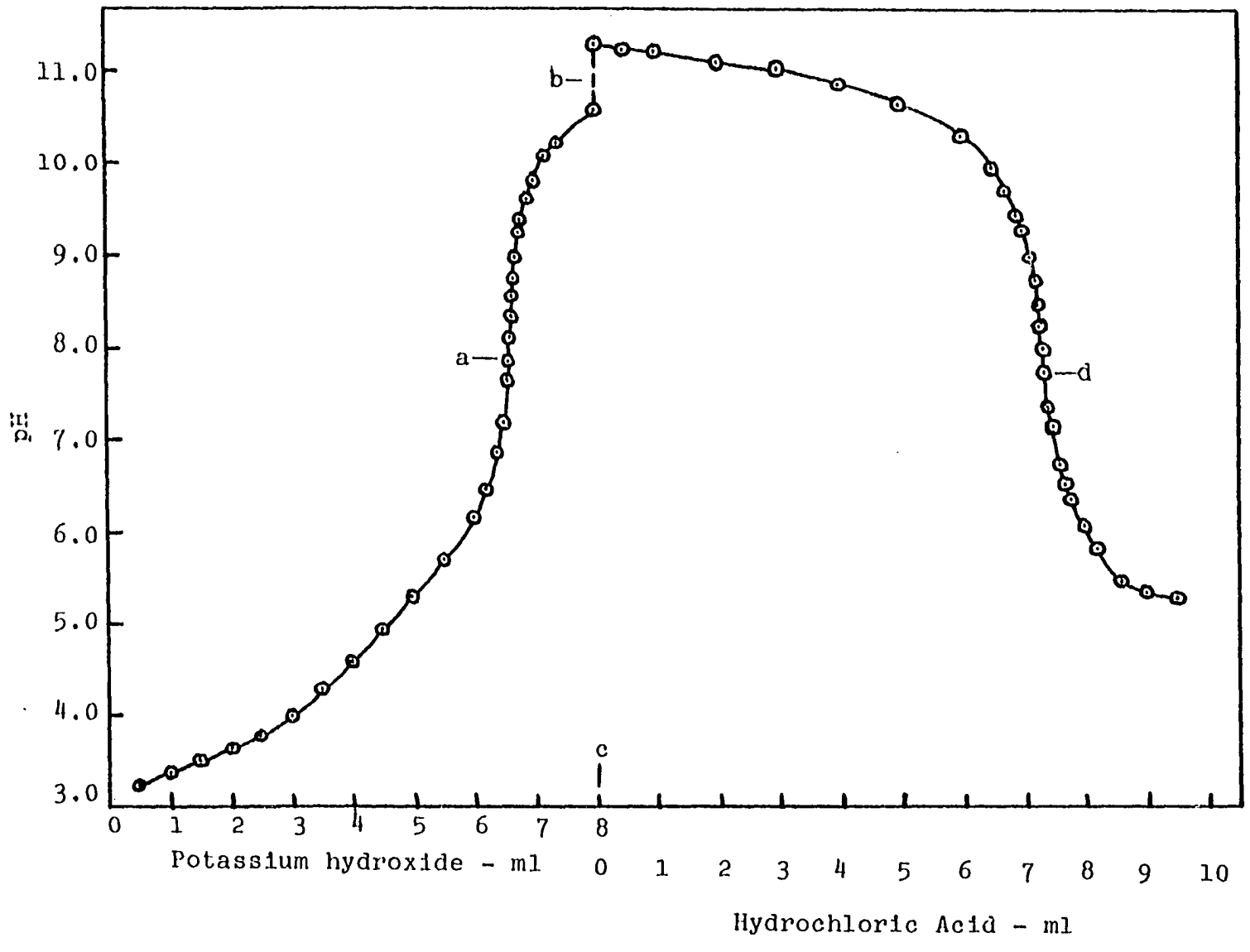
b. Potassium hydroxide added; excess calcium added

Concentration of potassium hydroxide: 0.0910 M
Volume of potassium hydroxide added: 10.00 ml
Concentration of calcium chloride: 0.437 M
Volume of calcium chloride added: 5.00 ml

c. Start of back-titration of excess potassium hydroxide with hydrochloric acid

d. Potentiometric back-titration of excess potassium hydroxide with hydrochloric acid

Concentration of hydrochloric acid: 0.0960 M


11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
pH
a
b
c
d
0 1 2 3 4 5 6 7 8
Potassium hydroxide - ml
0 1 2 3 4 5 6 7 8 9 10
Hydrochloric Acid - ml

slowness with which replacement of the last two hydrogen ions of Calcein by calcium occurs. Calcein does not react with calcium quantitatively except in basic solution. When a 12-fold excess of calcium is added to an alkaline solution of Calcein, as in the above titrations, the pH drops to between pH 4 and pH 5. An orange solid, the sodium form of Statocalcein ($K_2Ca_5Cal_2$), is present. At this pH, however, not all of the Calcein is in this form. Statocalcein precipitates at pH 8.3 which is very close to the pH at the end-point in the potentiometric titration of Calcein with sodium hydroxide. As this pH is continually approached with each drop of base, more Statocalcein is formed, releasing more hydrogen ions, and the pH drops. This is a continual process and the reaction appears to be fairly slow.

The solution to the problem of drift in the pH at the second end-point was found in Titration 3 reported above. The addition of base after passing the first end-point raises the pH of the solution to a level where the reaction of Calcein with calcium is quantitative, and the complete removal of the fifth and sixth replaceable hydrogen atoms of the Calcein molecule is thus effected. The back titration of the excess potassium hydroxide proceeds in normal fashion with essentially no drift.

The equivalent weight found for one sample of Calcein by this method was 164.5 g per equivalent; the value is good

to about ± 5 ppt. For establishing the purity of Calcein to be used as an analytical reagent, this is sufficiently good.

B. Recommended Procedure for Determining the Purity of Calcein by the Two-End-Point Method

Prepare a standard solution, approximately 0.1 M, of carbonate-free potassium hydroxide as follows. Weigh approximately twice the amount of potassium hydroxide pellets necessary to make 2 liters of 0.1 M potassium hydroxide. Add deionized water to the pellets, swirl, and then immediately decant the solution. This will remove the carbonate present on the surface of the pellets. Dissolve the portion of the pellets remaining and dilute to 2 liters with deionized water which has been boiled and cooled. Protect the solution from the atmosphere by a test tube containing some of the potassium hydroxide and fitted with a Bunsen valve. Standardize the solution using weighed samples of dried, primary standard potassium acid phthalate.

Weigh accurately a sample of Calcein, of about 100 mg, and suspend the material in deionized water in a beaker. Pass nitrogen gas, previously passed through ascarite in order to remove any carbon dioxide present, through the solution for 10 to 15 minutes prior to the titration. Titrate the Calcein potentiometrically with standard, carbonate-free,

potassium hydroxide. Pass nitrogen gas over the solution during the entire course of the titration.

After reaching and passing the first end-point, pipette a 10-ml aliquot of the standard, carbonate-free, potassium hydroxide into the beaker. Add a 12-fold molar excess of calcium in the form of calcium chloride. Titrate the excess potassium hydroxide potentiometrically with standard hydrochloric acid. Calculate the equivalent weight based on the difference in the two end-points obtained. This difference will correspond to the removal of the fifth and sixth replaceable hydrogen atoms present in the Calcein molecule. This calculated equivalent weight must then be doubled to obtain the equivalent weight of Calcein.

1. Notes on the procedure

The purpose of passing carbon dioxide-free nitrogen through the solution before the titration and over the solution during the titration is to avoid the interference of carbonate in the back-titration of the excess potassium hydroxide. This procedure should be used when small samples of Calcein, of the order of 100 mg, are titrated. A small error resulting from the presence of carbonate would be a serious error in the calculation of the equivalent weight. If sufficient Calcein is available such that a sample of 0.5 g or more can be taken for analysis, the use of nitrogen

during the titration is not necessary. The larger sample size means, of course, that a proportionally larger aliquot of standard potassium hydroxide must be added after reaching and passing the first end-point.

IV. DIPOTASSIUM DIHYDROGEN ETHYLENEDIAMINETETRAACETATE AS A TITRIMETRIC REAGENT FOR CALCIUM

Calcium is determined most commonly by titration with disodium dihydrogen ethylenediaminetetraacetate (EDTA). In the presence of magnesium, the titration is made in a solution of pH 12.5 to 13.0 with Calcein as indicator. At this pH magnesium is present as the unreactive, slightly-dissociated hydroxide, $Mg(OH)_2$, and the Calcein is present as the fluorescent calcium derivative. During the titration, EDTA unites with the calcium in solution forming disodium calcium ethylenediaminetetraacetate and when this reaction is complete the EDTA extracts the calcium from the calcium-Calcein compound causing the fluorescence to disappear, thus marking the end-point.

Unfortunately, the stock solution of the indicator, which is made up by dissolving Calcein in sodium hydroxide, does not keep well and must be made up fresh every few days. A stable form of the indicator, Statocalcein, $K_2Ca_5Cal_2$, was synthesized by Hoyle and Diehl (40). Statocalcein is readily soluble and solutions of it are fairly stable on storage. A solution of high pH containing a little EDTA and Statocalcein (corresponding to the end-point in the EDTA titration of calcium) is nonfluorescent; when a solution of Calcein dissolved by the addition of sodium hydroxide is used as indicator some fluorescence remains, sufficient to cause a

disturbing background. This "residual" fluorescence of Calcein resulting from the presence of sodium was first observed by Körbl, Vydra and Pribil (54) who recommended that potassium hydroxide rather than sodium hydroxide be added to raise the pH of the solution to be titrated and that the unnecessary introduction of sodium salts be avoided. Essentially the same findings, with variations in the experimental work, were made by Diehl and Oulmann (23) and Bozhevol'nov and Kreingol'd (8,9).

The use of Statocalcein eliminated another source of sodium and it became of interest to learn if a still further reduction in the background fluorescence could be obtained by substituting dipotassium dihydrogen ethylenediaminetetraacetate for disodium dihydrogen ethylenediaminetetraacetate as the titrant. As will be shown below, the improvement was negligible but an incidental observation may prove of value. Curiously, students in the beginning course in quantitative analysis have trouble preparing the 0.0100 M EDTA solution. Disodium dihydrogen ethylenediaminetetraacetate dihydrate dissolves slowly, the student will often fail to observe undissolved solid in the volumetric flask in which he is preparing the solution and will proceed to standardize the solution with, of course, wildly varying and baffling results. Dipotassium dihydrogen ethylenediaminetetraacetate dissolves almost instantaneously and should prove better in the hands of students.

A. Experimental Work

1. Reagents

A solution approximately 0.010 M in dipotassium ethylenediaminetetraacetate was prepared by dissolving 4.0 g of the salt in 1 liter of deionized water. The solution was standardized by titrating aliquots of standard 0.01000 M calcium solution prepared from Baker "Analyzed Reagent" grade calcium carbonate and found to be 0.01068 M.

A solution approximately 0.010 M in disodium dihydrogen ethylenediaminetetraacetate was prepared from the dihydrate salt in the usual way (25). The solution was standardized by titrating aliquots of standard 0.01000 M calcium solution and found to be 0.01141 M.

A solution of Statocalcein was prepared by dissolving 10 mg of the material in 100 ml of deionized water.

Potassium hydroxide, 5 M, was prepared by diluting 12 M potassium hydroxide obtained from the Hach Chemical Company, Ames, Iowa.

A 0.2 per cent solution of ascorbic acid and a 2.5 per cent solution of potassium cyanide were prepared.

2. Measurement of fluorescence

All measurements of relative fluorescence were made with the Turner Model 110 Fluorometer. The primary filter used was a Corning 5850 filter; the secondary filter was a

combination of a Wratten 1 per cent neutral density filter with a Turner 2A-15 yellow filter. The slit setting was 3x.

3. Fluorometric titrations

A series of solutions in 100-ml volumetric flasks was prepared. To each flask was added 5.00 ml of 0.01000 M calcium, 5.0 ml of 0.2 per cent ascorbic acid, 3.0 ml of 2.5 per cent potassium cyanide, and 5.0 ml of 5 M potassium hydroxide. The solution in the first flask was diluted to volume with deionized water and used as a reagent blank. To the remaining flasks was added 1.00 ml of the Statocalcein solution; to the third and succeeding flasks was added increasing volumes of 0.01141 M disodium dihydrogen ethylenediaminetetraacetate. A similar series of solutions was prepared with increasing volumes of 0.01068 M dipotassium dihydrogen ethylenediaminetetraacetate. All solutions were diluted to volume with deionized water. The relative fluorescence of each solution was measured.

The data obtained in these fluorometric titrations is plotted in the graphs of Figure 6.

B. Results and Discussion

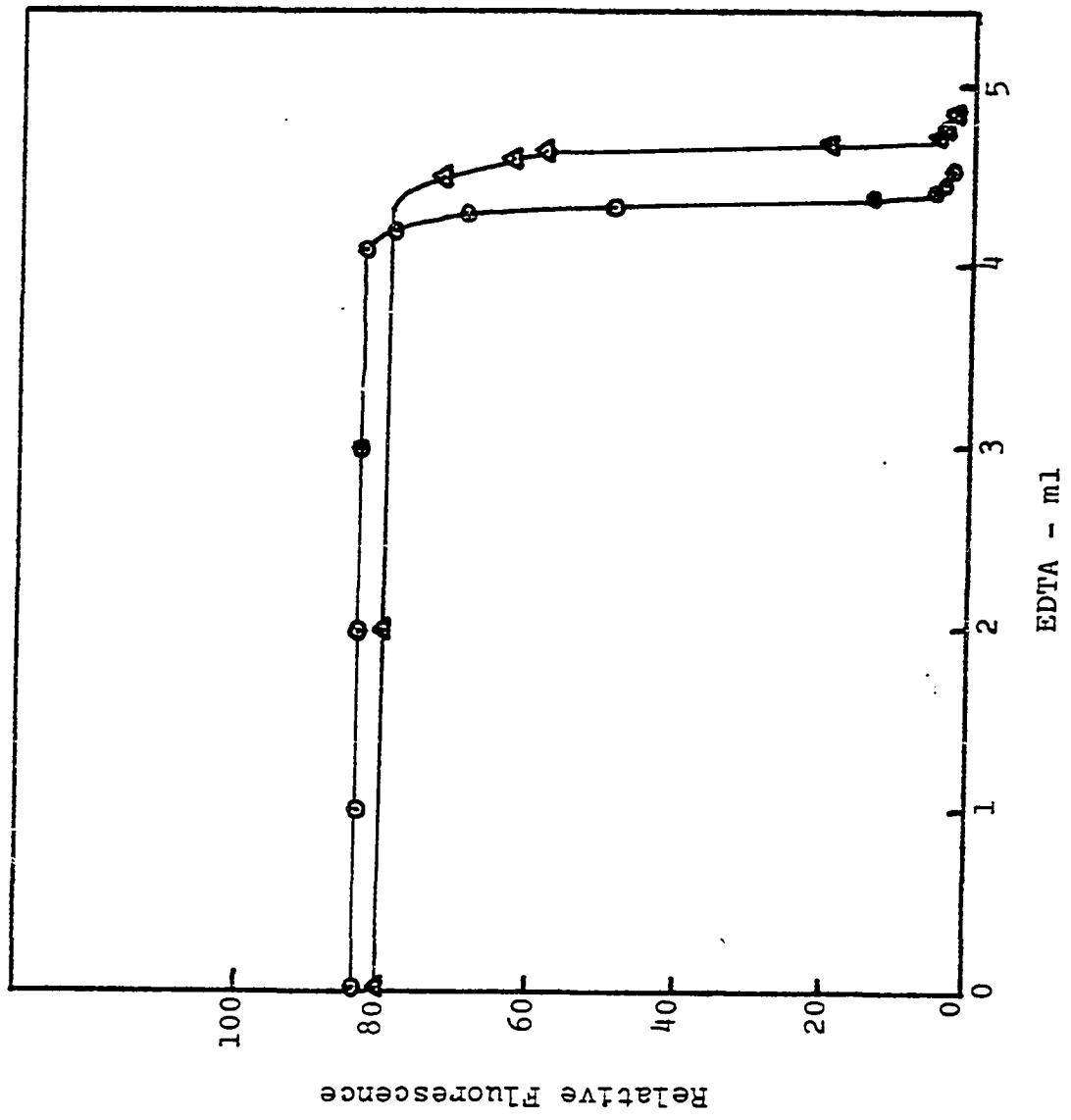
In the determination of calcium in limestone and other materials, ascorbic acid and potassium cyanide are added as masking agents for iron and copper (25); these materials were

Figure 6. Fluorometric titration of calcium using disodium and dipotassium ethylenediaminetetraacetate as titrants and Statocalcein as the indicator

Concentration of calcium: 0.01000 M
Volume of calcium taken: 5.00 ml
Volume of Statocalcein taken: 1.00 ml

⊙ Titration using disodium ethylenediaminetetraacetate as titrant.
Concentration of disodium ethylenediaminetetraacetate: 0.01141 M

△ Titration using dipotassium ethylene diaminetetraacetate as titrant.
Concentration of dipotassium ethylenediaminetetraacetate: 0.01068 M


Relative Fluorescence
100
80
60
40
20
0
0
1
2
3
4
5
EDTA - ml

included in the present work to make the solutions conform to the conditions encountered in actual practice.

The titrations show that there is no significant difference in the background fluorescence in the region of the end-point using as the titrating agent the disodium or the dipotassium salt of ethylenediaminetetraacetic acid. Measurements with the fluorometer did indicate that the background fluorescence was slightly lower with the potassium salt but this was not detectable by eye.

V. PROCEDURE FOR THE DIRECT FLUOROMETRIC DETERMINATION OF CALCIUM. LINEAR CALIBRATION CURVE

Shortly after the initial papers on Calcein, Diehl and Ellingboe (18), 1956, and Körbl and Vydra (51), 1958, attempts were made to use the reagent for the direct fluorometric determination of calcium: Körbl, Vydra and Pribil (53), 1958; Wallach and Steck (83), 1963; Kepner and Hercules (48), 1963. Although the method has been extensively used, particularly for the determination of calcium in serum, because of the speed and apparent simplicity, the results in the hands of most workers have been less than satisfactory. Differences in the composition of the Calcein from lot to lot give rise to variations in the calibration curves, and to curves which are S-shaped and do not pass through the origin. Uncertainty as to the exact composition of Calcein and of the nature of the reaction with calcium made it impossible to discern the source of the trouble. Some workers claimed to have obtained linear calibration curves, for example Körbl and Vydra (51), who obtained a linear calibration curve over the range 0.08 to 0.64 µg of calcium per ml by a procedure now known to be low in sensitivity toward calcium. The same linear calibration curve of limited range and sensitivity was also obtained by Wallach, Surgenor, Soderberg and Delano (82), Kepner and Hercules (48), and Bozhevol'nov and Kreingol'd (8,9), all of whom believed that calcium and Calcein react

in the ratio of one atom of calcium to one molecule of Calcein. Wallach and Steck (83) did recognize that a compound in the ratio of two atoms of calcium to one molecule of Calcein might be formed but stated that the change in fluorescence occurred only with the binding of the second atom of the metal. Numerous other workers, including the original authors (24), were unable to obtain linear and reproducible calibration curves.

Hefley (35,36) was the first to synthesize a pure Calcein. Her studies showed conclusively that first one and then a second atom of calcium combines with each molecule of Calcein. She also showed that each atom of calcium added caused a change in fluorescence and that overall the response of fluorescence to calcium added consisted of two distinctly different, linear portions. The slope of the second linear portion was just twice that of the first. That is to say, the fluorescence of Calcein increases linearly as the first atom of calcium is added, and increases at twice this rate as the second atom of calcium is added. Addition of calcium beyond the two atoms of calcium per molecule of Calcein caused no further increase in fluorescence. This finding was confirmed by later workers: Hill (38), Uemura (77), and Bandrowski and Benson (2).

The nonreproducible and nonlinear features of the calibration curves obtained in early work were traced by Hefley

to the presence in the Calcein of fluorescein and of heavy metals; the residual fluorescein may amount to several per cent and the metals found were mercury, zinc, iron and aluminum. The Calcein used in the present work was essentially free of these impurities.

In the present work, attention has been directed to the direct fluorometric determination of calcium and in particular the procedure has been modified so that only the fluorescence caused by the second atom of calcium to unite with the Calcein is used. Two ways of preparing a suitable reagent solution were developed.

The first approach was to mix the proper amounts of Statocalcein ($K_2Ca_5Cal_2$) and Calcein such that the resulting mole ratio of calcium to Calcein was one. The amounts used were 0.006040 g of Statocalcein and 0.008191 g of Calcein, weighed on the Mettler Model M5 Microbalance. These were dissolved in a minimum of 1 M potassium hydroxide and diluted to 100.0 ml. The calibration curve obtained with this reagent was linear. Although this approach has the merit that a solid reagent is obtained which can be stored indefinitely and used at will, two reagents are required, Statocalcein and fluorescein-free Calcein, neither of which are currently available commercially.

The second approach to obtaining a linear calibration curve was simply to mix stock solutions of calcium chloride

and Calcein such that the mole ratio of calcium to Calcein in the final solution was one-to-one. In practice a slight excess of calcium over the one-to-one ratio was added; failure to do so gave rise to a calibration curve having an intercept below zero on the fluorescence axis.

A. Recommended Procedure for the Fluorometric Determination of Calcium Using the One-to-One Calcium-Calcein Reagent

1. Reagents

a. 0.0100 M Calcium chloride Weigh accurately exactly 1.000 g of primary standard calcium carbonate, transferring the material directly into a 1-liter volumetric flask. Add 6 ml of concentrated hydrochloric acid to dissolve the calcium carbonate. Dilute to volume with deionized water and mix.

b. 5.00×10^{-5} M Calcium chloride Pipette 5 ml of 0.0100 M calcium chloride into a 1-liter volumetric flask. Dilute to volume with deionized water and mix.

c. One-to-one calcium-Calcein solution Weigh accurately on an analytical balance approximately 30 mg of Calcein. Transfer to a beaker. Add sufficient 1 M potassium hydroxide to dissolve the Calcein. Calculate the volume of 0.0100 M calcium chloride solution to be added so that the molar ratio of calcium to Calcein is exactly one;

the molecular weight of Calcein is 640.8 g. Add this volume of 0.01000 M calcium chloride solution plus an excess of 0.15 ml. Transfer the solution to a 250-ml volumetric flask and dilute to volume.

2. Apparatus

The measurements of relative fluorescence made in this work were made on the Turner Model 110 Fluorometer. Other fluorometers should give satisfactory results. The primary filter used was a Corning 5850 filter, maximum transmittance at 365 nm; the secondary filter was a combination of a Wratten 1 per cent, neutral density filter and a Turner 2A-15 filter, the latter filter being a sharp cut-off filter passing light of wavelengths greater than 520 nm.

3. Preparation of calibration curve

Pipet into each of a series of 100-ml volumetric flasks, 10 ml of 1 M potassium hydroxide, 5 ml of the one-to-one calcium-Calcein reagent, and varying amounts of 5.00×10^{-5} M calcium chloride. Dilute to volume with deionized water and mix. The first solution prepared should contain no calcium and be used to set the fluorometer to zero. Measure the relative fluorescence of the other solutions.

B. Advantages of the One-to-One Calcium-Calcein Reagent

The calibration curve obtained with the one-to-one calcium-Calcein reagent prepared as described above is shown in Figure 7.

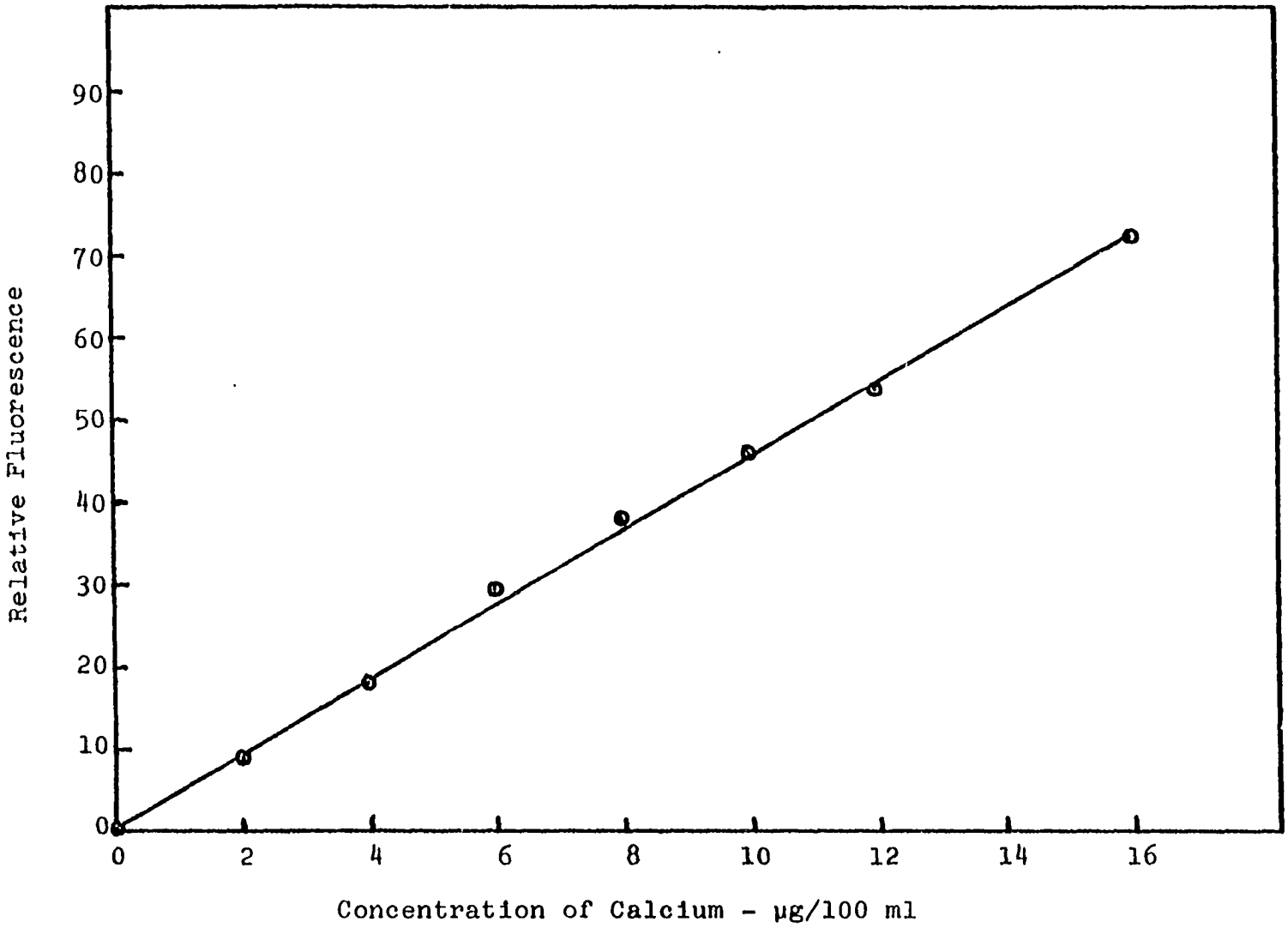
The main advantage in using a calibration curve as described in this work is that any addition of calcium to the calcium-Calcein reagent results in a linear increase in the fluorescence. The problems of the S-shaped calibration curves found in other work are circumvented.

The reagent proved more stable in alkaline than in acid or neutral solution. The relative fluorescence of standard solutions prepared using the one-to-one calcium-Calcein reagent which had been prepared in acid or neutral solution was not reproducible over any length of time. Solutions of the reagent prepared at alkaline pH were stable for about two hours. This is probably related to the fact that the formation of the slightly-dissociated compound of Calcein and calcium is favored in alkaline solution.

The standard solutions, pH 13, prepared from the one-to-one calcium-Calcein reagent were stable for two hours, regardless of the pH of the solution of the reagent. After two hours, some departures from the initial readings were observed. The stability is about the same as that of the

Figure 7. Fluorescence of Calcein as a function of the concentration of calcium using the one-to-one calcium-Calcein reagent

Concentration of Calcein: 9.3×10^{-6} M
Reagent: pH 11.7


Relative Fluorescence
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
Concentration of Calcium - µg/100 ml

indicator Statocalcein as reported by Hoyle and Diehl (40). This is a decided advantage over the earlier method.

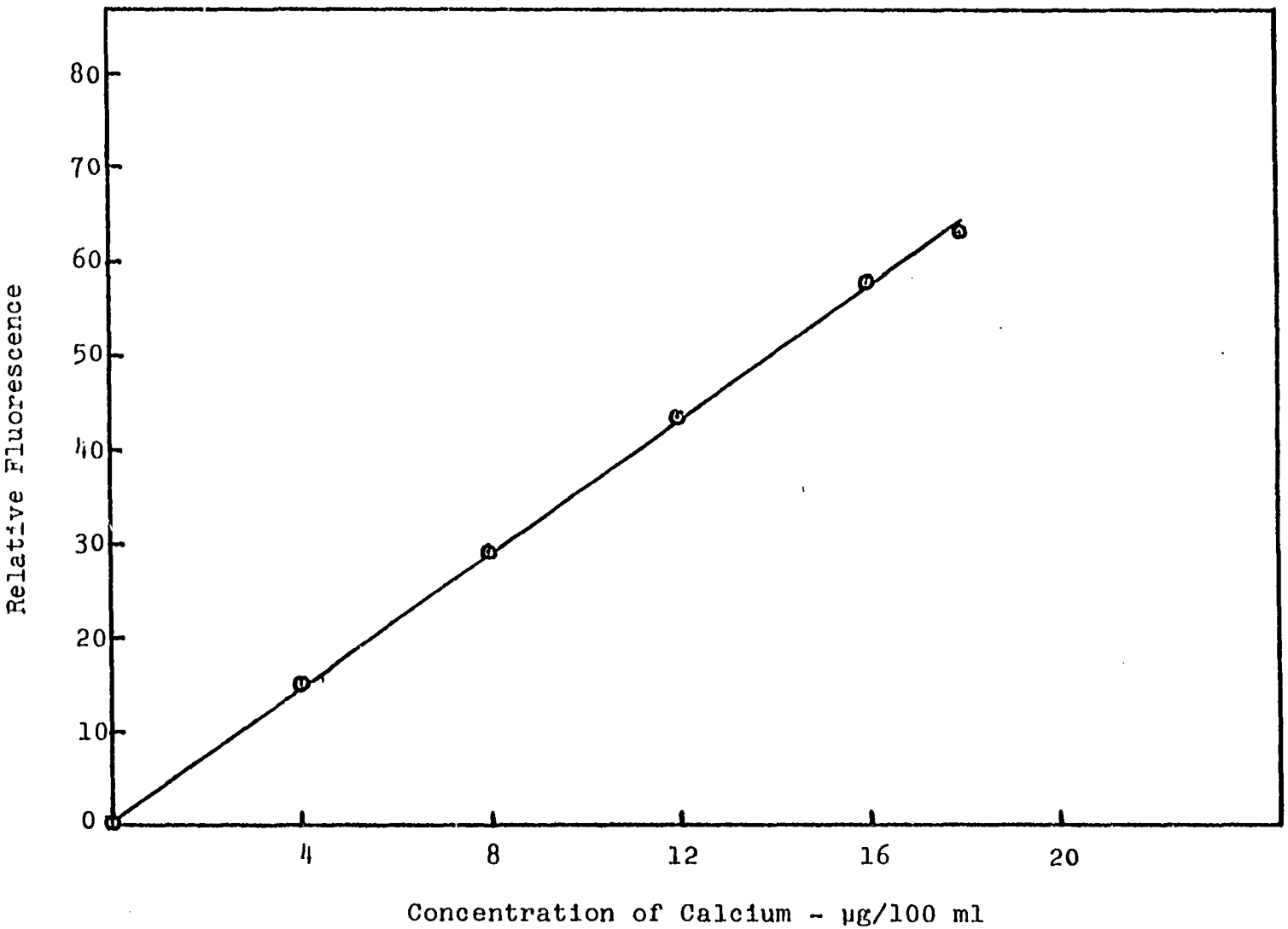
Although the stability of the one-to-one calcium-Calcein reagent prepared in acidic solution is not as great as the stability of reagent prepared in alkaline solution, the calibration curve prepared from such a reagent (acid solution) exhibits a linear response in the fluorescence of Calcein on addition of calcium. The standard solutions used to prepare the calibration curve presented in Figure 8 were prepared from a one-to-one calcium-Calcein reagent, the reagent being at pH 2.95.

Hefley and Jeselskis (34) reported using a few crystals of ascorbic acid added to the reagent solution of Calcein to increase the stability. Application of this technique to the present work indicated that the presence of ascorbic acid decreased the fluorescence of the standard solutions. Further studies were not made.

One final advantage is that of having the zero point of the calibration curve correspond to the fluorescence of the calcium-Calcein reagent. Metal-free Calcein is difficult to obtain, and solutions of Calcein usually have a varying background fluorescence. In the present method the background fluorescence is adjusted for in setting the instrument blank at zero using the reagent itself. This does not, however, lessen the desirability of obtaining Calcein in a metal-free, pure state for other applications of Calcein.

Figure 8. Fluorescence of Calcein as a function of the concentration of calcium using the one-to-one calcium-Calcein reagent

Concentration of Calcein: 7.4×10^{-6} M
Reagent: pH 2.95


Relative Fluorescence
80
70
60
50
40
30
20
10
0
4
8
12
16
20
Concentration of Calcium - µg/100 ml

VI. THE ELECTROCHEMICAL REDUCTION OF CALCEIN

Only one paper appears in the literature on the electrochemical behavior of Calcein, that of Nomura (61) who studied the alternating current polarography of various organic analytical reagents and the metal derivatives of these reagents. The tensammetric wave obtained with the organic reagent disappeared on the addition of the metal and reappeared when the metal was pulled away by a stronger chelating agent. This phenomenon was used to detect the endpoint in the titration of the metal with the chelating agent. Calcein was among the organic reagents studied but little is reported beyond the fact that a tensammetric wave was obtained.

Quite in contrast to Calcein, the literature on the electrochemistry of fluorescein is extensive. The earliest paper is that of Delahay (16) who studied the polarographic reduction of fluorescein at the dropping mercury cathode as a function of pH. The later investigations of Gollmick and Berg (31) and of Issa, Abd-el-Halim and Hasanein (45) also deal with the polarographic behavior of fluorescein as a function of pH. In these early studies no attention was paid to the composition of the electrolyte or to control of the ionic strength. It is now known that both of these factors play important roles in the electroreduction of organic molecules. Later studies of the electrochemistry of

fluorescein were made with strict control of the ionic strength, Bannerjee and Vig (3,4), Vig and Bannerjee (79), Vig (80).

In the present work, the differential pulse polarogram of Calcein at the dropping mercury cathode was obtained in solutions of varying pH. It was found that Calcein was reduced. Coulometric experiments were then made to learn the number of electrons involved in the reduction. Finally, a study was made of the reduction wave of Calcein in alkaline solution containing calcium.

A. Differential Pulse Polarography of Calcein as a Function of pH

The electrochemical reduction of Calcein at the dropping mercury cathode was studied by differential pulse polarography. The reduction wave was obtained at various pH and in solutions of constant ionic strength. Corresponding reduction waves of fluorescein were obtained in solutions of the same composition.

1. Experimental work

The Princeton Applied Research Corporation Model 174A Polarographic Analyzer, provided with a Hewlitt-Packard XY recorder, was used. The working electrode was the dropping mercury electrode (dme); the reference electrode used was a

saturated calomel electrode (sce); the counter electrode was a platinum wire.

The polarographic cell used, constructed in the glass shop of the Department of Chemistry, Iowa State University, was patterned after one commercially available from the Princeton Applied Research Corporation. A diagram of this cell is shown in Figure 9.

The buffers used were prepared from disodium hydrogen phosphate, citric acid, and potassium chloride. All were prepared to have an ionic strength of 0.5. The range of pH covered by these particular buffers was pH 2.2 to pH 8.0. The composition of the buffers is given in a paper by Elving, Markowitz, and Rosenthal (28). Buffers prepared for work in more acidic solutions were prepared from hydrochloric acid and potassium chloride. Buffers prepared for work in more alkaline solutions were prepared from sodium carbonate, sodium bicarbonate, and potassium chloride, or potassium hydroxide and potassium chloride. The ionic strength was 0.5.

Differential pulse polarograms were obtained using 1.0-ml aliquots of 2×10^{-3} M solutions of fluorescein and Calcein in 15 ml of buffer. The cathodic polarographic scans were generally over a range of 0.00 V to -1.50 V *versus* the sce. The drop time for the dme was 0.5 sec.

Figure 9. Cell for polarography

A. Nitrogen inlet

B. Dropping mercury electrode

C. Sample inlet

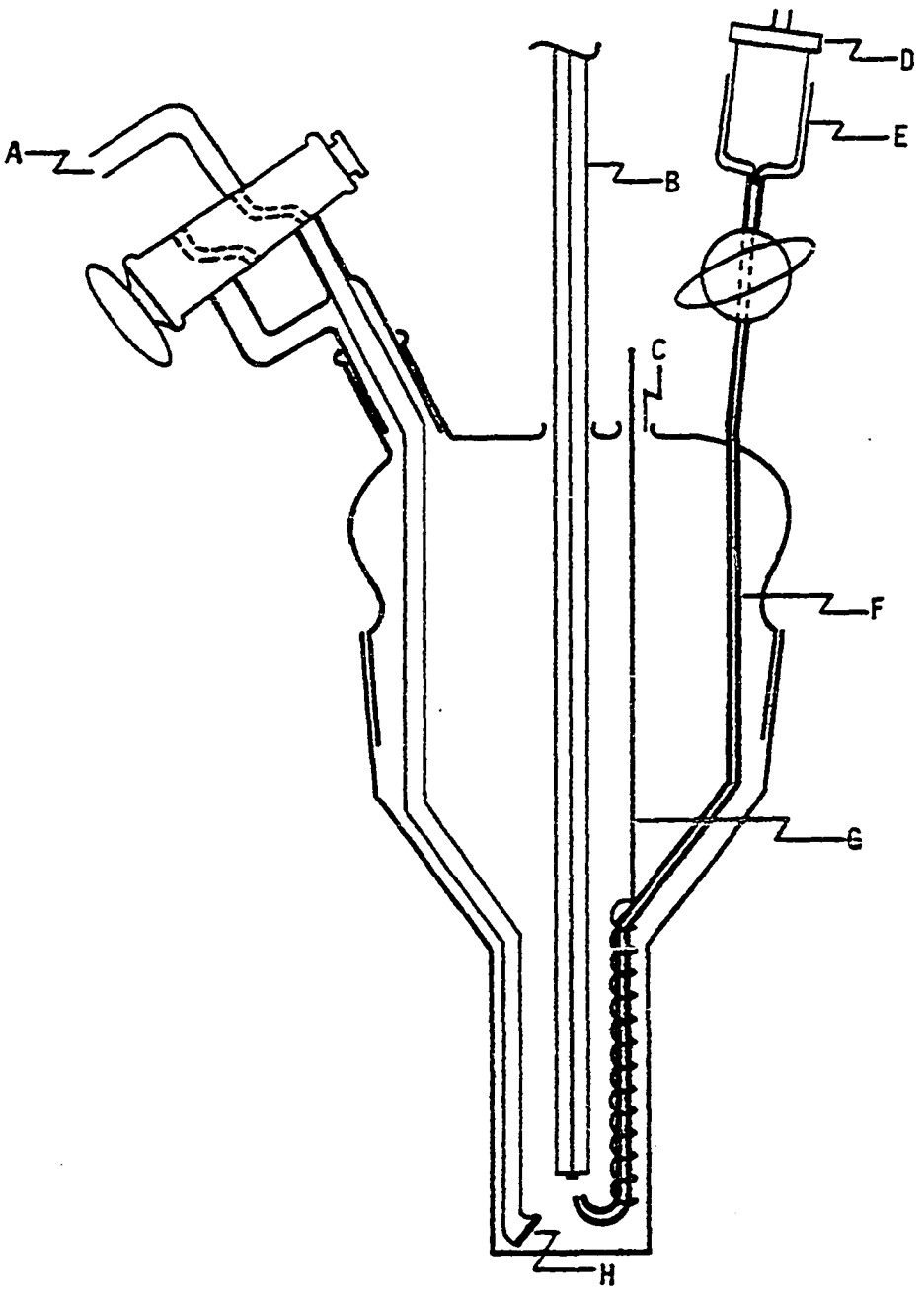
D. Saturated calomel reference electrode

E. Reservoir for saturated potassium chloride

F. Luggin capillary

G. Platinum counter electrode

H. Glass frit on bottom of nitrogen inlet tube


A
B
C
D
E
F
G
H

2. Results and discussion

The differential pulse polarograms of Calcein were obtained in solutions covering the range of pH 1 to pH 13. A reduction wave was observed at each value of pH studied. A second reduction wave was present in alkaline solution. This second wave was not studied in any detail. A plot of the half-wave potential as a function of the pH is presented in Figure 10. Points of inflection were found at pH 2.4, pH 5.4, pH 7.2, and pH 9.4.

The half-wave potential is a characteristic of each reducible species and thus shifts with pH as the nature of the reducible species changes in conformity with its acid-base nature. The height of the reduction wave is a measure of the amount of the reducible species present and thus the shape of the wave is determined by two quite different factors. In the Calcein molecule there are present seven replaceable hydrogen atoms (protons), starting with the protonated species, H_7Cal^+, present in very acid solution. The species and the acid dissociation constants associated with each are (57,10)

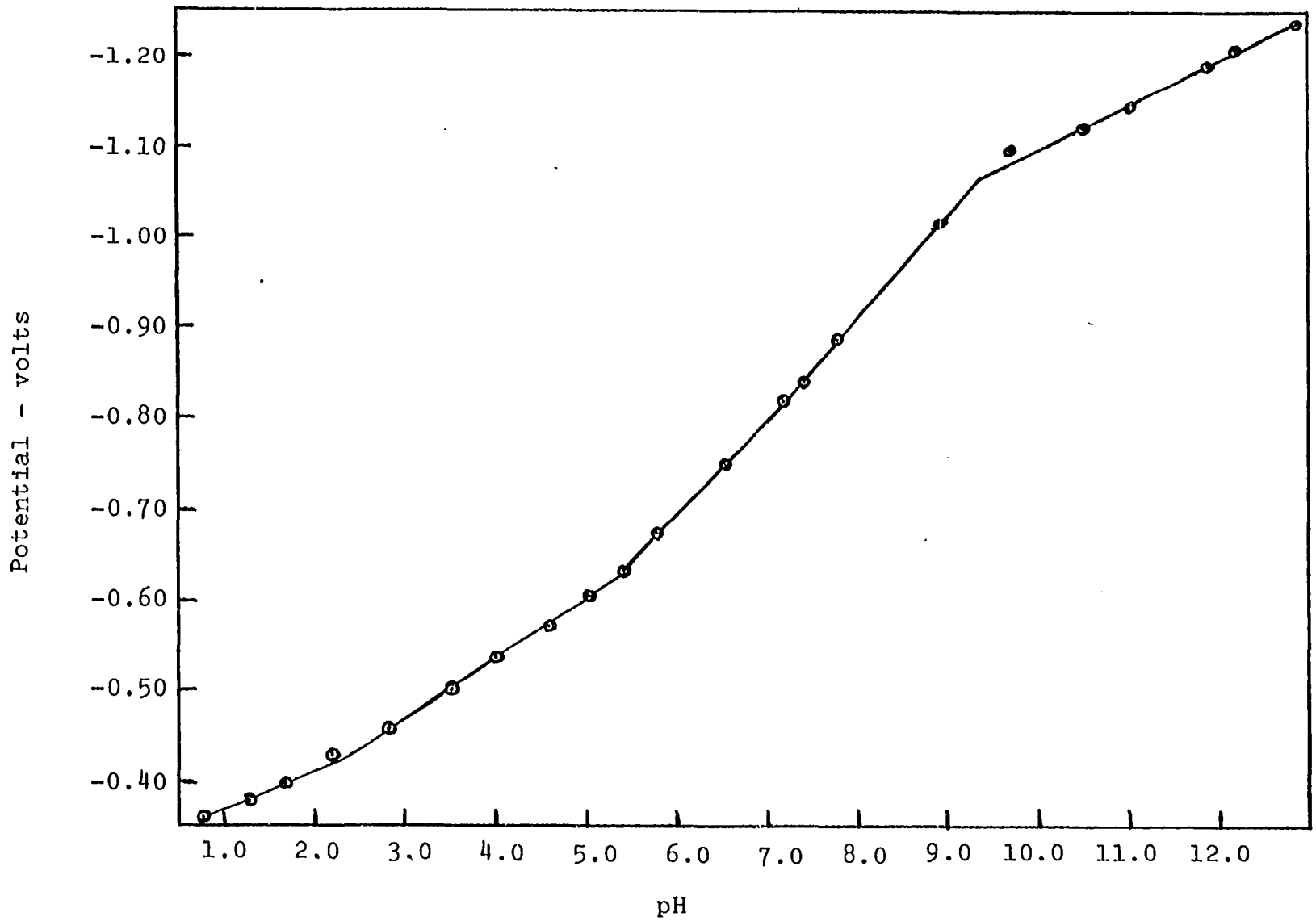
$K_{H_7Cal^+} = 1.75$ $K_{H_6Cal} = 2.74$ $K_{H_5Cal^-} = 3.53$

$K_{H_4Cal^{2-}} = 4.58$ $K_{H_3Cal^{3-}} = 6.19$ $K_{H_2Cal^{4-}} = 9.88$

$K_{HCal^{5-}} = 11.64$

Figure 10. Half-wave potential for the reduction of Calcein at the dropping mercury cathode as a function of pH

Concentration of Calcein: 1.3×10^{-4} M


-1.20
-1.10
-1.00
-0.90
-0.80
-0.70
-0.60
-0.50
-0.40
Potential - volts
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
pH

In the potentiometric titration of Calcein, one end-point only is observed, at four equivalents of base added per mole of Calcein; the values of the dissociation constants of H_6Cal, H_5Cal^-, H_4Cal^{2-}, and H_3Cal^{3-} are so close together that the titration of the various species overlap. In polarography a different principle is involved. The half-wave potential is determined in part by the charge carried, the charge in turn influencing the speed of migration to the electrode and the mechanism of the electron transfer taking place at the electrode; thus, the half-wave potentials of the positively charged species, H_7Cal^+, the neutral species, H_6Cal, and the negatively charged species H_5Cal^- might be expected to differ appreciably but those of the variously charged anions to differ from each other much less so. If a particular pH could be found at which a single species is present, the half-wave potential for this species would become known. For Calcein, the closest approach to such values are: 1) pH 2.25 at which the minimum in solubility occurs (10) and the species present is predominantly H_6Cal; and 2) pH 8.2, the pH at the end-point in the potentiometric titration of Calcein with sodium hydroxide, the species present being entirely H_2Cal^{4-}. The half-wave potential at pH 2.25 is -0.42 V. The half-wave potential at pH 8.2 is -0.93 V.

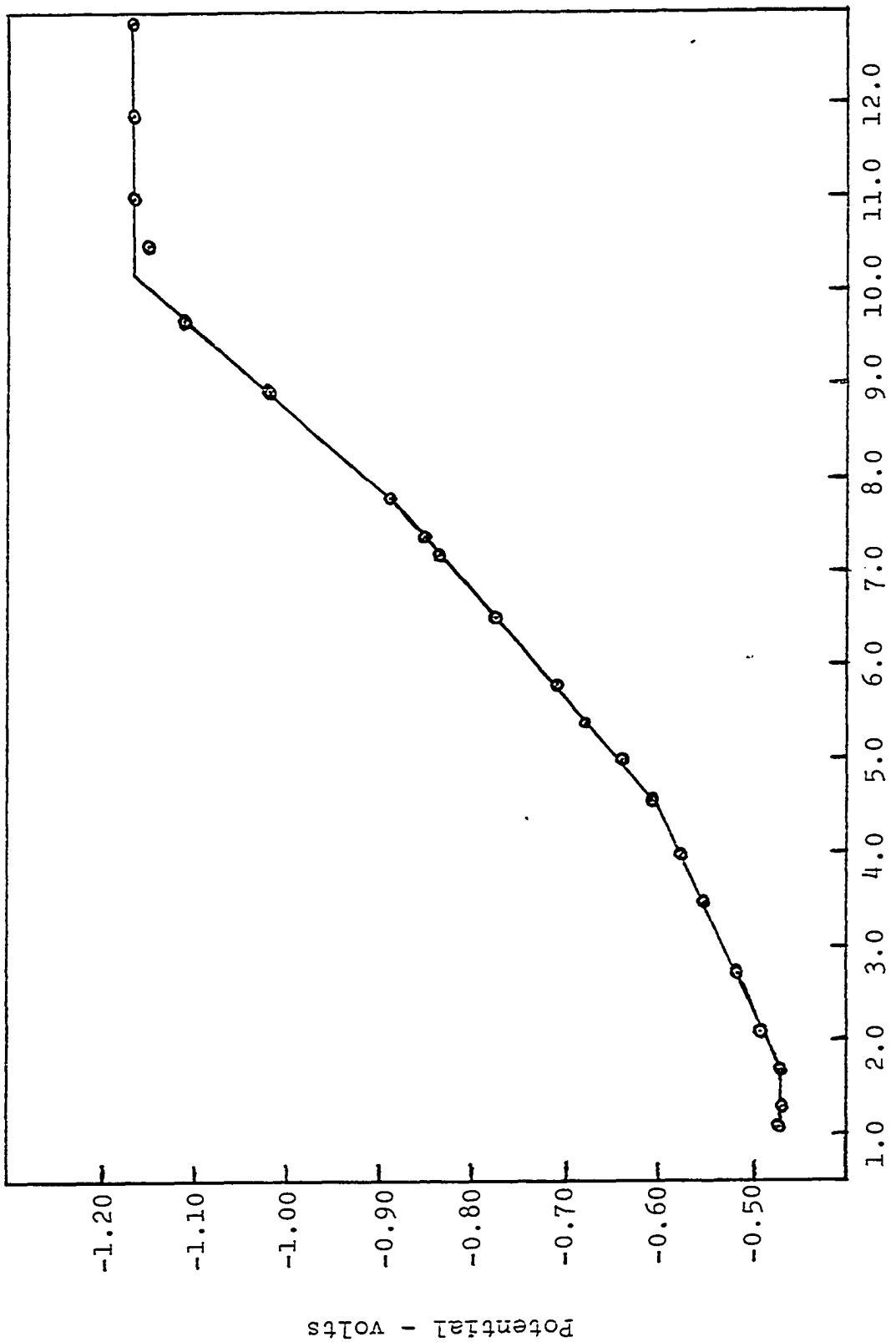
The values for the various acid dissociation constants of Calcein reported by Hefley (36), Markuszewski (57), and

Brayton (10) were based on data obtained from potentiometric titration and solubility, absorption in the ultraviolet and fluorescence as functions of pH; solubility, absorption and fluorescence differ markedly with the various ions, and made it possible to obtain values for the various acid dissociation constants. It is possible that all seven of the ionic forms of Calcein are reduced at the dropping mercury cathode and differentiation on the basis of the half-wave potential might seem impossible. On the other hand, four points of inflection are present in the curve of half-wave potential as a function of pH, and the problem remains a challenging one.

The results of a similar study with fluorescein are presented in Figure 11. Points of inflection were found at pH 1.7, pH 4.6, pH 7.8, and pH 10.5, in fair agreement with Bannerjee and Vig (3) who reported points of inflection at pH 2.0, pH 4.75, pH 7.0, and pH 9.2. The points of inflection do not coincide with the acid dissociation constants of fluorescein as reported by Markuszewski (57): $pK_{H_3Fl^+}$, 2.14; pK_{H_2Fl}, 4.75; pK_{HFl^-}, 6.55. The considerations advanced above for Calcein apply to fluorescein. Actually, the half-wave potentials of fluorescein will probably be the easier to sort out.

Figure 11. Half-wave potentials for the reduction of fluorescein at the dropping mercury cathode as a function of pH

Concentration of fluorescein: 1.3×10^{-4} M


Potential - volts
-1.20
-1.10
-1.00
-0.90
-0.80
-0.70
-0.60
-0.50
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
pH

B. The Number of Electrons Involved in the Electrochemical Reduction of Calcein

Three methods are available for determining the stoichiometry of a reaction occurring at an electrode, that is, for determining the number of electrons involved in an electrode reaction. One method makes use of the height of a polarographic wave and the so-called Ilkovic equation. A knowledge of the diffusion coefficient of the reducible species is required; this coefficient is difficult to obtain, the direct measurement being difficult experimentally and approximation methods giving uncertain results, and involving questionable assumptions. The Ilkovic equation itself is based on a debatable assumption and the polarographic wave method is used to determine the number of electrons involved in a reaction only when coulometric methods cannot be employed.

The coulometric method is based on measuring the total electric charge (current multiplied by time) passed for a given amount of chemical changed at the electrode. The experiment may be performed by the "constant current method" or the "controlled electrode potential method". In the constant current method of studying a reducible material, a second reducible species must be present in large amount in the solution to undergo reduction at the cathode and subsequently circulate through the solution and react with

the reducible species being studied. A way of measuring the amount of the reducible species reduced must be available; commonly, the reduction is carried to completion (coulometric titration) and the end-point is marked by one of the common indicator methods used in titrimetric work.

In the controlled cathode potential method, the single electrode potential of the cathode is set at a value which will cause the reducible species to be reduced as it is brought into contact with the cathode but the cathode potential is not allowed to increase thus precluding the reduction of a second species, such as water. The current drops steadily to zero as the concentration of the reducible species drops. This varying current must be integrated as a function of time. The polarograph is ideally adapted to the controlled cathode potential method inasmuch as the cell and all of the electrical apparatus is part of the instrument. The polarograph also provides a convenient analytical method for measuring the amount of the reducible species reduced in a given time.

In the present work the controlled cathode potential method was used. The study reported above of the half-wave potentials of Calcein at various pH provided a knowledge of the necessary cathode potential at which the electrolysis had to be performed. A parallel experiment with cadmium chloride, two-electron reduction to the metal, was performed as a check on the operation.

1. Experimental work

The constant potential coulometric studies were made on the Princeton Applied Research Corporation Model 174A Polarographic Analyzer provided with a Hewlitt-Packard strip chart recorder. The instrument was checked for current output at full-scale deflection of the recorder and found to agree with the current settings on the dial. The strip chart recorder was calibrated for use as a timer by measuring the amount of chart paper used per unit of time. This check agreed with the settings listed on the recorder.

A simple H-cell was used for the coulometric studies. The reference electrode used was a saturated calomel electrode; the working electrode was a mercury pool; the counter electrode used was a platinum wire. A diagram of the cell is shown in Figure 12.

Buffers were prepared in the same manner as those used in the polarographic studies in Part A, above. Buffers of values of pH of 2.1, 4.0, and 6.6, were prepared from disodium hydrogen phosphate, citric acid, and potassium chloride at an ionic strength of 0.5.

The calibration of the entire coulometric system was accomplished by electrolyzing an aliquot of a cadmium chloride solution in a buffer solution at pH 2.1. The cadmium chloride solution was prepared by dissolving 4.0440 g of hydrated cadmium chloride in one liter of water to give a concentration of cadmium of 0.0177 M. An aliquot of 1.00 ml of the

Figure 12. H-cell for coulometry

A. Nitrogen inlet.

B. Platinum counter electrode

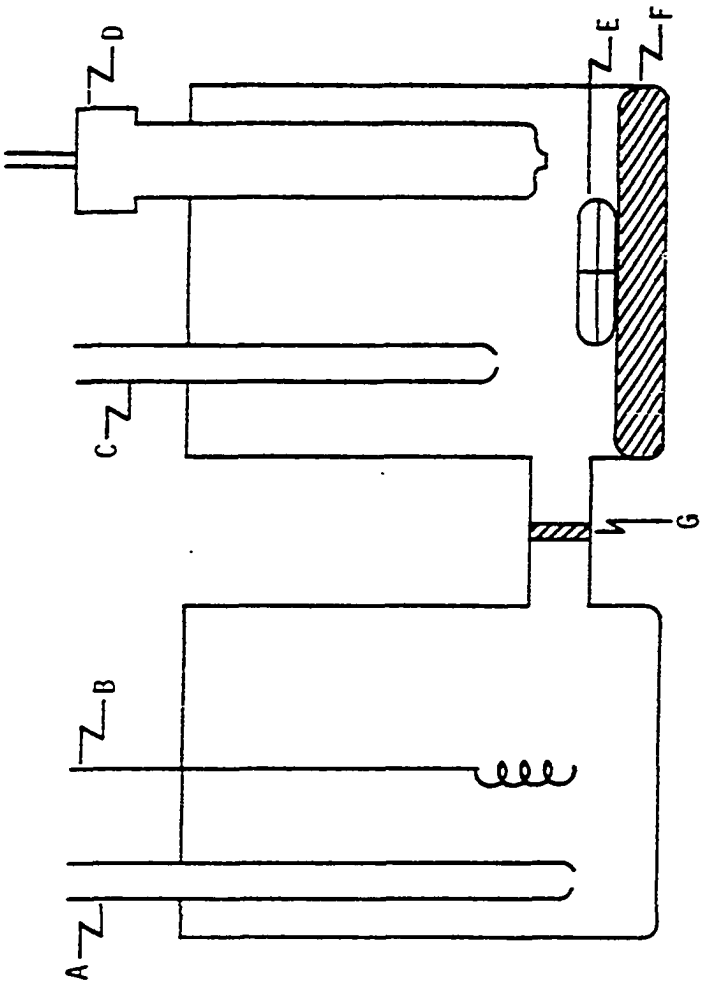
C. Nitrogen inlet

D. Saturated calomel reference electrode

E. Magnetic stirring bar

F. Mercury pool working electrode

G. Glass frit


D
E
F
C
G
B
A

cadmium solution was electrolyzed at a potential of -1.00 V. The solutions were deaerated with nitrogen gas and nitrogen gas was passed over the solution during the electrolysis.

After calibration of the coulometric system, aliquots of Calcein were electrolyzed in the same manner as the cadmium. The concentration of the solution of Calcein was 1.51×10^{-3} M. Aliquots of 1.00 ml were electrolyzed. The electrolyses were performed at pH 2.1, pH 4.0, and pH 6.6. The respective values of the half-wave potentials for Calcein at these values of pH are -0.42 V, -0.53 V, and -0.75 V. The potentials selected for electrolysis were selected so as to be at a potential where complete electrolysis of the Calcein in solution was expected. The potential was set at -1.00 V for the work done at pH 2.1 and pH 4.0, and set at -1.30 V for the work done at pH 6.6.

2. Results and discussion

The curves of current as a function of time showed an immediate increase in current upon an addition of an aliquot of the solution of cadmium. This current gradually decreased as the electrolysis occurred. The electrolysis was considered complete when the current reached a value equal to the baseline current. The baseline current was taken as that value of current shown on the recorder just before an aliquot of solution to be electrolyzed was added to the system.

Earlier calibration of the recorder for use as a timer and for measuring current enabled a determination of the total current passed during the electrolysis. The time of the electrolysis multiplied by the current gave a value for the total coulombs of electricity passed. The measurement was actually made by cutting out the portion of the curve on the strip chart recording and weighing the paper. This weight corresponded to the amount of electricity passed. From the total current and the weight of cadmium electrolyzed the size of the current was calculated using the Faraday laws of electrolysis.

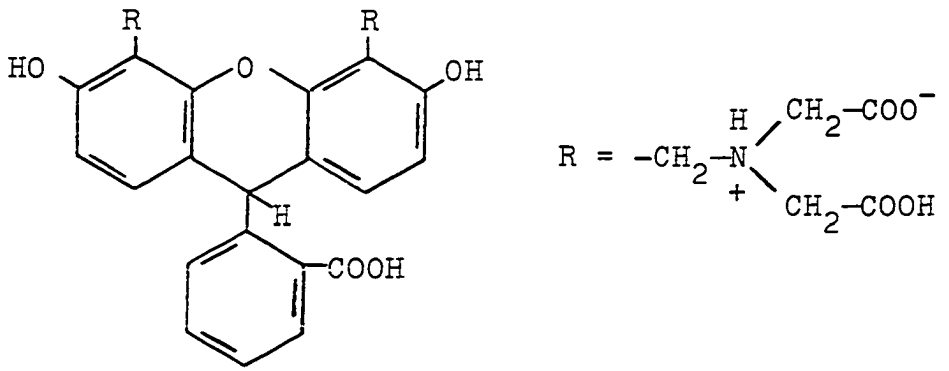
The values found for the number of electrons in the reduction of cadmium were 2.1, 2.1, 2.2, and 2.2, average 2.1.

The electrolysis of calcein was performed in the same manner as for cadmium. The number of electrons involved was also calculated in the same way. The average number of electrons involved in the reduction of Calcein at pH 2.2 was 1.9, at pH 4.0, 1.9, and at pH 6.6, 2.0. Thus, over the range of pH studied, the reduction of Calcein is a two-electron process.

At the beginning of the electrolysis the solutions were a bright yellow-green. As the electrolysis proceeded, the color and fluorescence decreased. At the completion of the electrolysis the solutions were a very pale green. To a

portion of the solution containing the reduced material was added calcium and sufficient potassium hydroxide to bring the pH to 13; under an ultraviolet lamp, the solution was non-fluorescent (13).

The reduction product of fluorescein is called fluorescin. Fluorescin is colorless and nonfluorescent. The coulometric reduction of Calcein indicated a reduction of Calcein to a form analogous to fluorescin. This reduction product is now designated as Calcin. The structure is undoubtedly:



C. The Polarography of Calcein in the Presence of Calcium

Calcein is primarily a fluorochromic indicator for the EDTA titration of metal ions and a reagent for the direct fluorometric determination of metals, chiefly so far for the metal calcium. Naturally, the alteration in the polarographic

behavior of Calcein on the addition of metal ions is of interest.

1. Experimental work

The polarographic instrument and cell used were those used for the differential pulse polarography study. The working electrode used was the dme with a drop time of 1 sec. The reference electrode used was a saturated calomel electrode; the counter electrode was a platinum wire.

A solution of Calcein was prepared by dissolving 0.0874 g of Calcein in one liter of water with a minimum of 1 M potassium hydroxide. The concentration of the Calcein was thus 1.33×10^{-4} M.

Standard solutions of calcium were prepared from primary standard calcium carbonate. Appropriate dilutions were made to obtain solutions of desired concentration.

A 2-ml aliquot of 1.33×10^{-4} M Calcein was pipetted into the polarographic cell and 12 ml of buffer of pH 13 added. The buffer was prepared from potassium hydroxide and potassium chloride. The solution was deaerated with nitrogen. Additions of 1.00×10^{-3} M calcium were made using a 50-µl Hamilton syringe. The solution was mixed after each addition by a gentle bubbling of nitrogen. A differential pulse polarogram was obtained after adding the Calcein and after each subsequent addition of calcium.

2. Results and discussion

The differential pulse polarogram of Calcein at pH 13 gave rise to two reduction peaks, the half-wave potentials being -1.24 V and -1.47 V. A slight shoulder was present on the peak at -1.24 V, the shoulder being at a potential of approximately -1.15 V.

Upon addition of calcium, the shoulder became a distinct peak at a potential of -1.15 V. Further additions of calcium caused an increase in the height of the peak with only a very slight shift of the half-wave potential in the negative direction. This peak continues to rise linearly until slightly less than one atom of calcium per mole of Calcein has been added, Figures 13 and 14. Addition of calcium beyond the ratio of one atom of calcium for every molecule of Calcein caused no change in the height of the peak. It is thus probable that the presence of the shoulder at -1.15 V in the polarographic wave of Calcein only is caused by the presence of trace amounts of calcium in the potassium hydroxide used to make up the buffer and that the compound undergoing reduction at -1.15 V is the monocalcium derivative of Calcein.

The peak at -1.24 V in the polarographic wave of Calcein alone at pH 13 is most probably caused by the reduction of the anion of Calcein, Cal^{-6}. This peak decreases, as expected, upon the addition of calcium, as the monocalcium

Figure 13. Electrochemical response of Calcein as a function of the concentration of calcium

Concentration of Calcein: 2.6×10^{-5} M

pH 13

Potential: -1.15 V

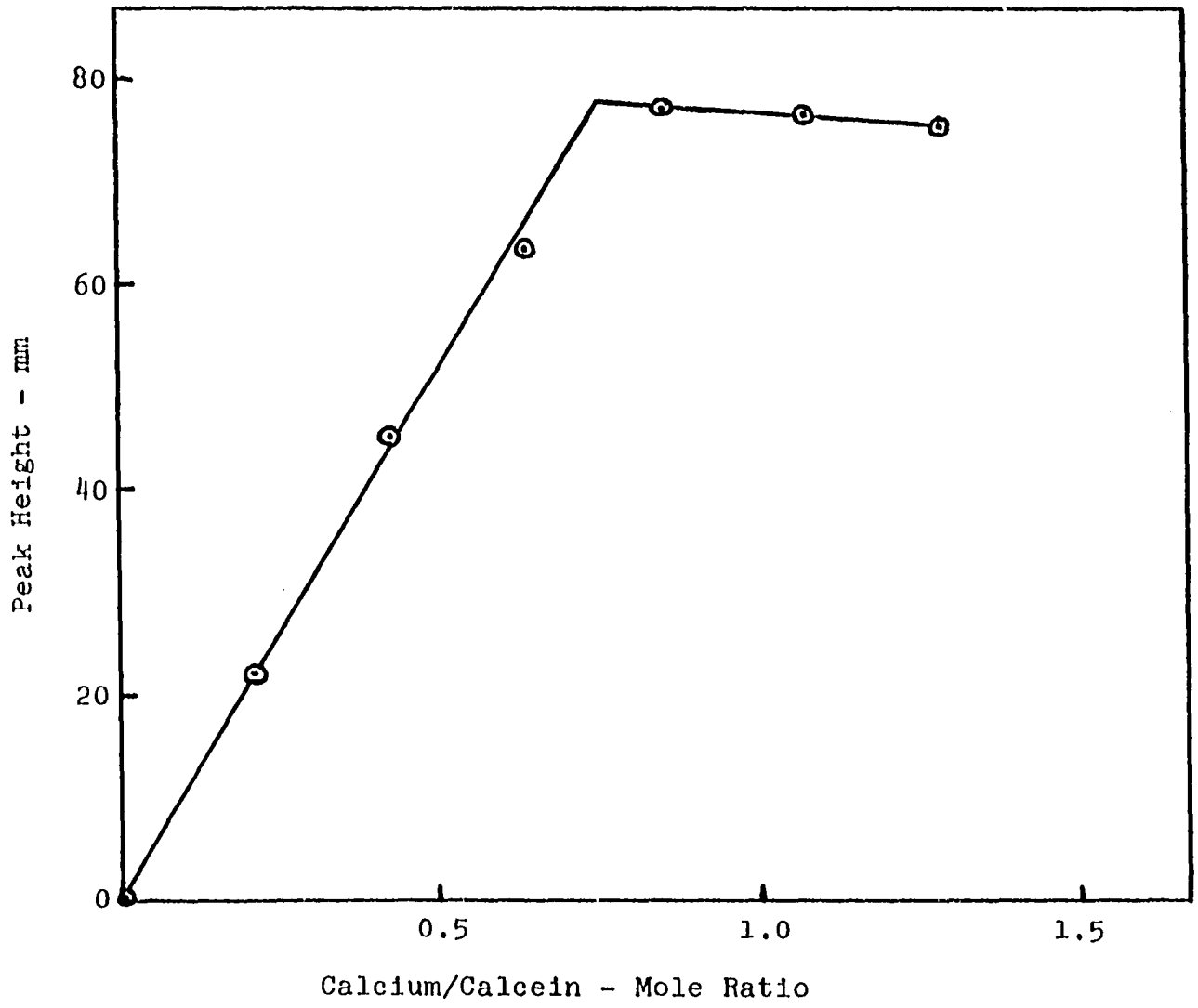
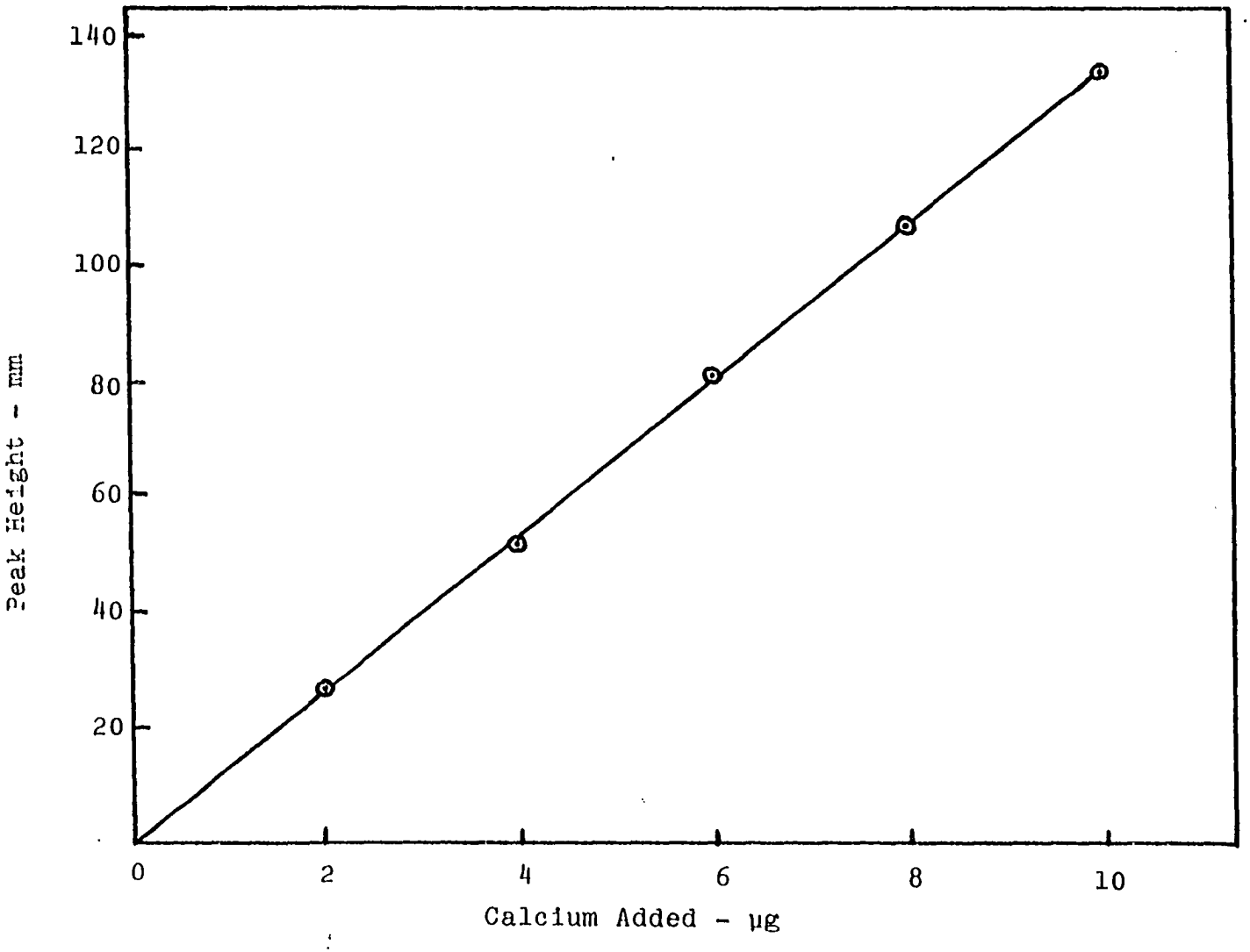

80
60
40
20
0
Peak Height - mm
0.5
1.0
1.5
Calcium/Calcein - Mole Ratio

Figure 14. Electrochemical response of Calcein as a function of the concentration of calcium

Concentration of Calcein: 2.2×10^{-5} M

pH 13

Potential: -1.15 V


140
120
100
80
60
40
20
Peak Height - mm
0
2
4
6
8
10
Calcium Added - µg

derivative of Calcein is formed and the amount of Cal^{-6} present in solution is reduced. The half-wave potential of the peak at -1.24 V is shifted slightly in the negative direction as calcium is added. As the amount of calcium added approaches one atom of calcium for every molecule of Calcein present, the height of the peak becomes constant and further additions of calcium after passing the end-point have no effect on the height.

Thus, the electrochemical response of Calcein to additions of calcium covers only the range in which the formation of the monocalcium derivative of Calcein occurs. Evidently the reduction of Calcein in the presence of calcium results in the formation of a species of Calcein in which one of the sites normally available for binding calcium is altered in such a way that binding calcium is no longer possible. This would explain the formation and reduction of only the monocalcium derivative of Calcein as observed in this work.

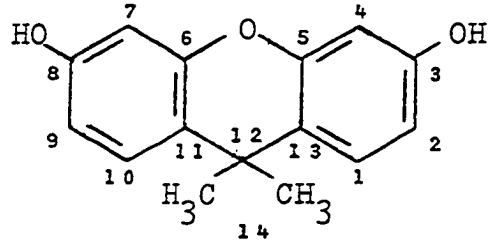
VII. CARBON-13 FOURIER TRANSFORM NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF FLUORESCEIN, CALCEIN, AND RELATED COMPOUNDS

A. Introduction

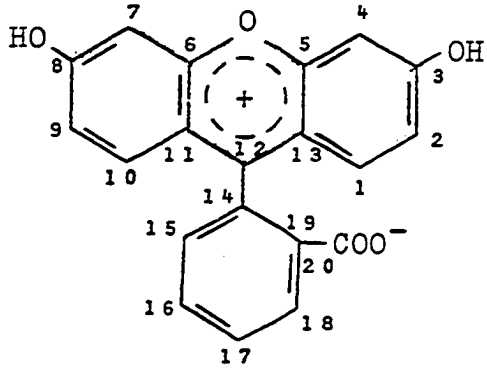
Carbon-13 Fourier transform nuclear magnetic resonance spectroscopy (C-13-FT-NMR) appeared to offer an independent method of establishing the structure of Calcein, in particular of establishing the positions in the molecule of the two substituent groups, the methyleneiminodiacetic acid groups. As in studies of the proton nuclear magnetic spectroscopy (NMR) of organic compounds, it was necessary to study at the same time other, closely related, compounds and to build up, so to speak, a library of spectra of compounds of the same basic structure.

For C-13-FT-NMR work, a new numbering scheme must be employed in describing organic molecules because all of the carbon atoms in the molecule come under study, not simply those bearing hydrogen atoms, those carbon atoms numbered in the conventional numbering schemes. The new numbering scheme for fluorescein, Calcein and related compounds is shown in Figure 15. Thus, the 4- and 5- positions in the conventional numbering system of fluorescein and its derivatives, the particular positions in the molecule of Calcein carrying the methyleneiminodiacetic acid groups according to Hefley

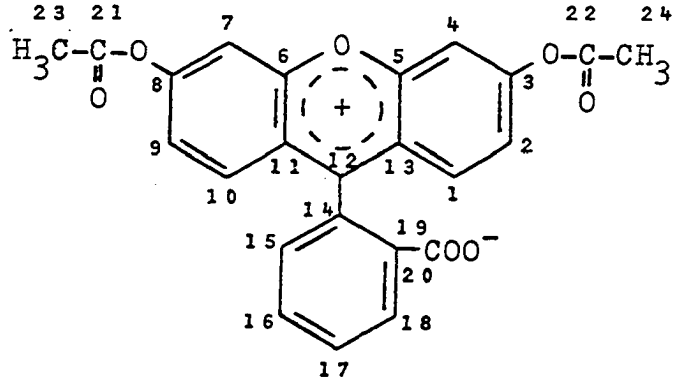
Figure 15. Structures and numbering scheme for carbon-13 Fourier transform nuclear magnetic work for fluorescein, Calcein and related compounds



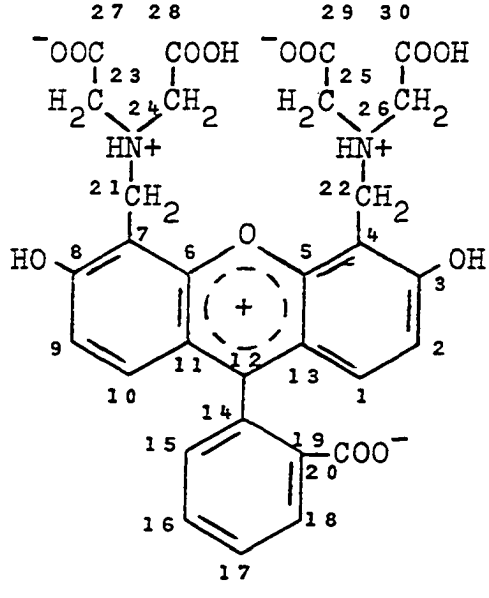
3,8-Dihydroxy-12,12-dimethylxanthene



Fluorescein



Diacetylfluorescein



Calcein

(36), become in the new numbering scheme the 4- and the 7-positions, respectively.

In order to observe nuclear magnetic resonance, a sample containing nuclear spins is placed in a static magnetic field and swept by an alternating field of radio-frequencies. When the applied radio-frequency matches the frequency at which a nucleus absorbs in the magnetic field, called the Larmor frequency, an absorption signal is recorded. In C-13-FT-NMR, a band of radio-frequencies which covers the entire range of Larmor frequencies of interest is applied as a short, intense pulse. The data from repetitive pulses is collected by the computer and converted to an absorption spectrum.

In atoms and molecules, a nucleus is shielded by electrons. When the nucleus under consideration is placed in the static magnetic field of an NMR spectrometer, the nucleus does not experience the applied magnetic field, but rather a field which is a combination of the applied field and an additional magnetic field induced by the shielding electrons. The strength of the induced field is related to the applied static magnetic field by a proportionality factor called the magnetic shielding constant. This constant characterizes the chemical environment of a particular nucleus and gives rise to what are called chemical shifts, that is to say, the peaks observed in an NMR spectrum vary in position owing to the effects of neighboring atoms and structures.

In order to measure chemical shifts, the absorption signal of a reference compound is assigned a chemical shift of zero. The chemical shifts of the chemically equivalent nuclei in the sample are then measured relative to the frequency of the absorption of the reference. Chemical shifts are proportional to the swept radio-frequency or the magnetic field strength. Therefore, chemical shifts obtained at different radio-frequencies, or field strengths, must be adjusted to the same radio-frequency (or field) before comparison. In order to obtain values of chemical shifts which are independent of the frequency (or field) used, the delta scale of chemical shifts is used. Values of delta (δ) are obtained by dividing the difference in the frequency between the absorption of the reference and the absorption of the nuclei in the sample by the frequency used. In C-13 NMR, the frequency used is 22.63 MHz (22.63×10^6 Hz). The equation for calculating values of δ is given by

$$\delta = \frac{\nu_S - \nu_R}{\nu_{App}} \times 10^{-6} ,$$

in which ν_S is the frequenc at which the nuclei in the sample absorb, ν_R is the frequency at which the reference absorbs, and ν_{App} is the radio-frequency applied.

The value of $\nu_S - \nu_R$ is usually on the order of several Hz, which is small compared to ν_{App}, which is on the order

of MHz. Thus, the values of δ are usually expressed as $\delta \times 10^6$ (ppm).

Tetramethylsilane (TMS) is a common compound used as a reference for calibrating C-13 spectra. The signal for the four equivalent methyl groups of TMS appears at very high field strength compared to the signals of other carbon atoms in organic molecules. Signals with a small value of delta relative to TMS are said to appear at high field and the corresponding nuclei are considered strongly shielded. Signals with values of delta that are large relative to TMS are said to be at low field and the corresponding nuclei are said to be weakly shielded or deshielded.

The chemical shifts which occur in C-13-FT-NMR are much larger than those occurring in proton NMR spectra. Carbon atoms which differ from each other only very slightly in chemical environment may quite often be distinguished from each other. This becomes of particular value in dealing with complicated molecules such as fluorescein and Calcein.

In general, in a C-13-FT-NMR spectrum, the number of peaks observed will equal the number of chemically non-equivalent carbon atoms in the molecule and the intensities of the signals can be used qualitatively as being proportional to the number of carbon atoms in a particular environment. A spectrum such as this is obtained under conditions that prevent any coupling of the carbon atoms and protons.

Decoupling of all protons from the carbon atoms is achieved by applying a strong, secondary, radio-frequency which covers the range of frequencies at which the protons absorb. This type of spectrum is referred to as a "proton broad band decoupled spectrum" in the literature. In the present work the term "spectrum" will be used instead.

More information can be obtained by allowing partial coupling of the carbon atoms and protons. This is achieved by applying the strong decoupling frequency discussed above as a pulse in between the pulse of the radio-frequency used to achieve resonance of the carbon atoms. This type of experiment gives rise to a so-called "gated decoupled spectrum". The spectrum which is obtained no longer consists of n peaks for n nonequivalent carbon atoms. A peak in the carbon-13 spectrum may give rise to a number of peaks in the gated decoupled spectrum. The number of peaks which appear in the gated decoupled spectrum is dependent on the number of protons attached to the carbon atom. This number can be predicted by the multiplicity rule:

$$2mI_x + 1 = \text{multiplicity},$$

in which m is the number of protons attached to the carbon atom and I_x is the spin quantum number for the proton (1/2). Thus, a singlet appears in the gated decoupled spectrum for a carbon atom which has no protons attached, a doublet for

carbon atoms with one proton attached, and so on. Both of the techniques described above were employed in the present work.

A detailed treatment of the theory, and in particular carbon-13-FT-NMR, can be found in the work by Breitmaier and Voelter (12).

During the course of the work, the initial goal was expanded in an attempt to identify as many of the peaks which appeared in the spectrum of Calcein as possible. The spectra of several similar xanthene derivatives were obtained. There was thus laid a framework for future workers in the field of xanthene chemistry. Taken together with data already in the literature, it was possible to assign each peak in the spectrum of Calcein and fluorescein to specific carbon atoms.

B. Experimental Work

1. Apparatus

Carbon-13 Fourier transform nuclear magnetic resonance spectra were determined at 22.63 MHz using a Bruker HX-90 Spectrometer equipped with a Nicolet 1080 Data System. The pulse width was 3.5 μsec with a repetition time of 2.000 sec. The limit of resolution was 0.94 Hz per data point. All initial carbon-13 spectra were obtained under such conditions that coupling of carbon atoms and protons did not occur. To obtain information about the number of protons attached to each carbon, a gated decoupling facility was used.

2. Materials

a. Calcein The Calcein used was synthesized and purified by a method described in my master of science thesis (58). The material contained less than 0.5 per cent fluorescein. The purity as determined by titration was 97.5 per cent.

b. Fluorescein The fluorescein was prepared by hydrolyzing diacetylfluorescein by the method of Dolinsky and Jones (26). The diacetylfluorescein was refluxed in ethanol with the appropriate amount of sodium hydroxide. The mixture was filtered, placed in a 4-liter beaker surrounded by ice, and 2.5 liters of glacial acetic acid were added to bring the solution to pH 4. The product was filtered and washed with deionized water. The product was then dissolved with sodium hydroxide and reprecipitated with glacial acetic acid. The yellow fluorescein was repeatedly filtered and washed with deionized water. The fluorescein was then dried for 30 hours at 60° in a vacuum oven. The equivalent weight as determined by titration was 166.3 g per equivalent. The theoretical equivalent weight based on two replaceable hydrogen atoms is 166.2 g per equivalent.

c. Diacetylfluorescein The material used was prepared by Mr. Richard C. Miller. The compound was crystalline white. It was used as received.

d. 3,8-Dihydroxy-12,12-dimethylxanthene This compound was prepared by Dr. Richard Markuszewski following the procedure of Hanousek (32,33), which involves the condensation of resorcinol and acetone in the presence of anhydrous zinc. The material was purified by Dr. Markuszewski by making the diacetyl derivative, recrystallizing, and then hydrolyzing in alcoholic sodium hydroxide and precipitating by the addition of dilute hydrochloric acid. The material received from Dr. Markuszewski was beige in color. The compound was reprecipitated once from hot ethanol and then air-dried. The product was ivory in color.

e. Hexadeuterated dimethylsulfoxide Hexadeuterated dimethylsulfoxide, 99.5 per cent deuterium, was obtained from Stohler Isotope Chemical Company.

f. 1,4-Dioxane 1,4-Dioxane was obtained from the Fisher Chemical Company and stored over molecular sieves.

C. Results and Discussion

The carbon-13-FT-NMR spectra obtained and the data for each are presented in Figures 16-22. All chemical shifts are reported with tetramethylsilane as the reference. In addition to the spectra obtained in this work, use was made of the spectra already reported by others of compounds containing carbon atoms having chemical environments similar to those found about the carbon atoms in xanthene compounds.

The assignments made for each of the peaks in the spectra are summarized in Table 2. The assignments considered tentative are marked with an asterisk. Assignments considered interchangeable with each other are marked with a cross.

The arguments for the assignments of the peaks in the spectra presented in this work are rather complex. The discussion will be broken down into a discussion of the assignments made in each of the spectra. The spectrum of 3,8-dihydroxy-12,12-dimethylxanthene will be discussed first, followed by a discussion of the spectra obtained for fluorescein, Calcein and diacetylfluorescein. In the following discussions, the term "spectrum" will be used to refer to the spectra obtained under conditions that prevent any coupling of protons and carbon atoms, or the so-called "proton broad band decoupled spectra".

The principal findings are that Calcein is a symmetrical molecule, that the two methyleneiminodiacetic acid groups occupy equivalent positions in the molecule, and more specifically that they occupy the 4- and 7- positions. It became possible to make this assertion because it was possible to assign all of the peaks of the carbon atoms present in the xanthene portion of the fluorescein molecule and then to account for the difference in the spectra of fluorescein and Calcein.

Table 2. Assignment of peaks in the carbon-13-FT-NMR spectra of 3,8-dihydroxy-12,12-dimethylxanthene, fluorescein, Calcein, and diacetylfluorescein (See Figure 15 for numbering of carbon atoms)

Position	Ppm from TMS[a] 3,8-Dihydroxy-12,12-dimethylxanthene	Fluorescein	Calcein	Diacetyl-fluorescein
C_1, C_{10}	127.2d	*129.0d	*127.6d	*128.7
C_2, C_9	110.8d	112.7d	112.6d	*117.8
C_3, C_8	156.3s	159.5s	159.0s	151.2
C_4, C_7	102.0d	102.5d	109.3s	110.1
C_5, C_6	150.1s	152.0s	149.9s	152.1
C_{11}, C_{13}	120.5s	109.8s	109.7s	*116.7
C_{12}	32.3s	83.3s	83.8s	80.8
C_{14}	32.8m	152.5s	152.3s	153.1
C_{15}	---	†124.6d	†124.6d	†124.7

[a]Tetramethylsilane used as a reference.

s = singlet m = multiplet
d = doublet * = tentative assignment
† = interchangeable

Table 2. (Continued)

Position	3,8-Dihydroxy-12,12-dimethylxanthene	Fluorescein	Calcein	Diacetyl-fluorescein
		Ppm from TMS		
C_{16}	---	135.3d	135.4d	135.1
C_{17}	---	†123.9d	†124.1d	†123.8
C_{18}	---	†129.9d	†130.0d	†129.7
C_{19}	---	126.3s	126.1s	126.0
C_{20}	---	168.7s	168.6s	168.1
C_{21}, C_{22}	---	---	46.9m	---
C_{23}, C_{24}, C_{25}, C_{26}	---	---	54.1m	---
C_{27}, C_{28}, C_{29}, C_{30}	---	---	172.6s	---

1. The spectrum of 3,8-dihydroxy-12,12-dimethylxanthene

The spectrum of 3,8-dihydroxy-12,12-dimethylxanthene (Figure 16) was obtained in hexadeuterated dimethylsulfoxide (DMSO-d_6). Eight distinct peaks are present. There should be present in the spectrum eight peaks, excluding the peaks caused by the solvent, because eight chemically nonequivalent carbon atoms are present in the molecule. The "gated decoupled spectrum" of 3,8-dihydroxy-12,12-dimethylxanthene is presented in Figure 17.

a. Assignments of peaks to the carbon atoms in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene The reader may refer to Figure 15 at this point for the numbering system employed in the following discussions. When two or more positions in the molecule are equivalent, only one of the positions will be referred to in order to simplify the discussion. The equivalent carbon atoms in all of the compounds are listed in the first column of Table 2.

The peak at 156.3 ppm in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene, Figure 16, has been assigned to the carbon atom attached to the hydroxy group, C_3. The peak at 150.1 ppm has been assigned to C_5, the carbon atom next to the ether oxygen.

The peaks at 127.2 ppm, 110.8 ppm, and 102.0 ppm have been assigned to C_1, C_2 and C_4, respectively. The peak at 120.5 ppm has been assigned to C_{13}.

Figure 16. Carbon-13-FT-NMR spectrum of 3,8-dihydroxy-12,12-dimethylxanthene

Peak	Position of Peak		Relative Intensity	Assignment[b]
	Frequency (Hz)	Ppm[a]		
1	3537.7	156.3	4.34	C_3, C_8
2	3396.8	150.1	4.46	C_5, C_6
3	2878.5	127.2	3.29	C_1, C_{10}
4	2727.6	120.5	3.45	C_{11}, C_{13}
5	2509.0	110.8	2.69	C_2, C_9
6	2308.1	102.0	3.35	C_4, C_7
7	743.4	32.8	2.36	C_{14}
8	731.2	32.3	2.14	C_{12}

[a]Tetramethylsilane used as a reference.

[b]See Section C, Subsection 1b.

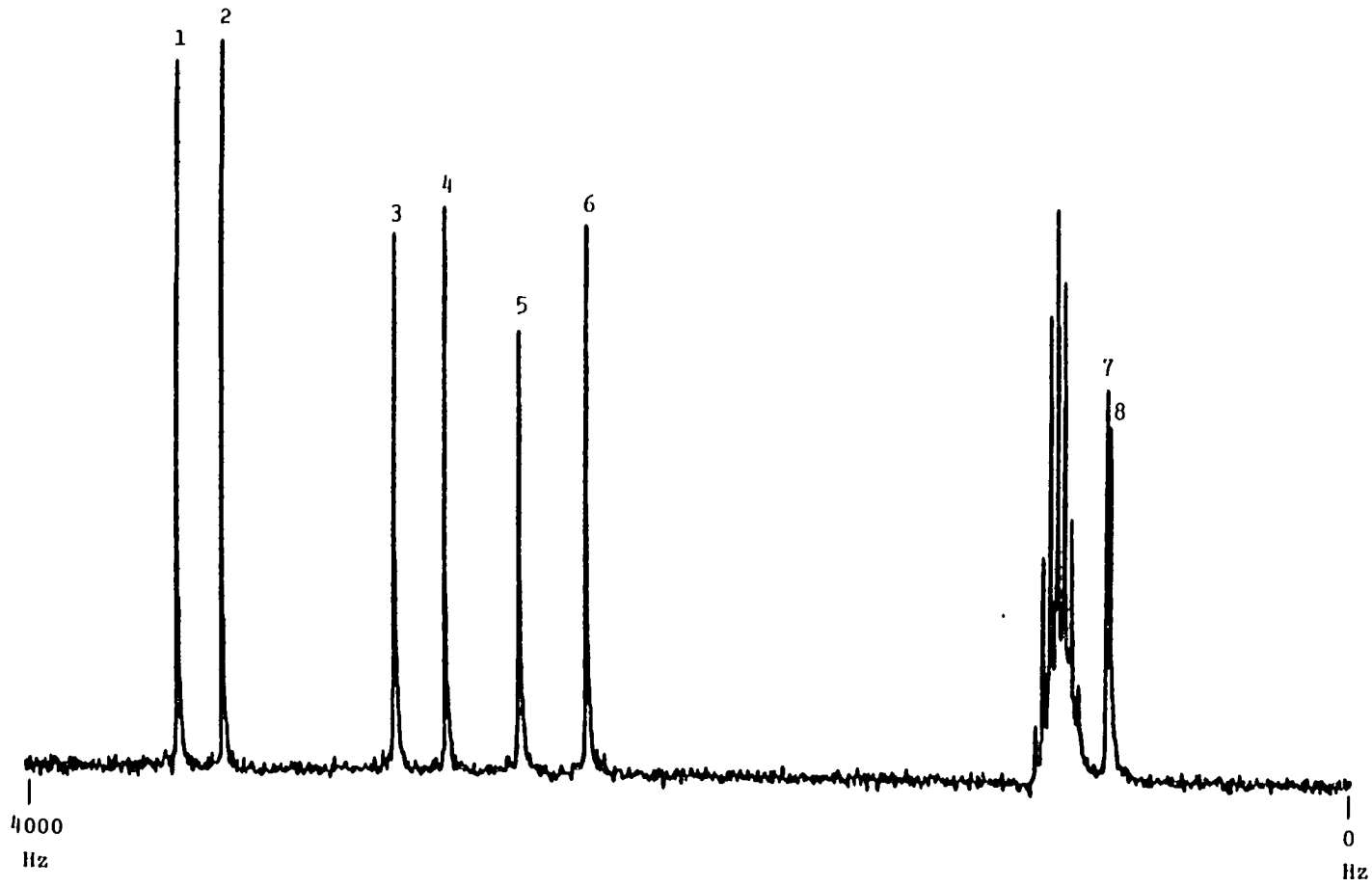

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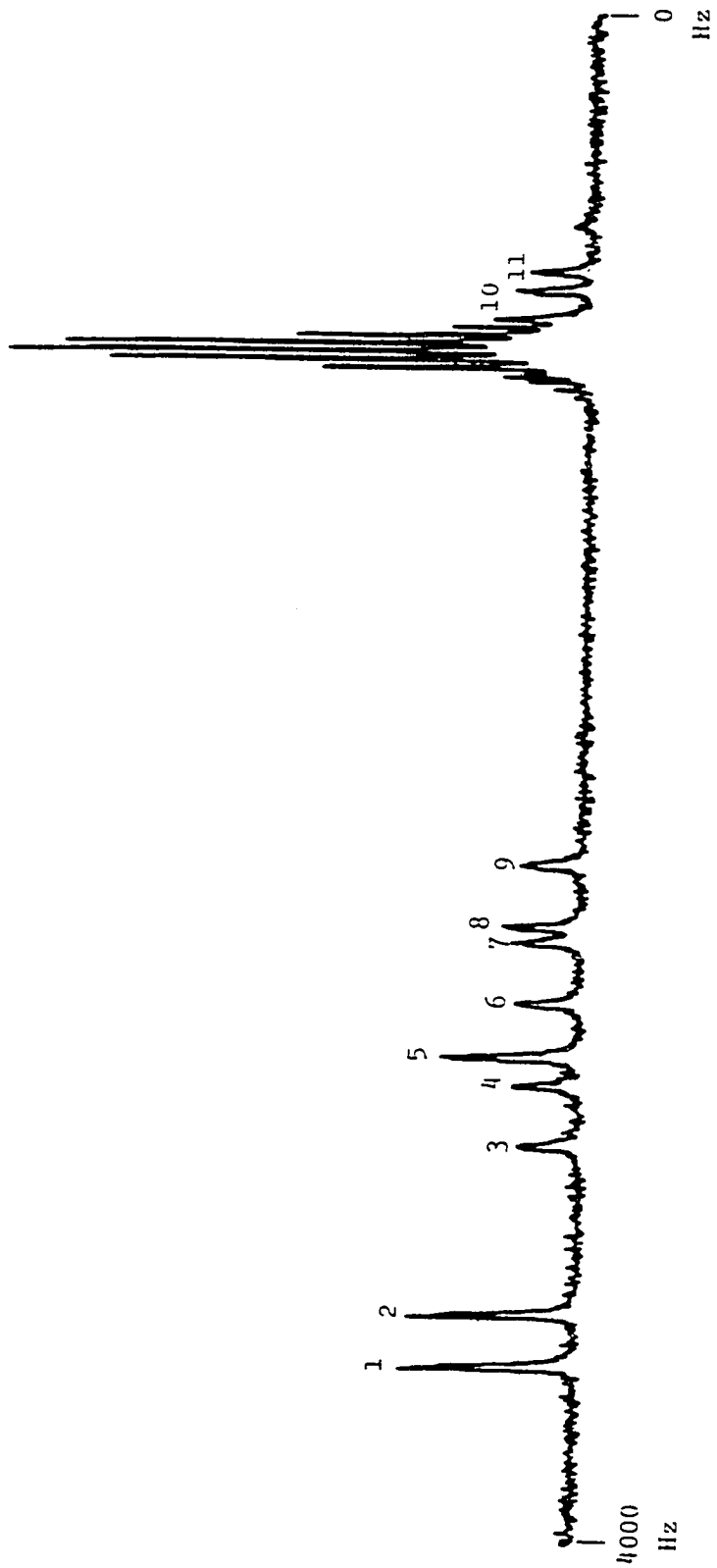
Figure 17. Gated decoupled carbon-13-FT-NMR spectrum of 3,8-dihydroxy-12,12-dimethylxanthene

Peak	Position of Peak		Intensity
	Frequency (Hz)	Ppm[a]	
1	3538.8	156.3	1.83
2	3397.9	150.1	1.73
3	2954.0	130.5	0.59
4	2797.5	123.6	0.66
5	2723.2	120.3	1.42
6	2584.5	114.2	0.65
7	2425.8	107.2	0.70
8	2382.5	105.3	0.81
9	2226.0	98.3	0.62
10	729.0	32.2	0.76
11	679.1	30.0	0.61

[a]Tetramethylsilane used as a reference.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The peak at 32.3 ppm has been assigned to C_{12}, while the peak at 32.8 ppm has been assigned to the methyl groups, C_{14}.

b. Justification for assignments of peaks to carbon atoms in the 3,8-dihydroxy-12,12-dimethylxanthene molecule

The carbon atoms labeled C_3 and C_5 were expected to give rise to the two peaks farthest downfield because these carbon atoms are the most deshielded atoms in the molecule. The peaks at 156.3 ppm and 150.1 ppm appeared as singlets in the "gated decoupled spectrum", as expected, because neither C_3 nor C_5 has any protons attached.

The assignments of the peak at 156.3 ppm to C_3 and the peak at 150.1 ppm to C_5 were based in part on comparison to coumarin and the hydroxycoumarins as given by Cussans and Huckerby (15). The carbon-13 spectrum of 7-hydroxycoumarin is of particular interest in that the peak at 156.4 ppm is assigned to the carbon atom next to the ether oxygen, while the peak at 162.4 ppm is assigned to the carbon atom attached to the hydroxy group.

It is necessary at this time to use a piece of evidence for the above assignments which appears in the discussion of the spectra of fluorescein and diacetylfluorescein later in this work. The peak at 159.5 ppm in the spectrum of fluorescein, Figure 18, appears as a singlet in the "gated decoupled spectrum", Figure 19, and is shifted to 151.2 ppm in the spectrum of diacetylfluorescein, Figure 22. Terui, Tori,

and Tsuji (76) have reported an upfield shift for a carbon atom of this type in the comparison of phenyl acetate with phenol. Because the only other peak farther downfield in the spectrum of fluorescein is due to the phthalate carboxyl group, the peak of the ether carbon atom must occur upfield from the peak of the carbon atom with the hydroxy group attached. Thus, the peak farthest downfield in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene has also been assigned to the carbon atom attached to the hydroxy group.

The peak at 32.3 ppm in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene has been assigned to C_{12}. This peak appears as a singlet in the "gated decoupled spectrum". Support for this assignment was found in the literature. Proulx and Smith (65) reported the peak of the alpha carbon atom of 1-phenylethane at 29.3 ppm and Miyajima and Nishimoto (59) found the peak of the carbon atom alpha to the phenyl group of 2-phenylisopropane at 33.8 ppm.

The peak at 120.5 ppm, the last peak that appears as a singlet in the "gated decoupled spectrum", has been assigned to C_{13} by elimination of the other singlets as possibilities.

The three peaks at 127.2 ppm, 110.8 ppm and 102.0 ppm have been assigned to C_1, C_2 and C_4, respectively. All three peaks appear as doublets in the "gated decoupled spectrum" as expected for carbon atoms with one proton attached. The argument for the assignments is based on the comparison to

the spectra reported for benzene (11), diphenyl ether (46), and phenol (46).

All of the carbon atoms in benzene absorb at 127.5 ppm. The introduction of a hydroxy group onto the benzene ring gives rise to a spectrum containing four peaks located at 154.9 ppm, 129.7 ppm, 121.0 ppm, and 115.4 ppm. The peak at 154.9 ppm is assigned to the carbon atom attached to the hydroxy group. The peak at 129.7 ppm is assigned to the carbon atom meta to the hydroxy group; the peak at 121.0 ppm is assigned to the carbon atom para to the hydroxy group; the peak at 115.4 ppm is assigned to the carbon atom ortho to the hydroxy group.

The carbon atom ortho to the hydroxy group thus gives rise to a peak which is at a quite different spot in the spectrum of phenol when compared with the spectrum of benzene. The carbon atom meta to the hydroxy group absorbs at a position quite close to the absorbance noted for benzene and thus is only slightly affected by the introduction of the hydroxy group. The carbon atom para to the hydroxy group is affected less than the ortho carbon atom but more than the meta carbon atom. This same trend is noted with the carbon atoms ortho, para, and meta to the carbon atom attached to the ether oxygen in diphenyl ether. The presence of both an ether oxygen and a hydroxy group in a structure such as 3,8-dihydroxy-12,12-dimethylxanthene should give rise to an additive effect of the above trend.

The carbon atom labeled C_1 in 3,8-dihydroxy-12,12-dimethylxanthene is meta to both the hydroxy group and the ether carbon atom of the xanthene skeleton. This carbon atom should be the least affected in analogy to benzene and thus the 127.2 ppm peak is assigned to it. The C_4-carbon atom is ortho to both the ether linkage and the carbon atom carrying the hydroxy group. The expected shift in analogy to the carbon atoms of benzene should be the largest of the three carbon atoms under discussion. Therefore, the 102.0 ppm peak has been assigned to C_4. The remaining peak, at 110.8 ppm, is intermediate in terms of chemical shift and has been assigned to C_2; C_2 is ortho to the carbon atom carrying the hydroxy group and para to the ether carbon atom, and thus should produce a peak at an intermediate point.

The only remaining peak to be discussed in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene, is the peak at 32.8 ppm. This peak has been assigned to the methyl groups, C_{14}, by elimination of all other possibilities. This peak appears as a multiplet of undetermined order in the "gated decoupled spectrum" because of the presence of peaks of the solvent in the same region.

2. The spectrum of fluorescein

The spectrum of fluorescein was obtained in DMSO-d_6. Fourteen distinct peaks are present in the spectrum. Thus, there are fourteen chemically nonequivalent carbon atoms

present in the fluorescein molecule. The carbon-13 spectrum is presented in Figure 18.

Seven carbon atoms are present in the fluorescein molecule which have no protons attached and seven carbon atoms which have one proton attached. Seven singlets and seven doublets were determined to be present in the gated decoupled spectrum, Figure 19.

a. Assignments of peaks to the carbon atoms in the spectrum of fluorescein

i) Assignments of peaks to the carbon atoms in the xanthene portion of the fluorescein molecule The peaks at 159.5 ppm and 152.0 ppm have been assigned to the carbon atom attached to the hydroxy group, C_3, and the carbon atom attached to the ether oxygen, C_5, respectively.

The peaks observed at 129.0 ppm, 112.7 ppm, and 102.5 ppm have been assigned to carbon atoms C_1, C_2, and C_4, respectively.

The peak at 83.3 ppm has been assigned to C_{12} and the peak at 109.8 ppm to C_{13}.

ii) Assignments of peaks to the carbon atoms in the phthalate ring portion of the fluorescein molecule The peak at 168.7 ppm has been assigned to C_{20}, which is the carbon atom of the phthalate carboxyl group. The peak at 126.3 ppm has been assigned to C_{19}, which is the carbon atom attached to the carboxyl group. The peak at 135.3 ppm has

Figure 18. Carbon-13-FT-NMR spectrum of fluorescein

Peak	Position of Peak: Frequency (Hz)	Position of Peak: Ppm[a]	Relative Intensity	Assignment[b]
1	3819.6	168.7	1.52	C_{20}
2	3610.9	159.5	2.53	C_3, C_8
3	3451.4	152.5	1.16	C_{14}
4	3441.6	152.0	3.15	C_5, C_6
5	3063.5	135.3	0.42	C_{16}
6	2939.5	129.9	0.43	C_{18}
7	2920.8	129.0	0.86	C_1, C_{10}
8	2859.8	126.3	1.29	C_{19}
9	2820.4	124.6	0.45	C_{15}
10	2805.6	123.9	0.46	C_{17}
11	2550.6	112.7	0.66	C_2, C_9
12	2484.7	109.8	2.63	C_{11}, C_{13}
13	2320.3	102.5	0.82	C_4, C_7
14	1885.2	83.3	0.84	C_{12}

[a] Tetramethylsilane used as a reference.

[b] See Section C, subsection 2b.

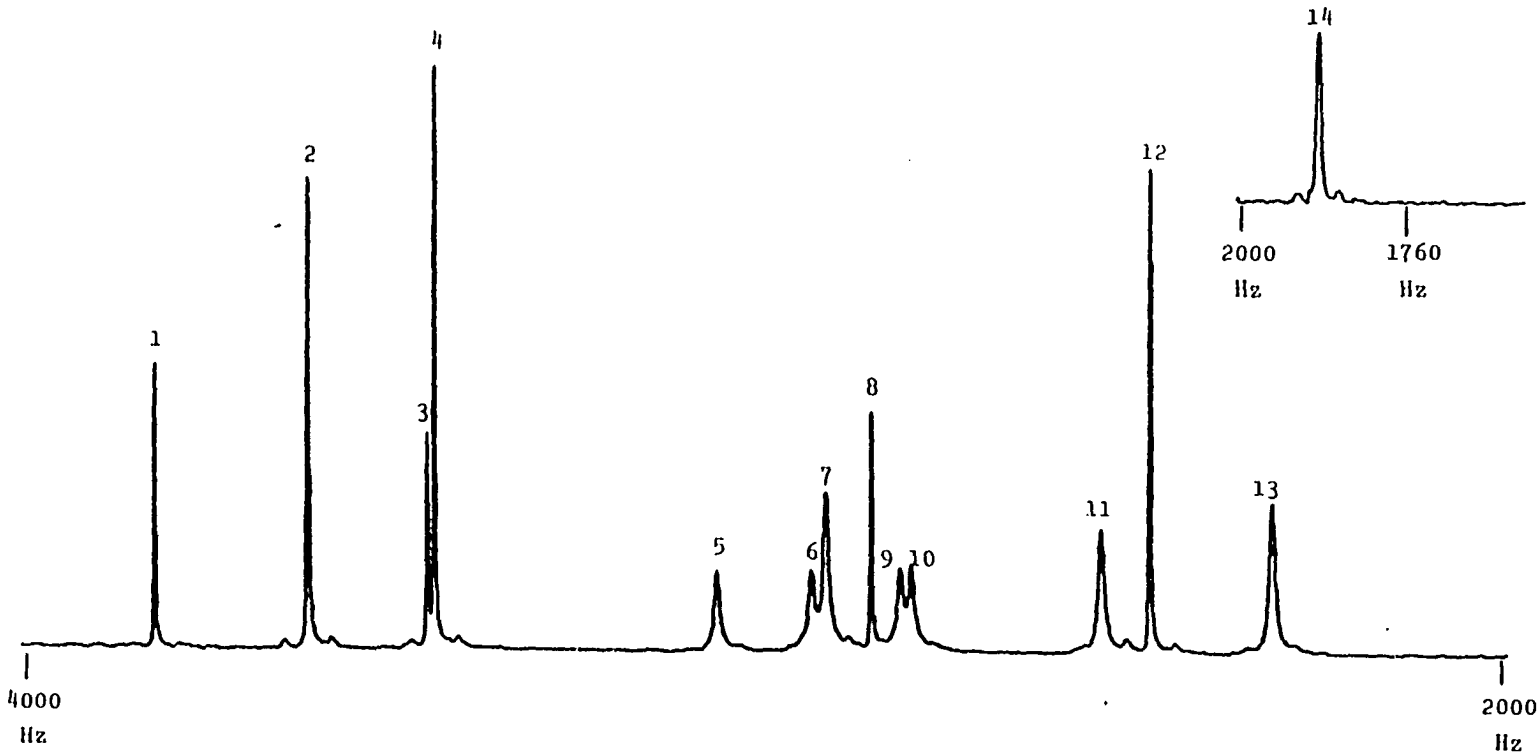
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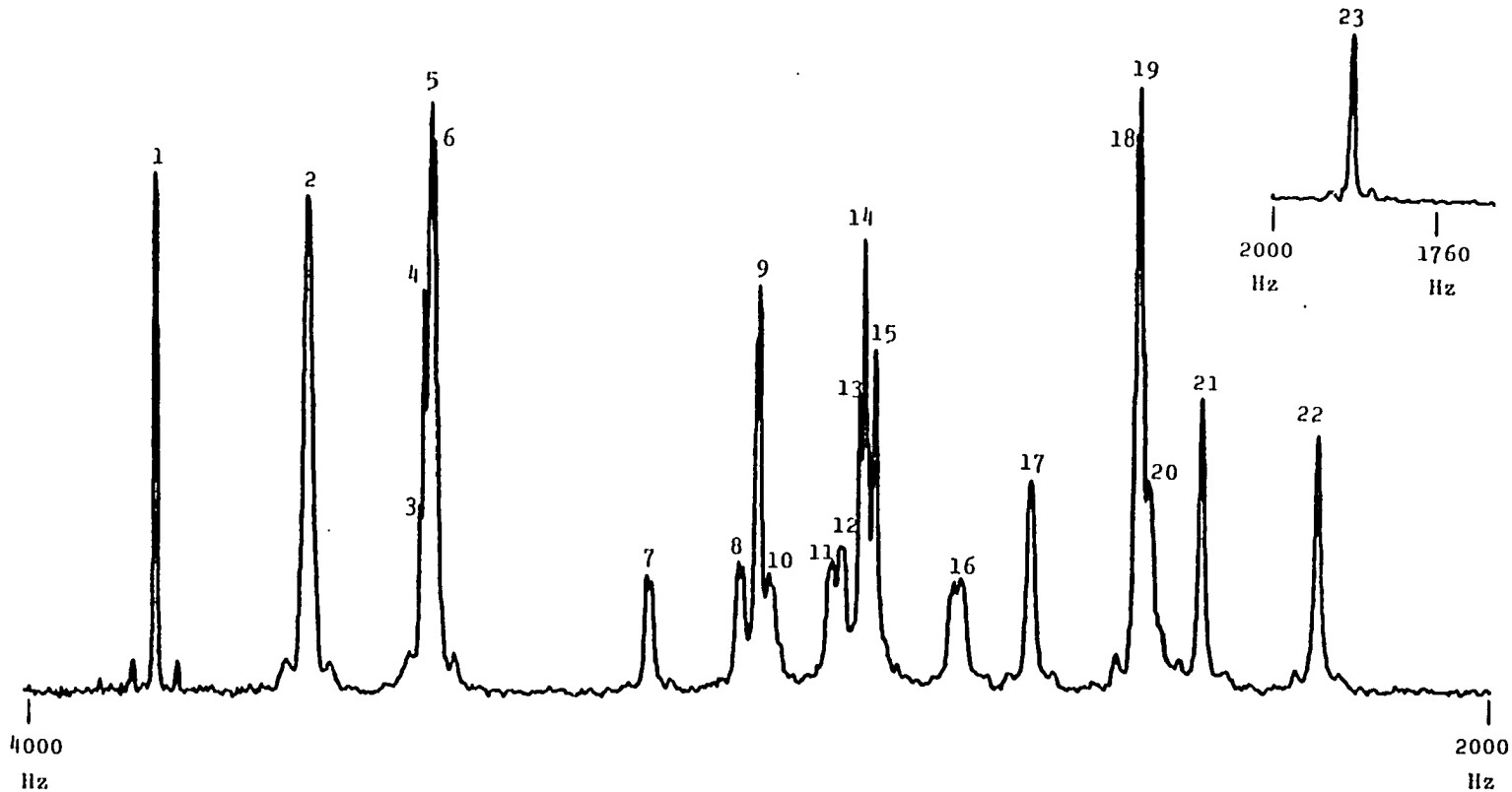
Figure 19. Gated decoupled carbon-13-FT-NMR spectrum of fluorescein

Peak	Position of Peak		Relative Intensity
	Frequency (Hz)	Ppm[a]	
1	3821.6	168.8	3.06
2	3609.9	159.5	2.95
3	3457.3	152.7	1.07
4	3452.4	152.5	2.36
5	3442.5	152.1	3.51
6	3438.6	151.9	3.29
7	3150.2	139.2	0.66
8	3026.1	133.7	0.75
9	3000.5	132.6	2.42
10	2987.7	132.0	0.68
11	2901.1	128.2	0.75
12	2890.3	127.7	0.84
13	2864.7	126.6	1.76
14	2857.8	126.2	2.71
15	2842.0	125.6	2.03
16	2724.9	120.4	0.66
17	2631.4	116.2	1.26
18	2484.7	109.8	3.38
19	2481.7	109.6	3.65
20	2471.9	109.2	1.27
21	2398.1	105.9	1.77
22	2237.6	98.9	1.56
23	1885.2	83.3	1.10

[a]Tetramethylsilane used as a reference.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been assigned to the carbon atom para to the carboxyl group, C_{16}.

The peak at 152.5 ppm has been assigned to C_{14}. This is the carbon atom to which the xanthene portion of the fluorescein molecule is attached.

The peaks at 124.6 ppm, 123.9 ppm, and 129.9 ppm have been assigned to C_{15}, C_{17}, and C_{18}. These assignments are interchangeable because enough data was not available to assign these three peaks to the specific carbon atom giving rise to the peaks.

b. Justification for assignments of peaks to the carbon atoms in the spectrum of fluorescein

i) Justification for assignments of peaks to the carbon atoms in the xanthene portion of the fluorescein molecule The peaks at 159.5 ppm and 152.0 ppm have been assigned to C_3 and C_5. These peaks appear as singlets in the "gated decoupled spectrum". This assignment was made by comparison with the spectrum of 7-hydroxycoumarin as reported by Cussans and Huckerby (15), and by comparison of the spectra of fluorescein (Figure 18) and diacetylfluorescein (Figure 19). The argument is analogous to that presented in Section C, subsection 1b, for the assignments of peaks to the carbon atoms in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene.

The spectra of fluorescein and diacetylfluorescein are very similar. The major difference between the two spectra

is in the pattern of peaks in the region of 150 and 160 ppm. The peak at 159.5 ppm in the spectrum of fluorescein appears at 151.2 ppm in the spectrum of diacetylfluorescein. This type of shift is expected when the substituent at a carbon atom in an aromatic system is changed from a hydroxy group to an acetyl group. As mentioned earlier, Terui, Tori, and Tsuji (76) have reported an upfield shift for a carbon atom of this type in the comparison of phenol with phenyl acetate. As will be discussed shortly, the only other peak farther downfield in the spectrum of fluorescein is due to the phthalate carboxyl group. Thus, the peak due to the ether carbon atom must occur upfield from the peak of the carbon atom with the hydroxy group attached. Therefore, the peak at 159.5 ppm in the spectrum of fluorescein is assigned to C_3 and the peak at 152.0 ppm is assigned to C_5.

It should be noted at this point that the peaks in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene assigned to C_3 and C_5 were located at 156.3 ppm and 150.1 ppm, and that the relative intensities of these peaks correlate well with the relative intensities of the peaks in the spectrum of fluorescein at 159.5 ppm and 152.0 ppm. This rules out any consideration of assigning the low intensity peak at 152.5 ppm in the spectrum of fluorescein to C_3 or C_5.

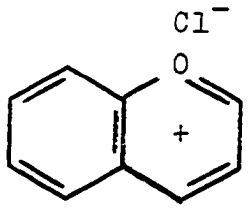
The peaks observed at 129.0 ppm, 112.7 ppm, and 102.5 ppm in the spectrum of fluorescein have been assigned to carbon

atoms C_1, C_2, and C_4, respectively. The assignment of the 129.0 ppm peak to C_1 is tentative because other peaks are present in the same region and make the assignment difficult. These three peaks were doublets in the gated decoupled spectrum. The corresponding peaks in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene were observed at 127.2 ppm, 110.8 ppm, and 102.0 ppm. Given the structural differences introduced by the phthalate ring, the correlation is good. Thus, the same argument used to assign the three peaks in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene to C_1, C_2, and C_4, discussed in Section C, subsection 1b, was used to assign the peaks in the spectrum of fluorescein to C_1, C_2, and C_4. The reader should refer to the above section for the details.

The peak at 83.3 ppm, a singlet in the "gated decoupled spectrum", has been assigned to C_{12}. The position of this peak is radically different from the peak at 32.3 ppm assigned to C_{12} in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene. This assignment is of interest because of the unique character of the chemical environment about C_{12}.

Markuszewski (57) made a detailed investigation into the determination of the structure of fluorescein. In the course of his work, he observed a sharp, well-defined band at 1536 cm^{-1} in the infrared spectrum of yellow fluorescein. The structure proposed for yellow fluorescein by Markuszewski

is presented in Figure 15. The structure contains a central, six-membered, oxygen-containing ring carrying a positive charge. This ring structure is identical with that in a class of compounds known as the pyrylium salts. One example is benzopyrylium chloride. The structure is



The infrared spectra of pyrylium ring compounds are characterized by two absorption bands, one of which is observed in the region of 1536 cm^{-1}. The presence of this band in both the infrared spectra of yellow fluorescein and of pyrylium ring compounds supports the structure of yellow fluorescein as presented in Figure 15.

The presence in fluorescein of a central, six-membered, oxygen-containing ring carrying a positive charge is the cause of the unique environment at C_{12}, and is the cause of the peak for C_{12} being found approximately 50 ppm farther downfield than the peak for C_{12} in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene. The positive charge is not localized at C_{12}, however, but rather is delocalized, probably over the central oxygen-containing ring. Localization of a positive charge at the carbon atom labeled C_{12} would result in a

chemical environment analogous to that found in the triphenylcarbonium ion. Ray, Kurland, and Colter (67) have reported that the carbon atom carrying the positive charge in a triphenylcarbonium ion gives rise to a peak in the carbon-13 spectrum at 210.6 ppm. This is convincing proof that the positive charge in the fluorescein molecule is not localized at C_{12}.

The peak observed at 109.8 ppm in the spectrum of fluorescein has been assigned to C_{13}. This peak appears as a singlet in the gated decoupled spectrum and is observed upfield from the analogous peak for C_{13}, at 120.5 ppm, in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene. The difference in chemical shifts is attributed to the difference in the environment about C_{12}. This assignment was based primarily on the elimination of other possibilities. The remaining three singlets in the "gated decoupled spectrum" of fluorescein have been assigned to carbon atoms in the phthalate ring portion of the molecule as discussed below.

ii) Justification for assignments of peaks to the carbon atoms in the phthalate ring portion of the fluorescein molecule The peak observed at 168.7 ppm has been assigned to C_{20}, the carbon atom of the phthalate carboxyl group. This assignment is supported by comparison with the spectrum of benzoic acid (46), in which the carbon atom of the carboxyl group is observed at 172.6 ppm. The peak at 126.3 ppm has

been assigned to C_{19}, which is the carbon atom attached to the carboxyl group. The peak for this carbon atom is a singlet in the "gated decoupled spectrum". The analogous carbon atom in the spectrum of benzoic acid is observed at 129.4 ppm. The peak at 135.3 ppm has been assigned to the carbon atom para to the carboxyl group, C_{16}. Again, the comparison is with the spectrum of benzoic acid where the analogous carbon atom is observed at 133.7 ppm.

The peak at 152.5 ppm is a singlet in the "gated decoupled spectrum" and has been assigned to the carbon atom to which the xanthene portion of the molecule is attached, that is, the carbon atom labelled C_{14}. This assignment made somewhat difficult because the exact nature of the environment at C_{12} is not known. However, this peak does not appear in the spectrum of 3,8-dihydroxy-12,12-dimethyl-xanthene, and thus must arise from a carbon atom in the phthalate ring.

The peak assigned to C_{14} is found farther downfield than might be expected. The effect, however, is supported by other examples. Introduction of a substituent into the benzene molecule has the greatest effect at the point of substitution while often causing little change in the rest of the spectrum. The carbon-13 spectrum of biphenyl is one example (11); the peaks of the carbon atoms at the point of substitution are observed at 141.5 ppm, while those of the

remaining carbon atoms fall between 127.4 ppm and 129.0 ppm. Another example is found in the spectrum of triphenylcarbinol, reported by Ray, Kurland, and Colter (67); the peak for the carbon atom at the point of substitution in the phenyl rings is found at 148.9 ppm while the remaining phenyl carbon atoms absorb at 127.4 ppm.

The three remaining peaks in the spectrum of fluorescein could not be definitely assigned to a specific carbon atom. The peaks at 124.6 ppm, 123.9 ppm, and 129.9 ppm fall in the range expected for benzenoid carbon atoms and have been collectively assigned to carbon atoms C_{15}, C_{17}, and C_{18}.

3. The spectrum of Calcein

The spectrum of Calcein was obtained in DMSO-d_6 (Figure 20). Seventeen distinct peaks are present. There should be present in the spectrum of a symmetrically substituted Calcein seventeen peaks, that is, excluding the peaks caused by the solvent, one peak for each of the seventeen chemically non-equivalent carbon atoms present in the molecule. Unsymmetrical location of the two methyleneiminodiacetic acid groups in Calcein is thus ruled out. The gated decoupled spectrum of Calcein is presented in Figure 21 and contains nine singlets as expected.

Figure 20. Carbon-13-FT-NMR spectrum of Calcein

Peak	Position of Peak: Frequency (Hz)	Position of Peak: Ppm[a]	Relative Intensity	Assignment[b]
1	3906.2	172.6	4.51	C_{27}, C_{28}, C_{29}, C_{30}
2	3816.6	168.6	1.05	C_{20}
3	3599.1	159.0	1.82	C_3, C_8
4	3447.5	152.3	0.96	C_{14}
5	3393.3	149.9	1.77	C_5, C_6
6	3065.5	135.4	0.50	C_{16}
7	2942.5	130.0	0.50	C_{18}
8	2889.3	127.6	0.79	C_1, C_{10}
9	2853.9	126.1	1.10	C_{19}
10	2819.4	124.6	0.54	C_{15}
11	2808.6	124.1	0.53	C_{17}
12	2547.1	112.6	0.71	C_2, C_9
13	2482.7	109.7	2.07	C_{11}, C_{13}
14	2474.8	109.3	2.26	C_4, C_7
15	1898.0	83.8	0.59	C_{12}
16	1224.6	54.1	1.14	C_{23}, C_{24}, C_{25}, C_{26}
17	1062.2	46.9	0.40	C_{21}, C_{22}

[a] Tetramethylsilane used as a reference.

[b] See Section C, subsection 3b.

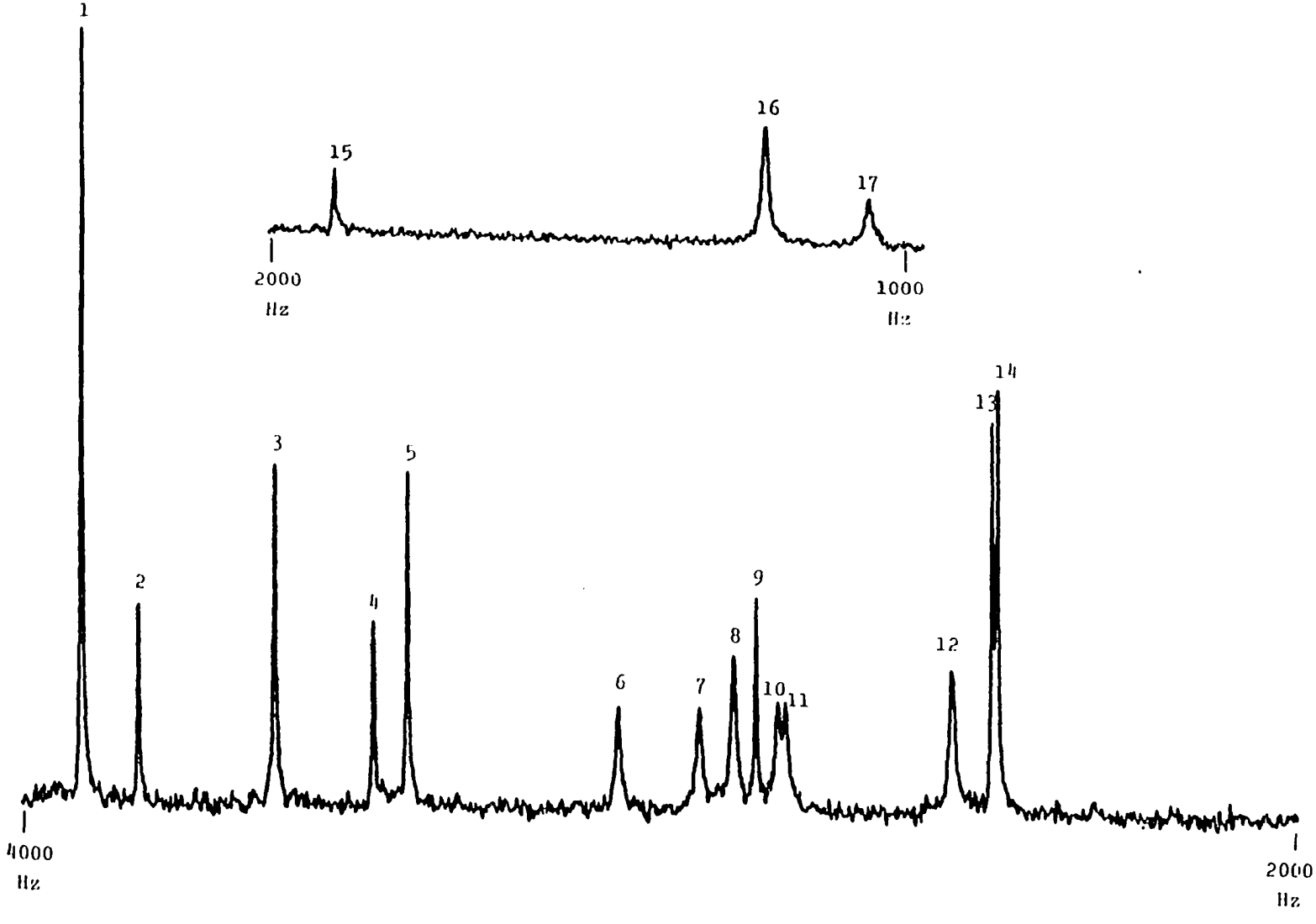

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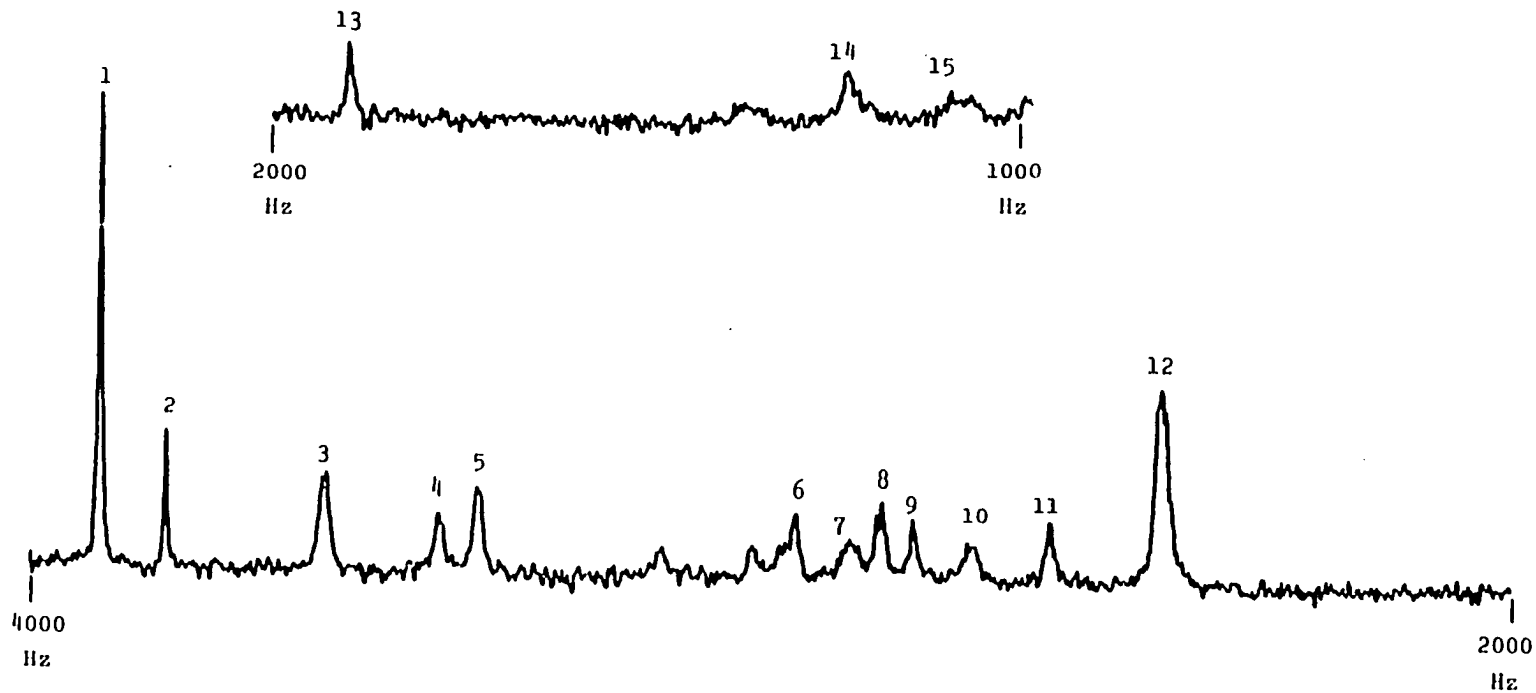
Figure 21. Gated decoupled carbon-13-FT-NMR spectrum of Calcein

Peak	Position of Peak		Relative Intensity
	Frequency (Hz)	Ppm[a]	
1	3908.2	172.7	5.63
2	3817.6	168.7	1.70
3	3597.1	158.9	1.24
4	3448.5	152.3	0.79
5	3395.3	150.0	1.09
6	2969.0	131.2	0.86
7	2897.2	128.0	0.57
8	2853.9	126.1	1.03
9	2812.5	124.2	0.82
10	2737.7	120.9	0.56
11	2627.4	116.1	0.81
12	2478.8	109.5	2.42
13	1898.0	83.8	0.96
14	1225.6	54.1	0.74
15	1087.8	48.1	0.55

[a]Tetramethylsilane used as a reference.


13
14
15
1
2000
Hz
1000
Hz
1
2
3
4
5
6
7
8
9
10
11
12
4000
Hz
2000
Hz

a. Assignments of peaks to the carbon atoms in the spectrum of Calcein

i) Assignments of peaks to the carbon atoms in the methyleneiminodiacetic acid groups of the Calcein molecule

The intense peak at 172.6 ppm has been assigned to the carbon atoms of the four carboxyl groups of the methyleneiminodiacetic acid groups.

The carbon atoms of the methylene groups attached to the carboxyl groups have been assigned the peak at 54.1 ppm, while the peak at 46.9 ppm has been assigned to the carbon atoms of the methylene groups attached to the xanthene portion of the molecule.

ii) Assignments of peaks to the carbon atoms in the xanthene portion of the Calcein molecule The peaks observed at 159.0 ppm and 149.9 ppm have been assigned to C_3 and C_5, respectively. The carbon atom labeled C_3 is attached to the hydroxy group while the carbon atom labeled C_5 is attached to the ether oxygen.

The peaks observed at 127.6 ppm, 112.6 ppm, and 109.3 ppm have been assigned to C_1, C_2, and C_4, respectively.

The peak at 83.8 ppm has been assigned to C_{12} and the peak at 109.7 ppm has been assigned to C_{13}.

iii) Assignment of peaks to the carbon atoms in the phthalate ring portion of the Calcein molecule The peak at 168.6 ppm has been assigned to C_{20}, which is the carbon atom of the phthalate carboxyl group. The peak at 126.1 ppm has

been assigned to C_{19}, the carbon atom attached to the phthalate carboxyl group. The peak at 135.4 ppm has been assigned to the carbon atom para to the carboxyl group, C_{16}.

The peak at 152.3 ppm has been assigned to C_{14}. This is the carbon atom in the phthalate ring to which the xanthene portion of the Calcein molecule is attached.

The peaks at 124.6 ppm, 124.1 ppm, and 130.0 ppm have been assigned to C_{15}, C_{17}, and C_{18}. These assignments are interchangeable because not enough data is available to assign each peak to a specific carbon atom.

b. Justification for assignments of peaks to the carbon atoms in the spectrum of Calcein

i) Justification for assignments of peaks to the carbon atoms in the methyleneiminodiacetic acid groups of the Calcein molecule The carbon atoms most deshielded in the Calcein molecule, and thus giving rise to peaks located farthest downfield, are those of the carboxyl groups of the methyleneiminodiacetic acid groups and of the phthalate ring. Because a carboxyl group is also present in the phthalate ring, relative intensities were used to make the assignments; thus, the intense peaks at 172.6 ppm in the spectrum of Calcein, a singlet in the "gated decoupled spectrum" (Figure 21) was assigned to the four carbon atoms of the four carboxyl groups. In the spectrum of disodium ethylenediaminetetraacetic acid (46) the corresponding peak appears at 171.5 ppm.

This is in good agreement with the value found experimentally for Calcein.

The peak in the spectrum of Calcein at 54.1 ppm has been assigned to the methylene groups attached to the carboxyl groups. The peak at 46.9 ppm has been assigned to the methylene groups attached to the xanthene portion of the molecule. The peak at 54.1 ppm is twice as intense as the peak at 46.9 ppm which indicates that twice as many carbon atoms are present. Comparison with the spectrum of disodium ethylenediaminetetraacetic acid (46) confirms the location of peaks for carbon atoms of this type in this range of chemical shifts.

ii) Justification for assignments of peaks to the carbon atoms in the xanthene portion of the Calcein molecule The peaks assigned to C_3 and C_5 were assigned using the same argument presented in Section C, subsection 1b, and in Section C, subsection 2bi. Thus, the carbon atom attached to the hydroxyl group has been assigned the peak at 159.0 ppm, which is farther downfield than the peak at 149.9 ppm assigned to C_5. Both peaks appear as singlets in the gated decoupled spectrum. The corresponding peaks in the spectrum of fluorescein were observed at 159.5 ppm and 152.0 ppm. The slight upfield shift has been attributed to the substitution of the methyleneiminodiacetic acid groups at C_4, the carbon atom ortho to both C_3 and C_5. Support for

this type of shift is found in the comparison of the carbon-13 spectrum of phenol with o-cresol (11). The carbon atom attached to the hydroxyl group in phenol is observed at 154.9 ppm. This same carbon atom is observed at 153.5 ppm in the spectrum of o-cresol.

The peaks at 112.6 ppm, 109.3 ppm and 127.6 ppm have been assigned to C_2, C_4, and C_1, respectively. Each assignment will be discussed separately.

The peak at 112.6 ppm in the spectrum of Calcein has a corresponding peak at 112.7 ppm in the spectrum of fluorescein. The same logic was used to assign each of these peaks to C_2 in the respective spectra. This argument was presented in detail in Section C, subsection 2bi.

The assignment of the peak at 109.3 ppm in the spectrum of Calcein to C_4 is critical because Markuszewski has proven that this is the site of substitution in Calcein. In comparing the spectrum of fluorescein with the spectrum of Calcein, it is apparent the peak at 102.5 ppm in the spectrum of fluorescein has shifted to 109.3 ppm in the spectrum of Calcein, and that the peak at 109.3 ppm is a singlet, unresolved from the singlet at 109.8 ppm, in the "gated decoupled spectrum" of Calcein. The singlet at 109.3 ppm has no corresponding peak in the "gated decoupled spectrum" of fluorescein, as expected, because the carbon atom labeled C_4 in fluorescein has one proton attached and appears as a

doublet in the gated decoupled spectrum. One would expect a shift of the 102.5 ppm peak in the spectrum of fluorescein upon substitution at the carbon giving rise to the peak. The shift is expected to be downfield. Support of this type of shift is found in comparison of the spectrum of phenol with the spectrum of o-cresol. The carbon atom ortho to the carbon attached to the hydroxyl group in phenol is observed at 115.4 ppm. The carbon atom in o-cresol attached to the methyl group and located ortho to the carbon atom attached to the hydroxyl group is observed at 124.0 ppm. Thus the peak at 109.3 ppm in the spectrum of Calcein has been assigned to C_4 and has been conclusively shown to be the site of the introduction of the methyleneiminodiacetic acid groups in the Calcein molecule.

The assignment of the peak at 127.6 ppm in the spectrum of Calcein to C_1 is considered tentative, as was the case with the assignment of the peak at 129.0 ppm to C_1 in the spectrum of fluorescein. Comparison of the spectrum of Calcein with that of fluorescein gives rise to evidence to support the assignment. Four peaks are observed in the region expected for benzenoid carbon atoms in the spectrum of Calcein. These peaks are at 130.0 ppm, 127.6 ppm, 124.6 ppm and 124.1 ppm. The analogous peaks in the spectrum of fluorescein are located at 129.9 ppm, 129.0 ppm, 124.6 ppm,

and 123.9 ppm. All but one of the four corresponding peaks are within 0.2 ppm of each other. The exception is the peak at 129.0 ppm in the spectrum of fluorescein which appears to have been shifted to a position at 127.6 ppm in the spectrum of Calcein. If the peak at 129.0 ppm in the spectrum of fluorescein is assigned to C_1, the upfield shift of the peak to 127.6 ppm in the spectrum of Calcein can be attributed to the introduction of the methyleneiminodiacetic acid groups into the fluorescein molecule at C_4.

The argument is plausible because of similar differences between the carbon-13 NMR spectra of phenol and o-cresol. The carbon atom meta to the carbon atom attached to the hydroxyl group in phenol is observed at 129.7 ppm. For o-cresol, the peak of the carbon atom meta to the carbon attached to the hydroxy group and para to the carbon attached to the methyl group is observed at 121.4 ppm.

The peak at 83.8 ppm in the spectrum of Calcein appears as a singlet in the gated decoupled spectrum of Calcein and has been assigned to C_{12}. The corresponding peak in the spectrum of fluorescein is at 83.3 ppm. The discussion of the structural factors that give rise to the chemical shift for C_{12} in the spectrum of fluorescein was presented in detail in Section C, subsection 2bi. The correlation of the chemical shift for C_{12} in the spectra of Calcein and fluorescein is excellent. Thus, the chemical environment about C_{12}

in the Calcein molecule is the same as the chemical environment about C_{12} in the fluorescein molecule, that is to say, both Calcein and fluorescein contain a central, six-membered oxygen-containing ring carrying a positive charge, which is delocalized over the central portion of the molecule.

The peak at 109.7 ppm in the spectrum of Calcein has been assigned to C_{13}. This peak is a singlet in the "gated decoupled spectrum". This assignment was based on elimination of other possibilities and on comparison with the spectrum of fluorescein where the corresponding peak assigned to C_{13} is observed at 109.8 ppm.

iii) Justification for the assignment of peaks to the carbon atoms of the phthalate ring portion of the Calcein molecule The peak at 168.6 ppm has been assigned to C_{20}, the peak at 126.1 ppm to C_{19}, the peak at 135.4 ppm to C_{16}, and the peak at 152.3 ppm to C_{14}. The corresponding peaks in the spectrum of fluorescein were observed at 168.7 ppm (C_{20}), 126.3 ppm (C_{19}), 135.3 ppm (C_{16}), and 152.5 ppm (C_{14}). The excellent correlation observed was the basis for the assignments made of the peaks in the spectrum of Calcein. The arguments for the assignments of the corresponding peaks in the spectrum of fluorescein were presented in Section C, subsection 2bii.

The peaks which appear in the spectrum of Calcein at 124.6 ppm, 124.1 ppm, and 130.0 ppm, have been assigned to

C_{15}, C_{17}, and C_{18}, and are in excellent correlation with the peaks in the spectrum of fluorescein assigned to C_{15}, C_{17}, and C_{18} which are at 124.6 ppm, 123.9 ppm, and 129.9 ppm. The assignments are interchangeable because not enough data is available to assign each peak to a specific carbon atom in the Calcein molecule.

4. The spectrum of diacetylfluorescein

The spectrum of diacetylfluorescein was obtained in a mixture of dioxane (90 per cent) and DMSO-d_6 (10 per cent). The solution was yellow in color. Sixteen peaks were present in the spectrum (Figure 22). Sixteen nonequivalent carbon atoms are present in the diacetylfluorescein molecule.

The discussion of the spectrum of diacetylfluorescein will not follow the format used in the previous discussions. The spectrum of diacetylfluorescein was obtained primarily to gain evidence for the assignment of the peak at 159.5 ppm in the spectrum of fluorescein to the carbon atom attached to the hydroxyl group. The evidence was presented in Section C, subsection 1b, and subsection 2bi.

No attempt was made to collect enough evidence to assign conclusively all of the peaks in the spectrum of diacetylfluorescein to specific carbon atoms. The assignments which are presented in Table 2 were made solely on correlations to the spectrum of fluorescein.

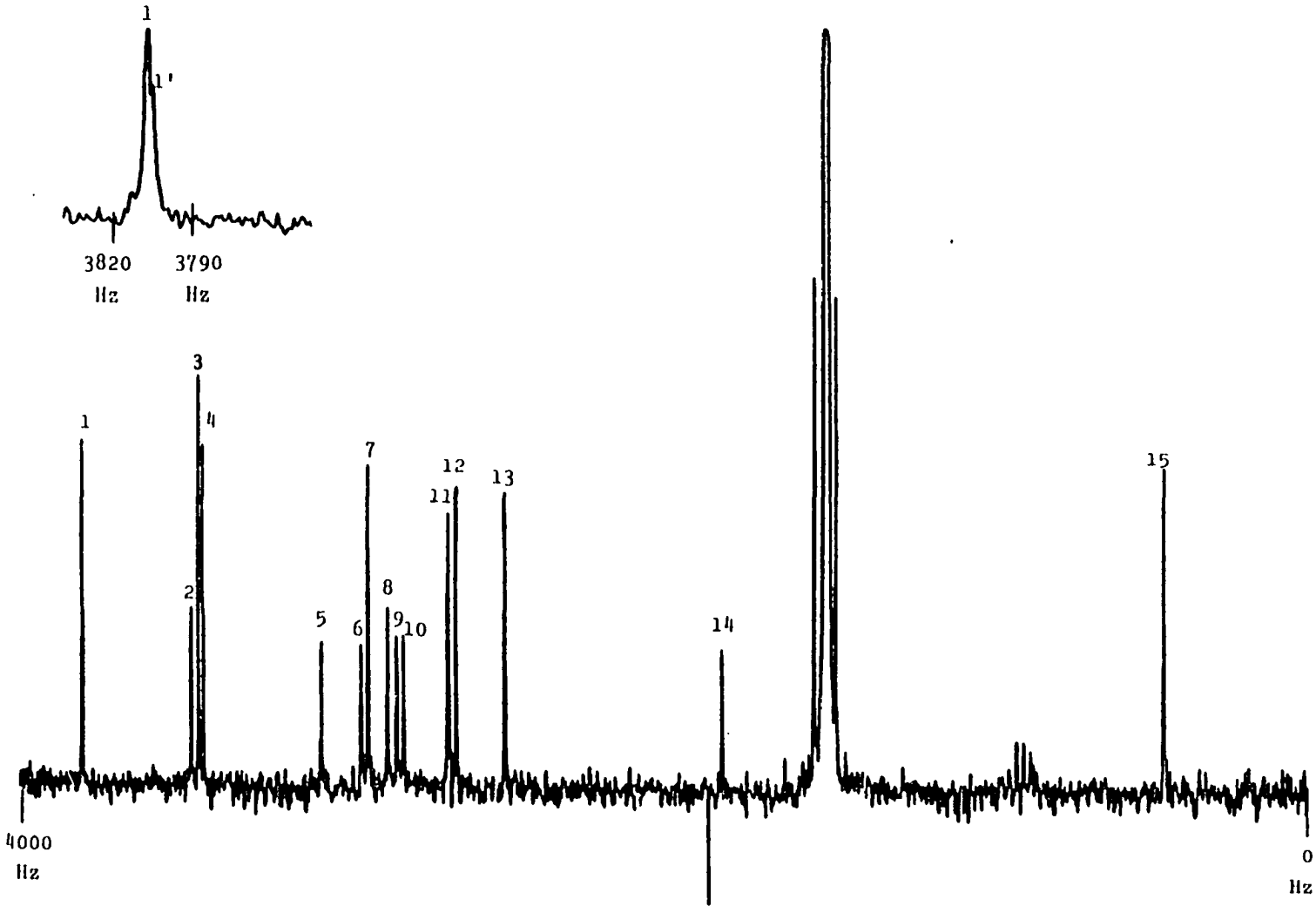
Figure 22. Carbon-13-FT-NMR spectrum of diacetylfluorescein

Peak	Position of Peak: Frequency (Hz)	Position of Peak: Ppm[a]	Relative Intensity	Assignment[b]
1, 1'[c]	3807.2	168.2	2.41	C_{21}, C_{22}
	3804.8	168.1	1.66	C_{20}
2	3465.6	153.1	1.25	C_{14}
3	3443.0	152.1	2.83	C_5, C_6
4	3422.3	151.2	2.36	C_3, C_8
5	3058.0	135.1	1.02	C_{16}
6	2936.0	129.7	1.00	C_{18}
7	2914.3	128.7	2.21	C_1, C_{10}
8	2852.3	126.0	1.25	C_{19}
9	2823.8	124.7	1.06	C_{15}
10	2803.1	123.8	1.09	C_{17}
11	2666.2	117.8	1.90	C_2, C_9
12	2641.6	116.7	2.07	C_{11}, C_{13}
13	2493.0	110.1	2.03	C_4, C_7
14	1828.5	80.8	0.98	C_{12}
15	450.3	19.9	2.23	$C_{23'}$, $C_{24'}$

[a] Tetramethylsilane used as a reference.

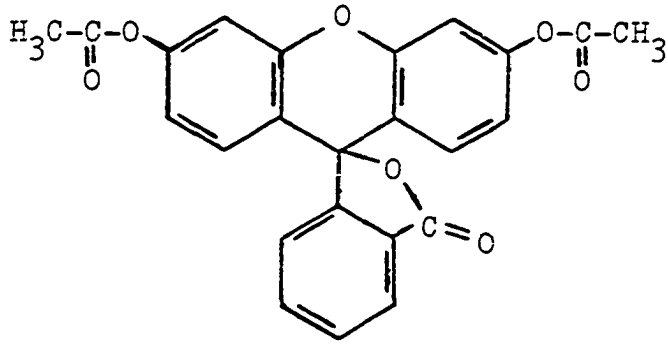
[b] See Section C, subsection 4.

[c] Two peaks present upon expansion of the spectrum.


1
1'
3820
Hz
3790
Hz
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
4000
Hz
0
Hz

The presence of a peak at 80.8 ppm deserves comment. This peak has been assigned to C_{12}. The corresponding peaks for C_{12} in the spectra of fluorescein and Calcein were observed at 83.3 ppm and 83.8 ppm. The excellent correlation is evidence for all three compounds having a similar environment about C_{12}. Thus, diacetylfluorescein in the solvent used above has a central, six-membered, oxygen-containing ring carrying a positive charge. The structure is presented in Figure 15.

Diacetylfluorescein in the solid state is a white crystalline material. Markuszewski noted that diacetylfluorescein dissolved in pure dioxane resulted in colorless solutions. He proved that the structure of diacetylfluorescein in dioxane is the structure presented below:



The presence of a lactone in the structure should change the environment at C_{12} and thus result in a different chemical shift for C_{12} when compared with the chemical shifts observed for C_{12} in fluorescein and Calcein.

The yellow color of the solution of diacetylfluorescein in dioxane-DMSO-d_6 is evidence that the presence of DMSO-d_6 probably resulted in the opening of the lactone ring. The excellent correlation of the chemical shifts for C_{12} in the spectra of diacetylfluorescein, fluorescein and Calcein is also evidence against the presence of a peak caused by the presence of a lactone.

D. Conclusion

The nature of the present work has demanded a necessarily complex discussion of the data. In reviewing the work, however, certain facts have been established.

Eight distinct peaks appeared in the spectrum of 3,8-dihydroxy-12,12-dimethylxanthene, excluding the peaks caused by the solvent. Thus, eight equivalent carbon atoms are present in the molecule. All of the peaks of the carbon atoms present in the 3,8-dihydroxy-12,12-dimethylxanthene molecule were assigned and justifications presented for all assignments.

Fourteen distinct peaks appeared in the spectrum of fluorescein, excluding the peaks due to the solvent. Fourteen equivalent carbon atoms are present in the fluorescein molecule. Using the assignments made for the peaks of the carbon atoms in the spectrum of 3,8-dihydroxy-12,12-dimethyl-xanthene as a foundation, it was possible to account for the

difference observed in the spectra of fluorescein and 3,8-dihydroxy-12,12-dimethylxanthene. All of the peaks for the carbon atoms in the spectrum of fluorescein were thus assigned and arguments presented to support the assignments.

Analysis of the spectrum of fluorescein provided evidence to support the structure of yellow fluorescein proposed by Markuszewski in which fluorescein contains a central, six-membered, oxygen-containing ring carrying a positive charge, and that this positive charge is spread over the central ring rather than localized on any one carbon atom.

Once the assignments of the peaks of the carbon atoms in the spectrum of fluorescein had been made, it was possible to account for the difference in the spectra of fluorescein and Calcein. The spectrum of Calcein contained seventeen distinct peaks, excluding the peaks caused by the solvent. There should be present in the spectrum of a symmetrically substituted Calcein seventeen peaks. The spectrum of a non-symmetrically substituted Calcein should show more than seventeen peaks. Unsymmetrical location of the two methylene-iminodiacetic acid groups in Calcein was thus ruled out.

Thus, the principal findings were that Calcein is a symmetrical molecule, that the two methyleneiminodiacetic acid groups occupy equivalent positions in the molecule and specifically occupy the 4- and 7- positions.

One other significant result of the study of the spectrum of Calcein was the determination that Calcein, like fluorescein, contains a central, six-membered, oxygen-containing ring carrying a positive charge.

VIII. SUMMARY

An extensive review of the literature on Calcein has been made. Since the initial paper by Diehl and Ellingboe (18), almost two hundred papers have been published on Calcein; much of the work on the fundamental chemistry of Calcein has been done at Iowa State University and this work has been reviewed in some detail.

The history of the problems associated with the naming and structure of Calcein has been traced. The proliferation of names has been discussed and the errors in the assignment of a structure to the material have been traced to a possible error by a printer in one of the early publications. The further confusion generated by the revision of the naming of organic compounds by Chemical Abstracts has been discussed.

The use of Calcein as an indicator in the titrimetric determination of various cations and anions, and in particular the determination of calcium, has been reviewed. The history and problems associated with the direct fluorometric determination of calcium in serum have been traced. The novel use of Calcein as a marker of bone growth has been mentioned.

In the present work, improvements and extensions of an earlier investigation into the synthesis of Calcein have been reported, the direct fluorometric determination of calcium has been improved, and, in the principal work of

this dissertation, an independent proof of the structure of Calcein has been offered.

A method has been set up for the determination of formaldehyde; the method involves the reaction of formaldehyde with unsymmetrical dimethylhydrazine. The concentration of commercial formaldehyde has been determined and it has been shown that solutions of formaldehyde do not undergo change on standing exposed to air. The statement made in my master of science thesis that formaldehyde used for the synthesis of Calcein should be taken from unopened bottles has been withdrawn.

Further studies have been made of the so-called "two-end-point method" of determining the purity of Calcein reported in my master of science thesis. This method, which differentiates Calcein from fluorescein and is based on titrating the two hydrogen ions liberated when two ions of calcium unite with one molecule of Calcein, is subject to a certain difficulty; drift in pH is observed at the second end-point. This drift has now been found to be seriously detrimental as well as annoying and has been traced to the formation of a precipitate, probably $Na_2Ca_5Cal_2$, which forms slowly as the second end-point is approached. A solution to this problem has been found and a modified procedure has been offered.

It has been shown that replacing disodium dihydrogen ethylenediaminetetraacetate by dipotassium dihydrogen ethylenediaminetetraacetate as the titrant in the EDTA determination of calcium using Statocalcein as indicator does not significantly reduce the background fluorescence at the end-point. Dipotassium dihydrogen ethylenediaminetetraacetate dissolves quickly in water in contrast to disodium dihydrogen ethylenediaminetetraacetate dihydrate and should prove a better reagent in the hands of students.

An improved method for the direct fluorometric determination of calcium has been devised. The method involves the preparation of a stock solution of Calcein containing calcium in the ratio of one atom of calcium per molecule of Calcein. The analytical work is then performed in the range in which calcium and Calcein form a two-to-one compound and the sensitivity of Calcein for detecting calcium is greatest. The addition of calcium to this solution has been shown to cause a linear increase in the fluorescence, a marked improvement over earlier methods. Such solutions are stable for two hours. Two methods of preparing the reagent solution have been described. The effect of pH on the stability of the "One-to-One Calcium-Calcein" reagent has been investigated.

An investigation into the electrochemistry of Calcein has been made. A polarographic reduction wave has been found

for Calcein over the range of value of pH from 1 to 13. The half-wave potential of the polarographic reduction wave is shifted to more negative values with increasing pH. Four points of inflection have been observed in the plot of the half-wave potential as a function of pH. No correlation has been found between the half-wave potential of this reduction wave and the known dissociation constants of Calcein as an acid.

The reduction of Calcein at pH 2.1, pH 4.0 and pH 6.6 has been studied by coulometry using the controlled cathode potential method. The reduction of Calcein has been found to be a two-electron process. It has been shown that the product is nonfluorescent. The reduction product has been given the common name Calcin, in analogy to fluorescin, the reduction product of fluorescein.

An additional wave has been found in the polarogram of Calcein in alkaline solution on the addition of calcium. The height of this reduction wave is linear with calcium added up to one atom of calcium added per molecule of Calcein. The height of this peak has been found to remain constant upon addition of calcium beyond one atom of calcium added per molecule of Calcein.

The carbon-13 Fourier transform nuclear magnetic resonance spectroscopy of fluorescein, Calcein and related compounds has been studied. A new numbering system for the

compounds has been devised in which every carbon atom is numbered.

3,8-Dihydroxy-12,12-dimethylxanthene has been shown to contain eight nonequivalent carbon atoms, fluorescein to contain fourteen nonequivalent carbon atoms, Calcein to contain seventeen, and diacetylfluorescein to contain sixteen.

All of the peaks in the spectra of 3,8-dihydroxy-12,12-dimethylxanthene, fluorescein and Calcein have been assigned to specific carbon atoms. Justifications for the assignments have been presented in all cases. The justifications consist of intercomparison of the spectra obtained and correlation with evidence from carbon-13 spectra reported in the literature.

All of the fourteen peaks which appear in the carbon-13 NMR spectrum of fluorescein have been assigned to specific carbon atoms. The peaks due to the carbon atoms of the xanthene portion of the fluorescein molecule have been distinguished from those of the carbon atoms of the phthalate ring. Evidence has been found for the presence in the fluorescein molecule of a central, six-membered, oxygen-containing ring carrying a delocalized positive charge.

All of the seventeen peaks which appear in the carbon-13 NMR spectrum of Calcein have been assigned to specific carbon atoms. The peaks in the spectrum due to the carbon atoms in the methyleneiminodiacetic acid portion of the molecule,

the xanthene portion of the molecule, and the phthalate ring portion of the molecule have all been distinguished from each other. Evidence has been found that the two methyleneiminodiacetic acid groups occupy equivalent positions in the Calcein molecule, more specifically that they occupy the 4- and 7-positions (the 4'- and 5'- positions of the old numbering system). As was the case with fluorescein, evidence has been found for the presence in the Calcein molecule of a central, six-membered, oxygen-containing ring carrying a delocalized charge.

A framework of carbon-13 spectra and assignments has been laid for future workers in the field of xanthene chemistry.

IX. BIBLIOGRAPHY

1. R. L. Alexander, Jr. Evaluation of an Automatic Calcium Titrator. Clinical Chemistry, 17, 1171 (1971).

2. J. F. Bandrowski and C. L. Benson. Investigation of the Use of Calcium in the Ultramicro Fluorometric Determination of Calcium in Serum. Clinical Chemistry, 18, 1411 (1972).

3. N. R. Bannerjee and S. K. Vig. Polarographic Current-Voltage Curves of Fluorescein. Part III. Course of Electrode Process. Indian J. Chem., 9, 444 (1971).

4. N. R. Bannerjee and S. K. Vig. Polarographic Current-Voltage Curves of Fluorescein. Part I. Effect of Pressure Head of Mercury on Mean Current. J. Chem. Soc. B., 5, 484 (1967).

5. I. M. Bett and G. P. Fraser. A Rapid Micro-Method for Determining Serum Calcium. Clin. Chim. Acta, 4, 364 (1959).

6. I. Birze. Chelating Derivatives of Dimethyldihydroxyfluorans. Ph.D. Dissertation. Iowa State University, Ames, Iowa, 1970.

7. A. B. Borle and F. N. Briggs. Microdetermination of Calcium in Biological Material by Automatic Fluorometric Titration. Anal. Chem., 40, 339 (1968).

8. E. A. Bozhevol'nov and S. U. Kreingol'd. Kharakteristike Fluorestsentnykh Svoistv Fluorestseinkompleksona. Zh. Anal. Khim., 17, 291 (1962) [transl.: Characteristics of the Fluorescent Properties of Fluorescein Complexon. J. Anal. Chem., USSR, 17, 294 (1962)].

9. E. A. Bozhevol'nov and S. U. Kreingol'd. Fluorestsentnoye Kompleksonometricheskoe Opredelenie Mikrokolichesty Kal'tsiya. Zh. Anal. Khim., 17, 560 (1962) [transl.: Fluorescent Complexometric Determination of Calcium. J. Anal. Chem., USSR, 17, 558 (1962)].

10. S. Brayton. The Solubility of Calcein at Low pH. Unpublished undergraduate research at Iowa State University, Ames, Iowa,

11. E. Breitmaier, G. Haas and W. Voelter. "Atlas of Carbon-13 NMR Data." Heydon and Son, Ltd., London, 1975.

12. E. Breitmaier and W. Voelter. "^{13}C-NMR Spectroscopy. Monographs in Modern Chemistry." Verlag Chemie GmgH, Weinheim/Bergstr., 1974.

13. V. Brustier, P. Valdiquie and P. Blanc. Note on Decolorizing Fluorescein. Bull. Soc. Chim., 6, 548 (1939).

14. M. J. Bunk, S. L. Balloun and H. Diehl. Calcein as a Marker of the Deposition of Calcium in the Growing Chick Embryo. Unpublished work.

15. N. J. Cussans and T. N. Huckerby. Carbon-13 NMR Spectroscopy of Heterocyclic Compounds - IV. Tetrahedron, 31, 2719 (1975).

16. P. Delahay. La réduction polarographique de la fluorescéine. Bull. Soc. Chim. Fr., 5, 348 (1948).

17. F. De Witt and R. J. Parsons. The Autodilutor for Fluorometric Microestimation of Serum Calcium. Amer. J. Clin. Path., 53, 324 (1970).

18. H. Diehl and J. L. Ellingboe. Indicator for Titration of Calcium in the Presence of Magnesium Using Disodium Dihydrogen Ethylenediamine Tetraacetate. Anal. Chem., 28, 882 (1956).

19. H. Diehl. Development of Metallochromic Indicators. Anal. Chem., 39, 30A (1967).

20. H. Diehl. Calcein, Calmagite, and o,o'-Dihydroxyazobenzene. Titrimetric, Colorimetric, and Fluorometric Reagents for Calcium and Magnesium. G. Frederick Smith Chemical Company, Columbus, Ohio, 1964.

21. H. Diehl. Calcein as an Indicator in the Titration of Calcium and Magnesium. Reference 20, p. 25.

22. H. Diehl and R. C. Miller. Determination of Calcium and Magnesium in Limestone. Reference 20, p. 33.

23. H. Diehl and K. Oulmann. Fluorescence of Calcein in Solutions of Potassium, Sodium, Lithium and Tetramethylammonium Hydroxide. Reference 20, p. 64.

24. H. Diehl and K. Oulmann. Fluorescein as an Impurity in Calcein. Reference 20, pp. 66-68.

25. H. Diehl. "Quantitative Analysis. Elementary Principles and Practice." 2nd ed. Oakland Street Science Press, Ames, Iowa, 1974, p. 280.

26. M. Dolinsky and J. H. Jones. Studies on Coal-Tar Dyes IX. D and C Yellow No. 7; D and C Orange No. 5; D and C Red No. 27; and F, D, and C, Red No. 3. J. Assoc. Off. Agr. Chem., 34, 114 (1951).

27. M. Effenberger. Chelatometric Determination of Sulfates in Water with Calcein as Indicator. Chem. Listy, 52, 1501 (1958); Coll. Czech. Chem. Commun., 24, 682 (1959).

28. P. J. Elving, J. M. Markowitz and I. Rosenthal. Preparation of Buffer Systems of Constant Ionic Strength. Anal. Chem., 28, 1179 (1956).

29. L. A. Freytag. The Red and Yellow Forms of Fluorescein. M.S. Thesis. Iowa State University, Ames, Iowa, 1971.

30. K. J. B. F. Fröhlich and J. Siemroth. Ein einfaches quantitatives Nachweisverfahren für Calzium, Barium und Strontium, angewendt auf Ca-Bestimmungen in NaCl- und KCl-Kristallen. Wiss. Z. Martin Luther Univ., Halle, Wittenberg, Math.-Nat. Reihe, 22, 49 (1973).

31. F. A. Gollmick and H. Berg. Polarographische und Photoaktivierte Reduktion von Chinonen und Ketonen. Ber. Bunsenges Phys. Chem., 69, 196 (1965).

32. V. Hanousek. Nebenprodukte der Fluoreszeinschmelze. Coll. Czech. Chem. Commun., 24, 1061 (1959).

33. V. Hanousek. 3,6-Dihydroxy-9,9-dimethylsanthene. Czech. Pat. 88,272 (1959); Chem. Abstr., 54, 8855a (1960).

34. A. J. Hefley and B. Jaselskis. Fluorometric Determination of Submicrogram Quantities of Cadmium by Reaction with the Metallofluorochromic Reagent, Calcein. Anal. Chem., 46 (13), 2036 (1974).

35. A. J. Hefley. Preparation and Properties of Calcein. M.S. Thesis. Iowa State University, Ames, Iowa, 1965.

36. A. J. Hefley. Structure and Uses of Chelating Derivatives of Fluorescein. Ph.D. Dissertation. Iowa State University, Ames, Iowa, 1967.

37. R. G. Hermann. Adaption of Calcein Titration Method for Calcium Determination in Tissues Without Ashing. Proc. Soc. Exptl. Biol. Med., 99, 777 (1958).

38. J. B. Hill. Automated Fluorometric Method for Determination of Serum Calcium. Clinical Chemistry, 11, 122 (1965).

39. H. K. Howerton and J. C. Wasilewski. "A New Instrument for Automatic Colorimetric and Fluorometric Titrations" in Titrimetric Methods. Plenum Press, New York, 1961, p. 51. Reprint No. 141, American Instrument Co., Inc., Silver Spring, Maryland.

40. W. C. Hoyle and H. Diehl. Statocalcein, a Stable Calcein Indicator for the EDTA Titration of Calcium. Talanta, 19, 206 (1972).

41. G. M. Huitink. Preparation and Properties of Calcein Blue. M.S. Thesis. Iowa State University, Ames, Iowa, 1965.

42. G. M. Huitink. Substituted Coumarins as Metallofluorochromic Indicators. Ph.D. Dissertation. Iowa State University, Ames, Iowa, 1967.

43. G. M. Huitink and H. Diehl. Methyl Calcein Blue and Other Analogues of Calcein Blue. Talanta, 21, 1193 (1974).

44. G. M. Huitink, D. P. Poe and H. Diehl. On the Properties of Calcein Blue. Talanta, 21, 1221 (1974).

45. R. M. Issa, F. M. Abd-el-Halim and A. A. Hasanein. Polarographic Studies of Fluorescein in Solutions of Varying pH at the Dropping Mercury Electrode. Electrochim. Acta, 14, 561 (1969).

46. L. F. Johnson and W. C. Jankowski. "Carbon-13 NMR Spectra." John Wiley and Sons, New York, 1972.

47. M. Kariyama, M. Akai and S. Nishijima. Three-Colour Fluorescent Labelling Method for Calcified Tissues in a Reptile, *Caiman Crocodilus*. Arch. Oral. Biol., 14, 1349 (1969).

48. B. L. Kepner and D. M. Hercules. Fluorometric Determination of Calcium in Blood Serum. Anal. Chem., 35, 1238 (1963).

49. B. Klein, J. H. Kaufman and J. Isaacs. Automated Simultaneous Calcium and Phosphorus Analysis. Clinical Chemistry, 13, 1071 (1967).

50. W. F. Koch, D. L. Biggs and H. Diehl. Tris(hydroxymethyl)aminomethane -- A Primary Standard? Talanta, 22, 637 (1975).

51. J. Körbl and F. Vydra. Metallochromic Indicators IV. A Note on the Preparation and Properties of Calcein. Coll. Czech. Chem. Commun., 23, 622 (1958).

52. J. Körbl and R. Pribil. Analysis for Industry. Industrial Chemist, 34, 679 (1958).

53. J. Körbl, F. Vydra and R. Pribil. Beitrag zur Charakterisierung des Fluoresceinkomplexons. Talanta, 1, 138 (1958).

54. J. Körbl, F. Vydra and R. Pribil. The Use of Fluorescein Complexone. Talanta, 1, 281 (1958).

55. C. C. Mabry and P. Wyles. Micro Automated Parallel Measurements of Serum Calcium and Phosphorus. Clinical Chemistry, 37, 395 (1967).

56. V. A. Malevannyi. Kompleksonometricheskoe opredelenie okisi kaltsiya i magniya v dvuokisi titana. Zavod. Lab., 30, 1448 (1964) [transl.: The Complexometric Determination of Calcium Oxide and Magnesium Oxide in Titanium Dioxide. Industrial Laboratory, 30, 1807 (1964)].

57. R. Markuszewski. Structure, Fluorescence, and Chelating Properties of Calcein. Ph.D. Dissertation. Iowa State University, Ames, Iowa, 1976.

58. D. B. Martin. Improved Synthesis of the Metallofluorochromic Indicator Calcein. M.S. Thesis. Iowa State University, Ames, Iowa, 1974.

59. G. Miyajima and K. Nishimoto. Carbon-13 Nuclear Magnetic Resonance Spectroscopy VIII -- Aliphatic Hydrocarbon Derivatives. Org. Mag. Res., 6, 313 (1974).

60. A. G. C. Morris. Complexometric Determination of Calcium and Magnesium in Ferromanganese Slags. Anal. Chem., 33, 599 (1961).

61. T. Nomura. Complexometric Titration Using a Tensammetric Wave of Organic Reagents as an End-Point Detection. Nagoya Kogyo Daigaku Gakuho, 24, 97 (1972).

62. H. A. Ntailianas and R. McL. Whitney. Calcein as an Indicator for the Determination of Total Calcium and Magnesium and Calcium alone in the Same Aliquot of Milk. J. Dairy Sci., 47, 19 (1964).

63. S. Olerud and G. L. Lorenzi. Triple Fluorochrome Labeling in Bone Formation and Bone Resorption. J. Bone Joint Surg., 52-A, 274 (1970).

64. R. L. Olsen, H. Diehl, P. F. Collins, and R. B. Ellestad. Determination of Calcium in Lithium Salts. Talanta, 7, 187 (1961).

65. T. W. Proulx and W. B. Smith. The Carbon-13 NMR Spectra of Phenyl-Substituted Methanes, Ethanes, and Ethylenes. J. Mag. Res., 23, 477 (1976).

66. P. Rasmussen. Effect of Oxytetracycline and Purified Calcein (DCAF) on the Apposition and Mineralization of Rat Incisor Dentin. Scand. J. Dent. Res., 83, 233 (1975).

67. G. J. Ray, R. J. Kurland and A. K. Colter. Magnetic Resonance Studies of Triphenylcarbonium Ions - II. Carbon-13 Magnetic Resonance Studies of Charge Delocalization in para-Substituted Triphenylcarbonium Ions. Tetrahedron, 27, 735 (1971).

68. C. Robinson and J. A. Weatherell. The Micro Determination of Calcium in Mammalian Hard Tissues. Analyst, 93, 722 (1968).

69. B. A. J. Sedlacek and P. Dusek. Komplexometrische Schnellmethode zur Calciumbestimmung in Milch. Z. Lebens.-Untersuch. Forsch., 129, 333 (1966).

70. S. Siggia. "Quantitative Analysis via Functional Groups." 3rd ed. John Wiley & Sons, New York, 1963, pp. 87-91.

71. T. Solheim. Pluricolor Fluorescent Labeling of Mineralizing Tissue. Scand. J. Dent. Res., 82, 19 (1974).

72. P. G. Sullivan. The Value of 2,4-Bis[N,N'-di-(carboxymethyl)aminomethyl] Fluorescein as a Bone-Marking Dye. Arch. Oral Biol., 17, 1487 (1972).

73. H. K. Suzuki and A. Mathews. Two-Color Fluorescent Labeling of Mineralizing Tissue with Tetracycline and 2,4-Bis[N,N'(carboxymethyl)aminomethyl] Fluorescein. Stain Technol., 41, 57 (1966).

74. V. Svoboda, V. Chromy, J. Körbl and L. Dorazil. Metallochromic Indicators - X. A New Mixed Indicator for Chelatometric Determination of Calcium. Talanta, 8, 249 (1961).

75. K. N. Tandon and R. C. Mehrotra. Studies on Adsorption Indicators. V. Application of Metallochrome Indicators as Adsorption Indicators. Anal. Chim. Acta, 30, 407 (1964).

76. Y. Terui, K. Tori and N. Tsuji. Esterification Shifts in Carbon-13 NMR Spectra of Alcohols. Tetrahedron Letters, 8, 621 (1976).

77. T. Uemura. A Fluorometric Method for the Determination of Calcium Using Calcein. Sci. Rep. Tohoku Univ., Ser. IV (Biol.), 34, 31 (1968).

78. A. J. Van der Reyden and R. L. M. Van Lingen. Determination of Trace Amounts of Calcium in Caustic Soda. Complexometric Titration after Separation on Dowex A-1 Chelating Resin. Z. Anal. Chem., 187, 241 (1962).

79. S. K. Vig and N. R. Bannerjee. Polarographic Current-Voltage Curves of Fluorescein - IV. Effect of Concentration. Electrochim. Acta, 16, 157 (1971).

80. S. K. Vig. Polarographic Current-Voltage Curves of Fluorescein. Part II - Reversibility of Electrode Process. Indian J. Chem., 7, 501 (1969).

81. F. Vydra, V. Markova and R. Pribil. Verwendung vol Fluoreszein-Komplexon fur die Indikation Argentometrischer Titrationen. Coll. Czech. Chem. Commun., 26, 2449 (1961).

82. D. F. H. Wallach, D. M. Surgenor, J. Soderberg and E. Delano. Preparation and Properties of 3,6-Dihydroxy-2,4-bis-[N,N'-di-(carboxymethyl)-aminomethyl] Fluoran. Anal. Chem., 31, 456 (1959).

83. D. F. H. Wallach and T. L. Steck. Fluorescence Techniques in the Microdetermination of Metals in Biological Materials. Utility of 2,4-Bis-[N,N'-di-(carboxymethyl)aminomethyl] Fluorescein in the Fluorometric Estimation of Al^{+3}, Alkaline Earths, Co^{+2}, Cu^{+2}, Ni^{+2}, and Zn^{+2} in Micromolar Concentrations. Anal. Chem., 35, 1035 (1963).

84. D. H. Wilkins. The Chelometric Determination of Chromium, Cobalt(III) and Copper with a Metalfluorechromic Indicator. Anal. Chim. Acta, 20, 324 (1959).

85. D. H. Wilkins. The Chelometric Determination of Cobalt and Iron Using a Fluorescent End-Point. Talanta, 2, 12 (1959).

86. D. H. Wilkins. Apparatus for Titrations using Ultraviolet Light. Talanta, 2, 88 (1959).

87. D. H. Wilkins and L. E. Hibbs. The Determination of Copper with Triethylenetetramine Using a Metalfluorechromic Indicator. Talanta, 2, 201 (1959).

88. D. H. Wilkins. The Separation and Determination of Nickel, Chromium, Cobalt, Iron, Titanium, Tungsten, Molybdenum, Niobium and Tantalum in a High Temperature Alloy by Anion-Exchange. Talanta, 2, 355 (1959).

89. E. Wohlmann. Über die Anwendung neuer Indikatoren der Chelatometrie in der Erz- und Silikatanalyse. Z. Angew. Geol., 8, 263 (1962).

90. R. G. Yalman, W. Bruegemann, P. T. Baker and S. M. Garn. Volumetric Determination of Calcium in Presence of Phosphate. Anal. Chem., 31, 1230 (1959).

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