1967

Structure and uses of chelating derivatives of fluorescein

Alta Jean Hefley

Iowa State University

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Iowa State University, Ph.D., 1967
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STRUCTURE AND USES OF CHELATING DERIVATIVES OF FLUORESCIN

by

Alta Jean Hefley

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

Major Subject: Analytical Chemistry

Approved:

Signature was redacted for privacy. In Charge of Major Work

Signature was redacted for privacy. Head of Major Department

Signature was redacted for privacy. Dean of Graduate College

Iowa State University Of Science and Technology Ames, Iowa

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PART I. INTRODUCTION

The compound called Calcein was introduced in 1956 by Diehl and Ellingboe (1) as a metallofluorochromic indicator for the EDTA titration of calcium in the presence of magnesium. It has found considerable favor among analytical chemists for the current (Fall of 1967) bibliography on the reagent numbers some eighty papers, the majority of which deal with application of the indicator in chemical analysis. The literature on Calcein to 1964 is reviewed in the monograph by Diehl (2).

As first prepared Calcein was admittedly impure (1). The impure material served well as indicator but it has been the history of metallochromic indicators that although first proposed as indicators, they are sooner or later tried as direct colorimetric or fluorometric reagents for the determination of metal ions. When such fate overtook Calcein, the results were far from satisfactory; the proposal (3) was to use Calcein for the direct fluorometric determination of calcium in blood serum, a matter of considerable importance, and the failure of the method to work in the hands of others than the authors of the proposal prompted further investigation at Iowa State University into the purification and properties of the reagent.

Calcein is prepared by the Mannich condensation of fluorescein, formaldehyde and iminodiacetic acid. As carried
out by Diehl and Ellingboe (1), the condensation was effected in an aqueous solution of sodium hydroxide. Subsequent to the original paper three papers (4, 5, 6) mention improvements on the method of preparation but only one, that of Wallach, Surgenor, Soderberg and Delano (6) gives details. The product of Wallach and coworkers was designated "Compound A," but it most certainly was Calcein in a more or less pure form. The elemental analysis reported was barely satisfactory and the other proofs of purity offered are unconvincing. Although Wallach and his coworkers arrived at the correct composition of the material, that is fluorescein bearing two methyleneiminodiacetic acid groups, the structure assignment which they made is incorrect as will be shown in the present work.

In the present research program, attention was first directed to obtaining Calcein in pure form and to establishing its composition. Success in this phase of the work has already been reported, Hefley, M. S. Thesis (7). The basic trouble in the purity problem proved to be the presence of metals in the Calcein. All methods of purification tried failed to remove the considerable amounts of calcium, iron, aluminum, and in some preparations mercury and zinc. The source of these metals proved to be the fluorescein used. Calcein is a strong chelating agent and gathers up quantitatively and retains tenaciously all the metals it encounters.
Fluorescein proved also to have such scavenging characteristics and it was only by carefully eliminating all metals from the fluorescein and the other reagents used that a metal-free Calcein could be prepared. A systematic study was then made of the variables in the Mannich condensation, and a procedure was finally evolved by which Calcein could be prepared consistently of definite composition and high purity. The present work on the structure and properties of Calcein then became possible.

The question of structure is an important one and is certainly essential to an understanding of the nature of the metal derivatives. The first direct attack on the structure was the work of Wallach, Surgenor, Soderberg and Delano (6), mentioned above, who concluded on the basis of the ultraviolet absorption spectrum with pH that their "Compound A," was the unsymmetrical substituted 2',4'-bis[N,N'-di(carboxymethyl)aminomethyl]-3',6'-dihydroxyfluoran (more simply fluorescein-2',4'-bis(methyleneiminodiacetic acid), structure VI, page 9. Some supporting evidence was adduced from the infrared spectrophotometry of Calcein and the products derived from dimethyl- and diethylfluorescein by the introduction of methyleneiminodiacetic acid groups.

Aside from the aesthetically unpleasing appearance of the unsymmetrically substituted molecule, most chemists would intuitively think that once a group had entered one of
the resorcinol rings a second group would enter the other identical and empty resorcinol ring in preference to crowding a second, large and bulky group into an already substituted ring.

In any case, once a pure Calcein became available, an immediate attack was made on the problem of structure. In our own hands infrared spectrometry offered little promise, the spectrum consisting of many overlapping and poorly resolved bands. The NMR spectrum on the other hand was characterized by well resolved bands which, together with supplementary spectra from related compounds, could be interpreted clearly and led to a definitive assignment of structure. Calcein is the symmetrically substituted compound, 4',5'-bis[N,N'-di(carboxymethyl)aminomethyl]-3',6'-di-hydroxyfluoran (or more simply fluorescein-4',5'-bis(methyleneimidodiacetic acid); structure IX, page 9.

No less than six replaceable hydrogen atoms are present in the Calcein molecule. Using very rough approximations of the successive acid dissociation constants and employing arguments advanced earlier by Schwarzenbach, Anderegg and Sallmann(8) on phenolic compounds bearing one and two methyleneiminodiacetic acid groups (described in the very first of the papers in this field), Wallach and coworkers (6) decided that the six replaceable hydrogen atoms were neutralized successively from a carboxyl group, the second
carboxyl group, a phenol group, the second phenol group, an ammonium group and the second ammonium group. This interpretation appears correct but the present work puts a firm foundation under the argument by supplying careful evaluation of the respective acid dissociation constants and a detailed correlation of the absorption and fluorescence phenomenon with pH and structure changes. Four separate lines of attack were necessary to obtain values for all six of the dissociation constants.

The nature of the calcium derivative, of course, is the ultimate object of the work for Calcein is primarily a reagent for calcium. Wallach and co-workers (6) came to the conclusion that two calcium derivatives of Calcein were formed, the first by the union of one calcium atom with Calcein forming a non-fluorescent compound, the second by the attachment of a second atom of calcium, this compound being fluorescent. Wallach and Steck (9) later measured the formation constants of the two to one compound and reported a value of $10^{6.3}$. Russian work, reported somewhat later of Bozhevol’nov and Kreingol’d (10) concluded that only a 1:1 compound was formed, that it was fluorescent and that the formation constant is $10^{6.8}$. The presence of calcium and other metals as impurities, would seriously disturb measurements in this field, and in the present work, the subject was attacked again. Two atoms of calcium do
enter the molecule but both of the calcium compounds are fluorescent. The plot of relative fluorescence versus calcium added, using pure Calcein, is three successive straight lines, with intersections at the points where one mole and two moles of calcium have been added, the slope of the second line being greater than the first and the slope of the third line, zero. This has implications, of course, in the use of Calcein for the direct fluorometric determination of calcium.
PART II. DETERMINATION OF THE STRUCTURE OF CALCEIN
BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Structure of Calcein

Nine structures are possible for a disubstituted fluorescein and for Calcein; these are shown in the accompanying diagrams, Structures I through IX. Adopting the nomenclature of Chemical Abstracts for fluorescein, see structural formula X, the possible combinations for two substituents in the resorcinol rings of fluorescein are

<table>
<thead>
<tr>
<th>Structure</th>
<th>Designation</th>
<th>Structure</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1',2'-'</td>
<td>VI</td>
<td>2',4'-'</td>
</tr>
<tr>
<td>II</td>
<td>1',4'-'</td>
<td>VII</td>
<td>2',5'-'</td>
</tr>
<tr>
<td>III</td>
<td>1',5'-'</td>
<td>VIII</td>
<td>2',7'-'</td>
</tr>
<tr>
<td>IV</td>
<td>1',7'-'</td>
<td>IX</td>
<td>4',5'-'</td>
</tr>
<tr>
<td>V</td>
<td>1',8'-'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

One assignment of a structure for Calcein has already been made, that by Wallach, Surgenor, Soderberg and Delano (6) who believed the compound to be the unsymmetrically substituted structure VI with the two methyleneiminodiacetic acid groups in the 2'- and 4'-positions. The assignment was based on the nature of changes in the ultraviolet absorption spectrum with pH, on infrared spectrophotometry, and on observations on related compounds prepared from substituted fluoresceins.
\[ R = \text{CH}_2\text{NCH}_2\text{COOH} \]
R = \text{CH}_2\text{N}\text{CH}_2\text{COOH}
The argument based on the absorption in the ultraviolet is a complex one based on correlating the changes in absorbance with pH with specific acid dissociation steps and drawing by analogy on the behavior as acids of similar compounds of Schwarzenbach, Angeregg and Sallmann (8). The compounds of Schwarzenbach and co-workers are phenol-2-methyleneiminodiacetic acid, Structure XI, and phenol-5-methyl-2,6-bis(methyleneiminodiacetic acid), Structure XII. The phenolic group of phenol-5-methyl-2,6-bis(methyleneiminodiacetic acid), Structure XII, is much stronger, \( pK = 6.65 \), than that of normal phenols, \( pK = 8 \) to 10, and stronger than that of phenol-2-methyleneiminodiacetic acid, Structure XI, \( pK = 8.17 \). One of the strong acid functions of Calcein, one having \( pK = 5.4 \), was identified by Wallach and co-workers with the dissociation of the first phenolic group. To account for the unusual acidity of this phenolic group, Wallach and co-workers concluded that the methyleneiminodiacetic acid groups must be located one on each side of the phenolic group, that is, in the 2'-, and 4'-positions. Aside from the fact that other circumstances and factors can cause an increase in the acidity of an acidic group, the argument is based on a faulty interpretation of the titration and spectrophotometric data. No statement appears as to the end-points observed in the titration and how many equivalents of alkali were consumed at various stages in the titration; apparently
it was considered that the neutralization of the first phenol was the fifth acid neutralization step. Eight acid dissociation steps are listed including the titration of all four carboxyl groups at low pH indicating a faulty conception of the nature of the zwitter ions present. This picture is clarified in Part III of this thesis. The structure assignment for Calcein of Wallach and co-workers based on absorption in the ultraviolet and its behavior as an acid can only be considered suspect.

Some supporting evidence for the assignment of structure made for Calcein was adduced by Wallach and co-workers (6). They observed in the spectra of fluorescein and Calcein, but not in that of tetraiodofluorescein, a band at 800 to 860 cm.\(^{-1}\) which they attributed to unsymmetrical trisubstitution. They observed also a band at 920 cm.\(^{-1}\) attributed to a single aromatic hydrogen atom. Infrared spectra of Calcein obtained in the course of the present work were invariably characterized by broad, poorly resolved bands which provided no basis for studies on structure. The common techniques for improving infrared spectra were tried without profit and it was concluded that infrared spectra offered little if any reliable information about Calcein.

Quite in contrast to the infrared spectra, the nuclear magnetic resonance spectra of Calcein, fluorescein and substituted calceins and fluoresceins were characterized by
numerous sharp bands from which the structure of Calcein could be deduced. Some interference from bands of the solvent was encountered but this was confined to the portion of the spectra related to the methyleneiminodiacetic acid parts of the molecules leaving the aromatic portions free for what proved to be an unambiguous assignment of structure.

Experimental work

**Nuclear magnetic resonance spectrometer.** The instrument used to obtain the spectra was the Varian Associates A60 Nuclear Magnetic Resonance Spectrometer. This is a 60-megacycle instrument and is limited to proton work. With the radio frequency oscillator set at 60-megacycles per second, the sweep generator periodically sweeps the main magnetic field in the immediate vicinity of 14,092 gauss, the approximate energy necessary to cause a proton to flip from its low energy state to its high energy state. For protons the range of sweep is of the order of 1000 cycles per second. Calibration of chemical shifts is in dimensionless units (δ, parts per million) from a reference marker, usually tetramethylsilane. Chemical shifts, δ, are obtained by dividing the distance of the shifts from the reference, in cycles per second, by the applied frequency and multiplying by 10⁶.
\[ \delta = \frac{\text{cycles per second} \times 10^6}{60 \times 10^6} \]  

where the denominator is the value of the applied radio frequency field, \(60 \times 10^6\) cycles per second. Peak areas are measured by the integrator, which on the A60 NMR Spectrometer superimposes a series of steps on the absorption peaks. The step heights are proportional to the number of protons under the respective peaks.

**Cells.** Clear, precision ground glass NMR tubes, 0.5 cm. o.d. were used.

**Materials.** Dimethyl sulfoxide. The dimethyl sulfoxide used was distilled over calcium hydride under reduced pressure and stored over molecular sieves.

**Reference standard.** Tetramethylsilane was used as the internal reference in all spectra. About two milliliters of tetramethylsilane (TMS) vapor was added to each sample and the sample was shaken well and the NMR tube capped to ensure that the TMS would not escape from the sample. The TMS peak was arbitrarily set at zero parts per million and all subsequent measurements of proton peak positions were referred to this in terms of part per million downfield shift.

**Chemicals used.** Calcein. The Calcein used was the highly purified material prepared in earlier work (7).

2-Methylresorcinol. Commercial 2-methyl resorcinol was purified by sublimation. The sublimation was carried out
in a large evaporating dish, the crude product being covered with a sheet of perforated filter paper. An inverted glass funnel placed over the product and paper served as a condenser. The 2-methylresorcinol formed large, white needles, m.p. 117-118°, reported 116-121° (11).

4',5'-Dimethylfluorescein. 4',5'-Dimethylfluorescein was synthesized by the following procedure. A thoroughly blended mixture of 400 g. of 2-methylresorcinol and 275 g. of phthalic anhydride was heated with stirring until the mixture melted. The temperature was held between 120° and 150° until the melt solidified (about two hours). The melt was dissolved in sodium hydroxide solution and the crude 4',5'-dimethylfluorescein was precipitated by adding concentrated hydrochloric acid. The product was again dissolved in sodium hydroxide and reprecipitated by addition of dilute hydrochloric acid (1:1). The product was dried and further purified by acetylation.

To 200 g. of impure 4',5'-dimethylfluorescein was added 100 g. of anhydrous sodium acetate and one liter of acetic anhydride. The mixture was refluxed for 2.5 hours. A white solid and dark brown liquid were produced. The white solid was water soluble and did not possess the properties expected of the diacetate of 4',5'-dimethylfluorescein. The liquid product of the reaction was poured into three liters of
deionized water which precipitated the diacetate of 4',5'-dimethylfluorescein. The precipitate did not form immediately and appeared first as an oil which crystallized on standing. The diacetate was recrystallized from 95 percent ethanol using a Soxhlet extractor. The diacetate was then hydrolyzed by refluxing with sodium hydroxide in 95 percent ethanol. The resulting solution was poured into three liters of deionized water and dilute (1:1) hydrochloric acid was added which precipitated the 4',5'-dimethylfluorescein. This was dissolved in sodium hydroxide and reprecipitated with hydrochloric acid several times.

4',5'-Dimethylcalcein. This compound was prepared by the Mannich condensation of 1.0 mole of 4',5'-dimethylfluorescein, 2.5 mole disodium imonidiacetate dihydrate and 5.0 mole of formaldehyde using glacial acetic acid as solvent, the conditions and procedure being the same as those developed for the synthesis of Calcein (7).

The 4',5'-dimethylcalcein was similar in appearance to Calcein. It was yellow-orange and was not of definite crystalline structure but was fluffy in appearance. No melting point was obtained; rather, as with Calcein, decomposition occurred on heating above 100°. The NMR spectrum observed was that expected for a molecule bearing two methyleneiminodiacetic acid groups and an unsubstituted phthalate ring. Over the region of the spectrum related to
the resorcinol rings the spectrum was poor and although integration over the two diffuse peaks observed totaled two protons, the interpretation of the spectra leads to an unlikely unsymmetrical structure. More work is required.

2',7'-Dichlorocalcein. This compound was prepared by the Mannich condensation of 1.0 mole of 2',7'-dichloro-fluorescein, 2.5 mole disodium iminodiacetate dihydrate and 5.0 mole of formaldehyde using glacial acetic acid as solvent, the conditions and procedure being the same as those developed for the synthesis of Calcein (7).

The 2',7'-dichlorocalcein was orange-yellow in color. No melting point was obtained, rather decomposition was observed at temperature above 100°. The NMR spectrum was sharp and easily interpreted; see below for details. The compound is 3',6'-dihydroxy-2',7'-dichloro-4',5'bis[N,N'-di-(carboxymethyl)aminomethyl]fluoran.

2',7'-Dichlorofluorescein. Obtained from Eastman Organic Chemicals and used as received for the preparation of 2',7'-dichlorocalcein.

4',5'-Dibromofluorescein. Obtained from Eastman Organic Chemicals and used as received.

4',5'-Diiodofluorescein. Obtained from Eastman Organic Chemicals and used as received.

2',4',5',7'-Tetrabromofluorescein. Obtained from Eastman Organic Chemicals and used as received.
2',4',5',7'-Tetraiodofluorescein. Obtained from Eastman Organic Chemicals and used as received.

Selection of solvent. NMR spectra are obtained on solutions of the material under examination in a suitable solvent. The concentration must be quite high, preferably of the order of 100 mg. per milliliter of solvent. Calcein, unfortunately, is only slightly soluble in the usual solvents. Large amounts of Calcein will dissolve in aqueous solutions of sodium hydroxide and potassium hydroxide but such solutions are unsatisfactory for NMR work. A large band would be introduced by the water, the position of which is variable and the neutralization of the six replaceable hydrogen atoms would complicate the picture.

Two solvents, trifluoroacetic acid and dimethylsulfoxide, proved useful but each suffered some disadvantages. The replaceable hydrogen atom of trifluoroacetic acid and those of Calcein exchanged so rapidly that the carboxyl and phenolic peaks of the spectrum were lost under the solvent peak.

The peak of dimethylsulfoxide when used as solvent is broad and several ringing side peaks are present at high sensitivity so that some of the peaks of Calcein were covered; this solvent peak covers the region 1.5 to 4.0 p.p.m. and makes interpretation in this area quite difficult. It is in this area that the peaks of aliphatic protons
usually appear; fortunately the part of the spectrum where aromatic protons appear is not affected. Because the region of interest in this work was mainly the aromatic region, dimethylsulfoxide was used as the solvent.

Results and Discussion

The nuclear magnetic resonance spectra were obtained of fluorescein, $2',7'$-dichlorofluorescein, $4',5'$-dibromofluorescein, $4',5'$-diiodofluorescein, $4',5'$-dimethylfluorescein, $2',4',5',7'$-tetrabromofluorescein, $2',4',5',7'$-tetraiodofluorescein, Calcine, $2',7'$-dichlorocalcein, and $4',5'$-dimethylcalcein. The results are summarized in Tables 1 and 2, the chemical shift in cycles per second together with the results of integrations being given in Table 1 and in terms of δ in parts per million (p.p.m.) in Table 2. The NMR spectra are shown for fluorescein, Figure 1; $2',7'$-dichlorofluorescein, Figure 2; $2',7'$-dichlorocalcein, Figure 3; and Calcine, Figure 4.

For interpreting the NMR spectra, the nature of NMR spectroscopy makes it convenient to divide the spectra and the discussion into three parts: (1) that dealing with the phthalate ring; (2) that dealing with the methyleneimino-diaceic acid groups, and (3) that dealing with the resorcinol rings of the molecules.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Proton Position in Cycles per Second</th>
<th>(Integration)</th>
<th>a&lt;sup&gt;a&lt;/sup&gt;</th>
<th>b&lt;sup&gt;b&lt;/sup&gt;</th>
<th>c&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1' , 8'</td>
<td>2' , 7'</td>
<td>4' , 5'</td>
<td>4</td>
<td>5 , 6</td>
</tr>
<tr>
<td><strong>Fluorescein</strong></td>
<td>-400(4)</td>
<td>412(2)</td>
<td>483(1)</td>
<td>463(2)</td>
<td>438(1)</td>
</tr>
<tr>
<td><strong>4' , 5' - Dibromo-fluorescein</strong></td>
<td>-403(4)</td>
<td>483(1)</td>
<td>467(2)</td>
<td>446(1)</td>
<td></td>
</tr>
<tr>
<td><strong>4' , 5' - Diodo-fluorescein</strong></td>
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<td>485(1)</td>
<td>467(2)</td>
<td>440(1)</td>
<td></td>
</tr>
<tr>
<td><strong>4' , 5' - Dimethylfluorescein</strong></td>
<td>410</td>
<td>384</td>
<td>482(1)</td>
<td>460(2)</td>
<td>444(1)</td>
</tr>
<tr>
<td><strong>2' , 7' - Dichloro-fluorescein</strong></td>
<td>399(2)</td>
<td>416(2)</td>
<td>483(1)</td>
<td>468(2)</td>
<td>440(1)</td>
</tr>
<tr>
<td><strong>2' , 4' , 5' , 7' - Tetraiodo-fluorescein</strong></td>
<td>435(2)</td>
<td></td>
<td>499(1)</td>
<td>470(2)</td>
<td>450(1)</td>
</tr>
<tr>
<td><strong>2' , 4' , 5' , 7' - Tetrabromo-fluorescein</strong></td>
<td>427(2)</td>
<td></td>
<td>494(1)</td>
<td>457(2)</td>
<td>433(1)</td>
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<tr>
<td><strong>4' , 5' - Dimethylcalcein</strong></td>
<td>377</td>
<td>401</td>
<td>479(1)</td>
<td>462(2)</td>
<td>441(1)</td>
</tr>
<tr>
<td><strong>2' , 7' - Dichlorocalcein</strong></td>
<td>399(2)</td>
<td>-396(4)</td>
<td>482(1)</td>
<td>468(2)</td>
<td>442(1)</td>
</tr>
<tr>
<td><strong>Calcein</strong></td>
<td></td>
<td></td>
<td>478(1)</td>
<td>464(2)</td>
<td>436(1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Methylene protons in acetic acid part of methylene iminodiacetic acid group

<sup>b</sup> Methylene protons on carbon linking amino group to ring

<sup>c</sup> Acidic protons

<sup>d</sup> Acidic protons show up in the same position. This indicates rapid exchange of phenolic and carboxylic protons of Calcein in dimethylsulfoxide.
Table 2. Positions of proton peaks observed in NMR spectra

| Compound                        | Proton Position in Parts per Million Chemical Shift |  |  |  |  |  |  |  |
|--------------------------------|---------------------------------------------------|---|---|---|---|---|---|
|                                | 1',8' | 2',7' | 4',5' | 4   | 5,6 | 7  | a  |
| Fluorescein                    | -6.67-| 6.87  | 8.05  | 7.72 | 7.30 | 9.83 |
| 4',5'-Dibromo-fluorescein      | -6.72-| 8.05  | 7.78  | 7.43 |
| 4',5'-Diiodo-fluorescein       | -6.67-| 8.08  | 7.78  | 7.33 |
| 4',5'-Dimethylfluorescein       | 6.83  | 6.40  | 8.03  | 7.67 | 7.60 | 12.65 |
| 2',7'-Dichloro-fluorescein     | 6.65  | 6.93  | 8.05  | 7.80 | 7.33 |
| 2',4',5',7'-Tetraiodo-fluorescein | 7.25 | 8.32  | 7.83  | 7.50 |
| 2',4',5',7'-Tetrabromo-fluorescein | 7.12 | 8.28  | 7.62  | 7.22 | 2.38 | 3.40 |
| 4',5'-Dimethyl-calcein         | 6.68  | 7.98  | 7.70  | 7.35 | 4.28 | 3.57 |
| 2',7'-Dichloro-calcein         | 6.65  | 8.03  | 7.80  | 7.37 | 4.32 | 3.63 |
| Calcein                        | -6.60-| 7.97  | 7.73  | 7.27 | 4.32 | 3.63 |
|                                |        |       |       |       | 10.18 |    |

a  Methylene protons in acetic acid part of methylenediminodiacetic acid group
b  Methylene protons on carbon linking amino group to ring
c  Acidic protons
Figure 1. Nuclear magnetic resonance spectrum of fluorescein showing proton peak positions and integrations.

400 cycles per second: 1',2',7',8'-protons.
412 cycles per second: 4',5'-protons.
438 cycles per second: 7'-proton.
463 cycles per second: 5,6'-protons.
483 cycles per second: 4'-proton.
Figure 2. Nuclear magnetic resonance spectrum of 2',7'-dichlorofluorescein showing proton peak positions and integrations.

399 cycles per second: 1',8'-protons.
416 cycles per second: 4',5'-protons.
440 cycles per second: 7'-proton.
468 cycles per second: 5,6'-protons.
483 cycles per second: 4'-proton.
Figure 3. Nuclear magnetic resonance spectrum of 2',7'-dichloro-
calcein showing proton peak positions and integrations.
399 cycles per second: 1',8'-protons.
442 cycles per second: 7-proton.
468 cycles per second: 5,6 -protons.
482 cycles per second: 4 -protons.
214 cycles per second: Y -protons
257 cycles per second: X -protons.
Figure 4. Nuclear magnetic resonance spectrum of Calceïn showing proton peak positions and integrations.

218 cycles per second: Y-protons (methylene).
259 cycles per second: X-protons (methylene).
396 cycles per second: 1', 2', 7', 8'-protons.
436 cycles per second: 7'-protons.
464 cycles per second: 5', 6'-protons.
478 cycles per second: 4'-protons.
611 cycles per second: acidic protons.
The NMR spectrum of the phthalate ring portion of the molecule. In the NMR spectra of the ten fluorescein and calcein compounds listed in the second paragraph above and in Table 2 and 3, a portion of each spectrum is essentially constant in position and configuration and is assigned to the protons occupying the 4-, 5-, 6-, and 7-positions, that is to those protons in the phthalate ring. These peaks appear as a doublet centered at 480 to 500 cycles per second (δ = 8.00 to 8.33) with integration showing one proton as a triplet centered around 460 cycles per second (δ = 7.67) with integration showing two protons, and as a doublet centered about 440 cycles per second (δ = 7.33) with integration showing one proton.
The doublets at δ = 7.33 and at δ = 8.00 to 8.33, both of which show only one proton on integration indicate a carbon atom with a single proton adjacent to another carbon atom bearing a single proton; these are the protons in the 4- and 7-positions. The electron withdrawing properties of the oxygen atoms cause a deshielding of nearby protons shifting the peak of the proton downfield, that is, to a higher delta. The proton of the 4-position is closer to these oxygen atoms and accordingly the peak at δ = 8.00 to 8.33 is assigned to the proton in the 4-position and the peak at δ = 7.33 to the proton in the 7-position.

The center peak in this region appears as a triplet integrating to two protons in all ten spectra. A triplet indicates one or more protons under the influence of two adjacent protons, either two protons on an adjoining carbon atom (not possible in a benzenoid ring) or one proton on each of two adjacent carbon atoms. The integration observed indicates that two such protons are present; these must be in the 5- and 6-positions.

The NMR spectrum of the methyleneiminodiacetic acid portion of the molecule. In the NMR spectra of all three of the calceins studied, two singlet peaks appear in the region 120 to 240 cycles per second (δ = 2.0 to 4.0), the general region of aliphatic protons. In the spectrum of Calcein, these peaks appear at 218 cycles per second (δ = 3.63)
and 259 cycles per second ($\delta = 4.32$) and the integrations show eight and four protons, respectively. In the spectrum of 2',7'-dichlorocalcein these peaks appear at 257 cycles per second ($\delta = 4.28$) and at 214 cycles per second ($\delta = 3.57$) but the peaks could not be integrated because of interference of the ringing peaks of the dimethylsulfoxide solvent. The heights of the peaks were in the ratio two to one and presumably represent the eight and four protons of the two methyleneiminodiacetic acid groups. In the spectrum of 4',5'-dimethylcalcein these peaks appear at 143 cycles per second ($\delta = 2.38$) and 204 cycles per second ($\delta = 3.40$). Again the integrations could not be made because of interference from the peaks of the solvent but again the heights of the peaks were in the ratio of two to one.

The peaks integrating to four protons must be the protons on the methylene group between the resorcinol rings and the nitrogen atoms; no splitting occurs because there are no adjacent protons. The peaks integrating to eight protons must be the protons on the four methylene groups between the nitrogen atoms and the four carboxyl groups.

The NMR spectrum of the resorcinol rings portion of the molecule. In each of the NMR spectra of the four compounds, fluorescein, 2',7'-dichlorofluorescein, Calcein and 2',7'-dichlorocalcein, a singlet peak occurs at 400 cycles per second ($\delta = 6.67$) (actually at 400, 399, 396, and 399
cycles per second, respectively). Integration of this peak gives a value of four in the spectra of fluorescein and Calcein but of two in the spectra of 2',7'-fluorescein and 2',7'-dichlorocalcein.

This means that in fluorescein and Calcein four identical protons are present; these obviously must be those in the positions 1', 2', 7', and 8'. In 2',7'-dichlorofluorescein and 2',7'-dichlorocalcein only two such protons are present, in the 1'- and 8'-positions.

In the spectrum of fluorescein and in the spectrum of 2',7'-dichlorofluorescein appear a singlet peak, integrating to two protons, at 412 and 416 cycles per second (\(\delta = 6.87\) and 6.93), respectively, which is not found in the spectra of Calcein and 2',7'-dichlorocalcein. These protons can be either the 4',5'- protons or the 1',8'- protons. Because this peak lies farther downfield, it is apparent that the protons are deshielded; this can only result from the oxygen atoms which lie on either side of the 4'- and 5'-positions and the assignment of this peak is thus to the protons in the 4'- and 5'-positions.

An objection which may be raised to the above assignment of the peak at 400 cycles per second is that the peak should appear as a doublet because of splitting by the proton on the adjacent carbon atom, that is the 1'- by the 2'-, the 2'- by the 1'-, the 7'- by the 8'-, and the 8'- by the 7'-. 
The phenomenon generally referred to as "collapsing" probably is responsible for the peak appearing as a singlet. Collapsing is common although not invariable in the spectra of monosubstituted benzenes.

In the spectra of $4',5'$-dibromofluorescein and $4',5'$-diiodofluorescein there appears a singlet peak at 403 cycles per second ($\delta = 6.72$) and 400 cycles per second ($\delta = 6.67$), respectively. The integration in each case gave a value of four. These peaks must be those of the four protons in the $1'$-, $2'$-, $7'$-, and $8'$- positions inasmuch as these are the only protons present in the resorcinol rings. These peaks lie in the same position as the 400 cycles per second peak of the four compounds under discussion above and this supports the assignment of the 400 cycles per second peak in these compounds to the $1'$-, $2'$-, $7'$-, and $8'$- positions.

In the spectrum of $2',4',5',7'$-tetrabromofluorescein and of $2',4',5',7'$-tetraiodofluorescein there appears a singlet peak, at 435 cycles per second ($\delta = 7.25$) and 427 cycles per second ($\delta = 7.12$), respectively. The integration in each case gave a value of two. These must be the protons in the $1'$- and $8'$- positions. These peaks lie far down-field as expected because of the electron withdrawing effects of the various oxygen and halogen atoms.

Unlike the spectra of fluorescein, $2',7'$-dichlorofluorescein, $4',5'$-dibromofluorescein, $4',5'$-diiodofluorescein,
and Calcein, in all of which the spectrum of the four protons present collapsed to a singlet integrating to four, there was present in the spectrum of 4',5'-dimethylfluorescein two doublets, centered at 410 cycles per second (δ = 6.83) and 384 cycles per second (δ = 6.40), both integrating to two protons. These must, of course, be the protons in the 1', 2', 7' and 8' positions, but the expected two peaks appear rather than the collapsed singlet of the analogous compounds.

The NMR spectrum of 4',5'-dimethylcalcein obtained was that expected for a molecule bearing two methyleneiminodiacetic acid groups and an unsubstituted phthalate ring. The spectrum in the region of the resorcinol rings was very poor. Two diffuse bands with high noise level did appear, at 377 and 401 cycles per second, integrating to a total of two protons. Two singlet peaks of this kind indicate two adjacent protons each in a different environment. This implies two hydrogen atoms in the same ring and that the material may be the unsymmetrically substituted compound 3',6'-dihydroxy-4',5'-dimethyl-1,2-bis[N,N'-di(carboxymethyl)aminomethyl]-fluoran. The material may have been a mixture and more work is required.

**Review of the NMR spectroscopy of Calcein.** The discussion of the NMR spectrum of Calcein was made in three parts and was unavoidably interwoven with the discussion of the spectra of related compounds. In overall review, it should
be said that the spectrum of Calcein proved clean cut and interpretable in unambiguous fashion. The spectrum in one region was clearly that of an aromatic ring bearing four hydrogen atoms on successive carbon atoms (phthalate ring); the integration was that expected for four hydrogen atoms. The spectrum in a second region was exactly that, in number and position of peaks and in integration, expected for two methyleneiminodiacetic acid groups. In the region of greatest interest, that reflecting the substitution in the resorcinol rings, the pattern was the remarkably simple one of one singlet peak. This was readily interpretable in terms of symmetrical substitution, that is, one methyleneiminodiacetic acid group into each ring, and in the 4'- and 5'-positions. Calcein is therefore fluorescein-4',5'-bis(methyleneiminodiacetic acid) or more formally: 3',6'-dihydroxy-4',5'-bis[N,N'-di(carboxymethyl)aminomethyl]fluoran.

It is incumbent now to inquire if the spectrum observed could be equally well or even at all interpreted on the basis of one of the other eight possible structures. It is fitting too, to ask about the six replaceable hydrogen atoms in the molecule and about the molecule of water which is present in the crystalline material.

Other possible structures as an explanation of the NMR spectrum of Calcein. The singlet peak integrating to four protons in the portion of the NMR spectrum of Calcein related
to the resorcinol rings is simple and indicates high symmetry. In addition to the symmetrical 4',5'-structure (Structure IX, page 9) deduced above as the structure of Calcein, two other symmetrical structures are possible, 1',8'-(Structure V, page 8) and 2',7'-(Structure VIII, page 9).

In the 1',8'-structure, the remaining four protons in the resorcinol ring occupy the 2'-, 4'-, 5'-, and 7'-positions. The 2'- and 7'-protons are alike and the 4'- and 5'-protons are also alike; but the 2'- and 7'-protons are surrounded by a quite different environment than the 4'- and 5'-protons. The NMR pattern expected then would be two singlets, each with integrations showing two protons, the positions being about 400 and 412 cycles per second.

In the 2',7'-structure, the remaining four protons occupy the 1'-, 4'-, 5'- and 8'-positions. Again pairs of protons are alike but the environments of the 1'- and 8'- and the 4'- and 5'-protons are quite different. The expected pattern again would be two singlet peaks, each integrating to two protons and lying at about 400 and 412 cycles per second (as in fluorescein and 2',7'-dichlorofluorescein).

In the six unsymmetrically substituted structures, the four hydrogen atoms are distributed about the two rings in various ways. In the 1',2'-, 1',4'- and 2',4'-structures (Structures I, II, and VI, page 8,9), the lone hydrogen atoms
in one ring would produce a single peak, the remaining three in the second ring a variety of singlets and doublets. In the unsymmetrical structures 1',5'-, 1',7'- and 2',5'- (Structures III, IV and VII, page 8,9) two hydrogen atoms are present in each ring but again the environment is different about the various individual atoms and again a variety of singlet and doublet peaks would be expected for each ring. In general then, the spectra of the unsymmetric compounds would be expected to be a more complex pattern than the simple one actually found.

Thus, all eight of the other possible structures for Calcein are rejected on the basis that each would lead to more complicated spectra than the one actually observed. Further support is thus supplied for the 4',5'-structure for Calcein deduced by direct interpretation of the observed spectrum.

NMR and the six replaceable hydrogen atoms of Calcein.

A peak in the NMR spectrum of Calcein appears at 611 cycles per second ($\delta = 10.18$). Integration over this peak gives a value of six protons.

The position far downfield indicates, that these six protons are greatly deshielded as expected for protons which are ionic in character.

Because all six of the protons appear in the singlet at 611 cycles per second, all six of the protons are exchanging
rapidly with the solvent. When excess deuterium oxide is added to Calcein in dimethylsulfoxide the peak at 611 cycles per second is shifted to 387 cycles per second ($\delta = 6.04$), the position in which the peak caused by water appears. This also indicates that all of the protons in Calcein are exchanging rapidly in dimethylsulfoxide.

NMR and the molecule of water of crystallization of crystalline Calcein. No peak was observed in the NMR spectrum of Calcein which could be assigned to the molecule of water known to be present in crystalline Calcein when first prepared. It is possible that the expected peak of the proton of water is simply obscured by the broad peak of the solvent dimethylsulfoxide. It is also possible that the water of hydration was lost during storage of the material over anhydrous phosphorus pentoxide. Unfortunately no record was kept of loss on weight during storage.

Summary

The NMR spectra of fluorescein, Calcein, six substituted fluoresceins and two substituted Calceins have been obtained and interpreted in terms of the known and unknown structures of the various molecules.

One portion of the NMR spectrum of each of the ten compounds studied is essentially identical in character, number, position and proton integration of peaks observed and is attributed to four protons on successive carbons in a
benzene ring. Thus, no substitution into the phthalate ring of the Calcein compounds occurred during their synthesis.

Another portion of the NMR spectra of Calcein, 2',7'-dichlorocalcein and 4',5'-dimethylcalcein was essentially identical in character and position of the peaks in the region expected for aliphatic protons. Integration showed the presence of the eight methylene hydrogen atoms corresponding to those of the four acetic acid groups present, and four methylene hydrogen atoms of a second kind, as expected for the two methylene groups linking the amino acids to the rings.

In a third region of the spectra appeared a group of peaks attributed to the protons of the resorcinol rings. Particular attention was paid to this part of the spectra of fluorescein, 2',7'-dichlorofluorescein, 4',5'-dibromo-fluorescein, 4',5'-diiodofluorescein, 4',5'-dimethyl-fluorescein, Calcein, 2',7'-dichlorocalcein, and 4',5'-dimethylcalcein. The similarities and differences in the number, position and character of the peaks and the integration over each for the number of protons led to a consistent and complete assignment of the various peaks. The simple spectrum of Calcein in this region (one singlet peak integrating to four protons) made it clearly evident that the molecule is highly symmetrical, with one methyleneimino-diacetic acid group present in each resorcinol rings and in
the positions between the ring oxygen atom and the phenol
groups. That is, Calcein is fluorescein-4′,5′-bis(methylene-
iminodiacetic acid) or more formally 3′,6′-dihydroxy-4′,5′-
bis[N,N′-di(carboxymethyl)aminomethyl]fluoran. The NMR
spectra predicted for the other eight structures possible
for Calcein are all more complex than the simple spectrum
found.

The NMR spectrum shows that all six of the replaceable
hydrogen atoms in the molecule of Calcein exchange rapidly
with the solvent used, dimethylsulfoxide.

The two methylenediacetic acid groups in the Calcein
derived from 2′,7′-dichlorofluorescein was shown by NMR
spectroscopy to occupy the 4′- and 5′- positions.

The Calcein derived from 4′,5′-dimethylfluorescein
appears from the NMR spectrum to be the unsymmetrically
substituted compound 4′,5′-dimethylfluorescein-1′,2′-bis-
(methyleneiminodiacetic acid) but work is required on this
compound.
PART III. DETERMINATION OF ACID DISSOCIATION CONSTANTS

Titration of the highly purified material with alkali quickly revealed that the equivalent weight of Calcein is 160 and thus that Calcein acts as a tetrabasic acid. The endpoint could be located with precision and the titration afforded a useful and accurate criterion of purity.

Actually six replaceable hydrogen atoms are present in the Calcein molecule, the acidic functions being four carboxyl groups and two phenol groups, but actually probably acting, because of the zwitter ion structure, as two free carboxyl groups, two phenol groups and two ammonium ions. The fluorescence and the union with metal ions are pH dependent and thus a knowledge of the nature of the behavior of Calcein as an acid is of considerable interest. The problem of determining the acid dissociation constants is complicated by the low solubility of Calcein, the material not going into solution completely during titration with alkali until some 1.5 moles of alkali have been added and the pH raised to about 4. Success was achieved in determining all six of the acid dissociation constants of Calcein but it was necessary to use four lines of attack: (1) Potentiometric titration with alkali, which yielded values for $K_2$, $K_3$ and $K_4$; (2) Measurement of the solubility as a function of pH, which led to a value for $K_1$; (3) Absorption spectrophotometry, which gave values for $K_3$. 
$K_2 \cdot K_3$ and $K_3 \cdot K_4$; and (4) Fluorescence spectrophotometry, which gave values for $K_3 \cdot K_4$, $K_5$ and $K_6$. Each of these lines of attack is now discussed separately.

**Determination of Acid Dissociation Constants**

**By Potentiometric Titration**

Potentiometric titration with alkali using the glass electrode is the simplest and most direct method of obtaining the acid dissociation constants of an acid. Complications may arise in interpreting the titration curve if the acid under investigation has more than one replaceable hydrogen atom, particularly if the values of the successive dissociation constants are not widely separated. This is true of Calcein with the further complication that, because of low solubility, the titration data could not be used to determine the first acid dissociation constant. On titration of a sample of the customary size, 0.2 g. or so in 200 ml. of water, with sodium hydroxide, the material did not pass completely into solution until the pH had been raised to 4 or 4.5. Thus, depending on the speed with which the titration was carried out, the data up to about 2 to 2.5 moles of alkali added was not meaningful. By adding a measured excess of standard alkali and back titrating with standard hydrochloric acid, a titration curve was obtained identical to the direct titration curve but with a useful range down to pH 3 or lower because precipitation of free Calcein did not occur before this pH was reached. Even so,
it was necessary to evaluate the first acid dissociation
constant by the solubility method (following section).
Because of an interesting feature of the characteristics of
Calcein as an acid it became possible to evaluate the second,
third and fourth constants by a relatively simple interpreta-
tion of the titration data.

Experimental work

Materials. The Calcein used was the highly purified
material prepared in earlier research (7).

Apparatus. A Beckman Expanded Scale pH Meter was used.
A high alkalinity electrode was used. It was necessary to
apply corrections to the readings obtained on this electrode
because of deviations observed when calibrating against
standard buffers; when the pH meter was zeroed with the pH 7.00
buffer the reading with the pH 4.00 buffer was 3.82 and with
the pH 10.00 buffer, 10.18. Because of this, a correction was
applied over the entire range, the correction function being
assumed to be linear with a slope of 0.06 pH units per pH unit.

Titrations. The direct titration of Calcein was carried
out in the following manner. To 0.4675 g. of Calcein was added
about 200 ml. of water. The resulting mixture was titrated with
0.0835 N sodium hydroxide. Complete dissolution of the Calcein
did not occur until a pH of 4 was reached. The titration
curve obtained is shown in Figure 5.
Figure 5. Potentiometric titration of Calcein with sodium hydroxide.

Weight of Calcein taken: 0.4675 g.

Concentration of sodium hydroxide: 0.0835 N.
The indirect titration was carried out by the following method. To 0.2140 g. of Calcein was added 35.00 ml. of 0.0997 N sodium hydroxide (an excess of two times the necessary amount to titrate the Calcein). The resulting solution was then titrated with 0.1114 N hydrochloric acid using a total of 50.00 ml. of acid. Using the total amount of Calcein present and the amount of sodium hydroxide added, the volume of hydrochloric acid required to titrate the excess sodium hydroxide was calculated. The pH corresponding to this amount was considered 200 percent titration. The end-point value was taken from the titration curve and the 0 percent titration point was taken as the point corresponding to the same number of milliliters as from 200 percent titration to the end-point. The titration curve obtained in this manner is shown in Figure 6.

Results and discussion

The titration curve obtained by adding a measured excess of standard sodium hydroxide and back titrating with standard hydrochloric acid superimposed that obtained by direct titration with sodium hydroxide; the data was more regular in character and useful in the region pH 3 to 4.

The data obtained in the titration of Calcein by the addition of a measured excess of standard alkali and back titration with hydrochloric acid, Table 3 and Figure 6, was used to calculate the second, third and fourth acid
Figure 6. Titration of Calcein by addition of a measured volume of standard sodium hydroxide and back titration with standard hydrochloric acid.

Weight of Calcein taken: 0.2140 g.

Sodium hydroxide added: 35.00 ml. of 0.0997 N.

Concentration of hydrochloric acid: 0.1114 N.

Right inset: Theoretical titration over the buffer region of a monobasic acid.

Left inset: Theoretical titration over the buffer region of a dibasic acid.

Theoretical curves for monobasic acid superimposed on experimental curve at the midpoints of the first four stages.

1. Point where precipitation of free Calcein begins in the back titration.
a — SODIUM HYDROXIDE ADDED —

$1/2 \log \frac{2-a}{a}$

pH

HYDROCHLORIC ACID
a — SODIUM HYDROXIDE ADDED — moles

HYDROCHLORIC ACID — ml.
E ADDED — moles

HCLORIC ACID — ml.
dissociation constants, $K_2$, $K_3$ and $K_4$.

Table 3. Titration of Calcein. Addition of a measured amount of standard sodium hydroxide and back titration with standard hydrochloric acid; 35.00 ml. 0.0997 N sodium hydroxide; 200 ml. water; 0.1114 N hydrochloric acid

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<th>Hydrochloric acid, ml.</th>
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$^a$ Precipitation of free Calcein began between 28.00 and 29.00 ml. of hydrochloric acid added
As will be seen on inspection of Figure 6, a single break appears in the titration curve, at four moles of alkali. The material is a fairly strong acid and no suggestion of even minor breaks or more than one inflection point appear in the curve prior to the break. The pH at the various stages of the titration are given in Table 4.

Table 4. Titration of Calcein. pH at various stages in the titration. Data taken by interpolation of original data given in Table 3

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<th>Alkali added (moles per ml. of Calcein)</th>
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</table>
The significance of the absence of minor breaks and inflections in the curve is that there exists a fairly uniform distribution of the numerical values of the constants, that is, that the ratio of any two successive constants is probably less than 100. This means that considerable overlap occurs in the titration of two successive steps but also that only two steps are involved at any particular pH. This latter greatly simplifies the problem of obtaining the values of the constants from the titration data.

Theoretical titration curves over the buffer region for a monobasic acid and for a dibasic acid having $K_1 = K_2$ are shown in the inserts of Figure 6 and the theoretical curve for a monobasic acid is superimposed on the titration curve at various stages. Over the upper branch of the fourth stage of the titration, the experimental curve and the theoretical curve superimpose, that is, over the region $a = 3.4$ to $a = 3.8$, $a$ being the number of moles of base added per mole of calcium or the fraction of the four replaceable hydrogen atoms titrated. Over the third, second and first stages of the titration the curves do not superimpose, the departure becoming greater successively, but even over the first stage the slope at the mid-point ($a = 1.5$) is still greater than that of a dibasic acid having $K_1 = K_2$. 
For Calcein we adopt the symbol $H_6A$ and for the six dissociation steps define the successive acid dissociation constants, $K_1$, $K_2$, $K_3$, $K_4$, $K_5$, and $K_6$ by the equations

$$
H_6A == H^+ + H_5A^- \quad K_1 = \frac{[H^+][H_5A^-]}{[H_6A]} \quad (2)
$$

$$
H_5A^- == H^+ + H_4A^{2-} \quad K_2 = \frac{[H^+][H_4A^{2-}]}{[H_5A^{2-}]} \quad (3)
$$

$$
H_4A^{2-} == H^+ + H_3A^{3-} \quad K_3 = \frac{[H^+][H_3A^{3-}]}{[H_4A^{2-}]} \quad (4)
$$

$$
H_3A^{3-} == H^+ + H_2A^{4-} \quad K_4 = \frac{[H^+][H_2A^{4-}]}{[H_3A^{3-}]} \quad (5)
$$

$$
H_2A^{4-} == H^+ + HA^{5-} \quad K_5 = \frac{[H^+][HA^{5-}]}{[H_2A^{4-}]} \quad (6)
$$

$$
HA^{5-} == H^+ + A^{6-} \quad K_6 = \frac{[H^+][A^{6-}]}{[HA^{5-}]} \quad (7)
$$

Considering the fourth dissociation step as a monobasic acid, the mid-point ($a = 3.50$) pH may be taken as a value for the negative logarithm of the acid dissociation constant, $pK_4 = pH_{mid-point} = 6.19$. A somewhat elaborate way of doing the same thing is to use the observed pH at various stages of the titration and Equation (8) obtained by rearranging Equation (5).
\[ K_4 = \frac{[H^+][H_2A^{-4}]}{[H_3A^{-3}]} \]  

\[ [H^+] = K_4 \frac{[H_3A^{-3}]}{[H_2A^{-4}]} \]

\[- \log[H^+] = - \log K_4 - \log \frac{[H_3A^{-3}]}{[H_2A^{-4}]} \]

\[ pH = pK_4 - \log \left( \frac{[H_3A^{-3}]}{[H_2A^{-4}]} \right) \]  

For \([H_3A^{-3}]\), the untitrated acid, we use the successive values of \(a\), \(a = 3.30, 3.40, \ldots 3.70\); for \([H_2A^{-4}]\), the salt formed, \(4.00 - a\). The values obtained were

<table>
<thead>
<tr>
<th>(a)</th>
<th>(pH)</th>
<th>+log (\frac{4.00 - a}{a})</th>
<th>pK4</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.30</td>
<td>5.80</td>
<td>+0.37</td>
<td>6.17</td>
</tr>
<tr>
<td>3.40</td>
<td>6.02</td>
<td>+0.18</td>
<td>6.20</td>
</tr>
<tr>
<td>3.50</td>
<td>6.19</td>
<td>0</td>
<td>6.19</td>
</tr>
<tr>
<td>3.60</td>
<td>6.37</td>
<td>-0.18</td>
<td>6.19</td>
</tr>
<tr>
<td>3.70</td>
<td>6.57</td>
<td>-0.37</td>
<td>6.20</td>
</tr>
</tbody>
</table>

Average: \(pK_4 = 6.19\)

A plot of \(pH\) versus \(\log \left( \frac{4.00 - a}{a} \right)\) gave a straight line having the theoretical slope of 1.0.

Because the successive acid dissociation constants differ appreciably, it became possible to determine \(K_2\) and \(K_3\) by considering successive steps in pairs. For a dibasic acid, the ionization reactions and the two acid dissociation constants are
Multiplication of the respective sides of Equations (9) and (10) gives

\[ K_1K_2 = \frac{[H^+]^2[HA^-][A^-]}{[H_2A][HA^-]} \]  

\[ [H^+] = \sqrt{\frac{K_1K_2[H_2A]}{[A^-]}} \]  \hspace{1cm} (11)

As applied to the determination of the third acid dissociation constant of Calcein

\[ [H^+] = \sqrt{\frac{K_3K_4 H_2A^-}{H_4A^-}} \]  \hspace{1cm} (12)

At the point \( a = 3.00 \), theoretically only \( H_3A^- \) should be present; actually because of the overlap some \( H_2A^- \) has formed and some \( H_4A^- \) remains untitrated, the amounts of the two being equal:

\[ [H_4A^-] = [H_2A^-] \]

Equation (12) thus becomes

\[ [H^+](a = 3.00) = \sqrt{K_3K_4} \]

and

\[ \frac{1}{2} pK_3 = pH(a = 3.00) - \frac{1}{2} pK_4 \]
Using the data of Table 4, pH\(_{a = 3.00}\) = 5.39, and 
\(pK_4 = 6.19\), gives

\[
\frac{1}{2} pK_3 = 5.39 - \frac{1}{2}(6.19) \\
pK_3 = 4.58
\]

In a similar manner, a value was obtained for pK\(_2\):

\[
\frac{1}{2} pK_2 = pH\(_{a = 2.00}\) - \frac{1}{2} pK_3 \\
= 4.09 - \frac{1}{2}(4.58) \\
pK_2 = 3.60
\]

Another value for the second acid dissociation constant was obtained in a similar manner using the value for the first acid dissociation constant obtained by the solubility method (following section), pK\(_1\) = 2.53.

\[
pK_2 = pH\(_{a = 1.00}\) - \frac{1}{2} pK_1 \\
= 3.02 - \frac{1}{2}(2.53) \\
pK_2 = 3.47
\]

This calculation involves titration data taken after precipitation of Calcein began (on the addition of standard hydrochloric acid), the value obtained is probably as good as that obtained above by two successive calculations from pK\(_4\). The average of the two, 3.60 and 3.47 is adopted, that is, pK\(_2\) = 3.53.

In this work the activities of the various ions have been assumed to be equal to the concentration (activity coefficient equal to one). No attention was paid to making the solutions of definite and constant ionic strength. Thus, the values
obtained approximate only the thermodynamic constants and are probably reliable to only two significant figures. Summarizing then

\[
\begin{align*}
K_1 &= 2.9 \times 10^{-3} \quad &pK_1 &= 2.5 \\
K_2 &= 3.4 \times 10^{-4} \quad &pK_2 &= 3.5 \\
K_3 &= 2.6 \times 10^{-5} \quad &pK_3 &= 4.6 \\
K_4 &= 6.4 \times 10^{-7} \quad &pK_4 &= 6.2 \\
\end{align*}
\]

The ratios of these constants taken successively are

\[
\begin{align*}
\frac{K_1}{K_2} &= 8.7 \\
\frac{K_2}{K_3} &= 13. \\
\frac{K_3}{K_4} &= 42. \\
\end{align*}
\]

Summary

The potentiometric titration of Calcein with sodium hydroxide yields a titration curve with one break, at pH = 8.30, corresponding to the replacement of four hydrogen atoms. Because of the low solubility of Calcein, better data for the purposes of calculating acid dissociation constants is obtained by adding a measured excess of standard sodium hydroxide and back titrating with standard hydrochloric acid.

Because of the low solubility a value for the first acid dissociation constant could not be obtained from the titration data.

The titration curve shows no minor breaks or inflections
up to the end-point indicating that the values of the four dissociation constants are more or less uniformly separated. At any particular pH then, only two steps of the titration overlap and the calculation of the successive constants is simplified.

The fourth step of the neutralization is that of a mono-basic acid and a value of $K_4$ is obtained directly. The third dissociation constant was obtained from the fourth dissociation constant and titration data. Similarly the second was obtained from the third. A value for the second was also obtained from a value for the first constant and titration data.

The values obtained for the second, third and fourth acid dissociation constants are: $K_2 = 3.4 \times 10^{-4}$ ($pK_2 = 3.5$); $K_3 = 2.6 \times 10^{-3}$ ($pK_3 = 4.6$); $K_4 = 6.4 \times 10^{-7}$ ($pK_4 = 6.2$).

Determination of Acid Dissociation Constants

By Solubility Measurements

Introduction

During the course of a titration of Calcein with sodium hydroxide, the Calcein being titrated remains undissolved until the pH of the solution reaches 3.5 or 4 corresponding to the addition of 1.5 to 2 moles of sodium hydroxide. The titration curve up to this point cannot be used for assessing the acid dissociation constants beyond the general observation that the first dissociation constant is that of a fairly strong
A direct attack on the problem of measuring the first acid dissociation constant is available in measurements of solubility as a function of pH. The method was proposed by Krebs and Speakman (12) and developed for a monobasic acid. The method is straightforward in theory and experiment but for Calcein required extension because more than one replaceable hydrogen is involved in the early stages of the neutralization.

**Theory for a monobasic acid.** For the monobasic acid

\[ HA = H^+ + A^- \]

\[ K_A = \frac{[H^+][A^-]}{[HA]} \]  

(13)

of low solubility, the acid which has passed into solution is present in the two forms HA and A\(^-\), the relative amounts of the two depending on the pH. If an analytical method is available for determining the total material in solution, the solubility,
$S_o$, may then be determined in a series of solutions of varying pH.

$$S_o = [HA] + [A^-] \quad (14)$$

It is assumed that the solubility of the unionized acid remains constant over the pH range and this solubility is designated as the intrinsic solubility, $S_i$. That is,

$$S_i = [HA] \quad (15)$$

and

$$S_o = S_i + [A^-] \quad (16)$$

Making use of the mathematical equation defining the acid dissociation constant gives

$$S_o = S_i + \frac{K_A [HA]}{[H^+]} \quad (17)$$

and

$$S_o = S_i + \frac{\frac{K_A}{[H^+]}}{[H^+]} \quad (18)$$

$$S_o = S_i + \frac{K_A}{[H^+] \cdot [H^+]} \quad (19)$$

The activities of the acid and the ion are assumed to be one. Rearrangement of this equation and taking the logarithms of both sides of the resulting equation gives

$$\log \left[ \frac{S_o}{S_i} - 1 \right] = \log K_A + \log \frac{1}{[H^+]} \quad (20)$$

Two treatments of the data obtained from the measurements are made. A plot of $S_o$ versus $\frac{1}{[H^+]}$ is made; the function is a
a straight line, the intercept being the intrinsic solubility, $S_i$. Using the value of $S_i$ so obtained, a plot of $\log \left[ \frac{S_0}{S_i} - 1 \right]$ versus $\log \frac{1}{[H^+]}$ is made; the function is a straight line, the slope being one and the intercept, $\log K_A$.

Experimental work

Reagents. Calcein. The Calcein used was the highly purified material prepared earlier (7).

Buffer Solutions. All buffers were prepared from combinations of 0.1 M hydrochloric acid, 0.1 M potassium hydroxide, 0.1 M citric acid in 0.1 M potassium chloride and 0.1 M glycine in 0.1 M potassium chloride. The ionic strength of each solution was thus 0.1. The pH of each buffer was measured before and after equilibration with Calcein. The pH range of the buffers was pH 1 to 5, at 0.5 pH unit intervals before equilibration.

Solubility measurements. To 25.00 ml. of buffer in 50 ml. polyethylene bottles was added 0.10 ml. of 0.1 M ethylenediaminetetraacetic acid and sufficient solid Calcein to ensure complete saturation of the buffer. The bottles were then wrapped in aluminum foil to exclude light which causes photo-decomposition of Calcein. The bottles were then shaken for sixteen hours at 25°C to ensure complete equilibration. A standard solution was prepared and treated exactly as the buffer solutions to minimize effects of temperature variations.
and decomposition. The standard was a weighed sample of Calcein which was dissolved in 25.00 ml. of 0.1 M potassium hydroxide containing 0.10 ml. of 0.1 M ethylenediaminetetraacetic acid.

Although there was undoubtedly some difference in the total volumes of the various solutions owing to the dissolved Calcein, the maximum deviation was less than 0.1 percent which is within the experimental error of the analysis. Use of volumetric flasks was avoided as the mixing in flasks is less effective because of the narrow necks.

After equilibration of the Calcein-buffer mixtures, the excess Calcein was filtered off on filters of sintered glass frit. One-milliliter aliquots of the Calcein-buffer solutions were diluted to 50.00 ml. with 0.1 M potassium hydroxide and the absorbance measured at 493 μm. The absorbance of these solutions were then related to concentration by using the calibration curve prepared by making absorbance measurements on aliquots of the standard solution. The calibration curve is shown in Figure 7.

Results and discussion

The solubility of Calcein was measured over the pH range 1.85 to 3.50. The results are shown in Table 5.

Application of the theory of the solubility of a mono-basic acid as a function of pH as given above was made, first on the assumption that over this pH range (1.85 to 3.50)
Figure 7. Calibration curve used in the determination of Calcein.
Absorbance measured at 493 mμ.
Concentration in mg. Calcein per 100 ml.
\[ \text{pH} = 13.4 \text{ (0.1N KOH).} \]
Molar extinction coefficient: \( 4.0 \times 10^4 \).
Table 5. Solubility of Calcein as a function of pH

<table>
<thead>
<tr>
<th>pH</th>
<th>$S_0$, mg. 100 ml.</th>
<th>$[H^+]$</th>
<th>$S_0$, moles/liter</th>
<th>$\log [H^+]$</th>
<th>$\log \left( \frac{S_0}{S_1} - 1 \right)$</th>
<th>$\log \left( \frac{1}{[H^+] + K_2} \right)$</th>
<th>$\log \left( \frac{S_0}{S_1} - 1 - \frac{K_1}{[H^+]} \right)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.85</td>
<td>28.5</td>
<td>1.4 x 10^{-2}</td>
<td>71</td>
<td>-0.745</td>
<td>1.851</td>
<td>-1.52</td>
<td></td>
</tr>
<tr>
<td>2.29</td>
<td>32.1</td>
<td>5.14 x 10^{-3}</td>
<td>185</td>
<td>-0.482</td>
<td>2.267</td>
<td>-0.61</td>
<td></td>
</tr>
<tr>
<td>2.75</td>
<td>66.3</td>
<td>1.77 x 10^{-3}</td>
<td>563</td>
<td>0.243</td>
<td>2.752</td>
<td>-1.05</td>
<td></td>
</tr>
<tr>
<td>3.61</td>
<td>94.4</td>
<td>6.99 x 10^{-4}</td>
<td>145</td>
<td>0.465</td>
<td>3.165</td>
<td>+0.134</td>
<td></td>
</tr>
<tr>
<td>3.38</td>
<td>155.0</td>
<td>4.16 x 10^{-4}</td>
<td>240</td>
<td>0.736</td>
<td>3.380</td>
<td>+0.218</td>
<td></td>
</tr>
<tr>
<td>3.50</td>
<td>198.0</td>
<td>3.16 x 10^{-4}</td>
<td>517</td>
<td>0.858</td>
<td>3.501</td>
<td>+0.324</td>
<td></td>
</tr>
</tbody>
</table>

$S_1 = 3.77 \times 10^{-4}$

$K_2 = 0.5 \times 10^{-5}$

$K_1 = 2.95 \times 10^{-3}$
Calcein behaved as a monobasic acid (Calculation A) and second that it behaved as a dibasic acid (Calculation B).

Calculation A. Assumption that Calcein behaves as a monobasic acid over the pH range 1.85 to 3.50. Adopting for Calcein and the various anionic species of it the symbols

\[ \text{H}_3\text{A}, \text{H}_5\text{A}^-, \text{H}_4\text{A}^{2-}, \text{H}_3\text{A}^{3-}, \text{H}_2\text{A}^{4-}, \text{HA}^{-5}, \text{A}^{-6} \]

the assumption is that the only species present between pH 1.85 and 3.50 are \( \text{H}_3\text{A} \) and \( \text{H}_5\text{A}^- \). Using these symbols, Equations (18) and (20) become

\[
S_0 = S_1 + \frac{K_1 S_1}{[H^+]} \tag{21}
\]

and

\[
\log \left[ \frac{S_0}{S_1} - 1 \right] = \log K_1 + \log \frac{1}{[H^+]} \tag{22}
\]

Plots of the function of Equation (22) is shown in Figure 8. The value for \( S_1 \) is found from a plot of \( S_0 \) versus \( \frac{1}{[H^+]} \). This plot is shown in Figure 9. Extrapolation of the line in Figure 9 to \( \frac{1}{[H^+]} = 0 \) gave for the intrinsic solubility of Calcein

\[ S_1 = 24.1 \text{ mg. Calcein per 100 ml.} \]

\[ = 3.77 \times 10^{-4} \text{M(molecular weight = 640)} \]

This value was used in calculating \( \frac{S_0}{S_1} \) and the numbers plotted in Figure 8. The intercept of the line in Figure 8 is -2.53, that is \( pK_1 = 2.53 \) \( (K_1 = 2.95 \times 10^{-3}) \). The slope of the line was found to be 0.98.
Figure 8. Determination of the first acid dissociation of Calcein from solubility measurements.

\[ \log \left( \frac{S_0}{S_i} - 1 \right) \] versus \( \log \frac{1}{[H^+]}. \)

\( S_i = 24.1 \) mg. Calcein per 100 ml. (3.77 x 10^{-4} M).

Intercept (log \( \frac{1}{[H^+]} \) = 0) = -2.53.

pK\( _i \) = 2.53.
Figure 9. Determination of the intrinsic solubility, $S_i$, of Calcein from solubility measurements. Solubility of Calcein as a function of $\frac{1}{[H^+]}$

$S_i = 3.77 \times 10^{-4}$ M.

= 24.1 mg./100 ml.
$S_0$ (MOLES CALCEIN PER LITER) $\times 10^4$
Thus, the first replaceable hydrogen atom of Calcein is a fairly strong acid and the value obtained for the slope is so close to 1.0 that the calculation of $pK_1$ is probably not seriously disturbed by the second ionization step, that is, that $K_2$ is considerably smaller than $K_1$.

It was noticed in measuring the solubilities of the various Calcein-buffer solutions that below a pH of about 1.5 the solubility of Calcein increased rapidly. This was also noted in the previous work (7) and was used as a means of purifying Calcein. Because at low pH the Calcein forms apparently the hydrochloride (in hydrochloric acid) which is soluble in aqueous solution, the pH was lowered to a value of about 1 and then raised with sodium hydroxide to pH 2.3 thus the metal ions which were present in the Calcein were left in solution. Because of this increase in solubility at low pH the plot of $S_0$ versus $\frac{1}{[H^+]}$ did not include values for $\frac{1}{[H^+]}$ below pH 1.5, but extrapolation was made to a value of $\frac{1}{[H^+]} = 0$ using the values between pH 1.85 and 3.50.

Calculation B. Assumption that Calcein behaves as a dibasic acid over the pH range 1.85 to 3.50. Under this assumption the total Calcein as measured by the spectrophotometric method in the respective solutions of varying pH, Table 5, is the sum of the three species, $[H_2A]$, $[H_5A^-]$ and $[H_4A^{2-}]$. Again the assumption is made that the amount of unionized acid
is independent of pH, that is \([\text{H}_2\text{A}] = S_1\), \(S_1\) being the intrinsic solubility. Then

\[
S_0 = [\text{H}_2\text{A}] + [\text{H}_2\text{A}^-] + [\text{H}_4\text{A}^{--}].
\]  

(23)

and using the equations defining \(K_1\) and \(K_2\)

\[
S_0 = [\text{H}_2\text{A}] + \frac{K_1[\text{H}_2\text{A}]}{[\text{H}^+]^2} + \frac{K_1K_2[\text{H}_2\text{A}]}{[\text{H}^+]^2}
\]  

(24)

\[
S_0 = S_1 + \frac{K_1S_1}{[\text{H}^+]} + \frac{K_1K_2S_1}{[\text{H}^+]^2}
\]  

(25)

The data of Table 5 was handled three ways: B-1. by the solution of simultaneous equations which lead to values for \(S_1\), \(K_1\) and \(K_2\); B-2. by applying a correction for the \(\frac{1}{[\text{H}^+]^2}\) term to the calculation made in the section above, which led to the same value for \(K_1\); and B-3. by still a further variation on the method of plotting, which lead to another value for \(K_2\).

**Calculation B-1. Method using simultaneous equations.**

The three variables, \(S_1\), \(K_1\) and \(K_2\) in Equation (25) were evaluated by solving three simultaneous equations, the data of lines 2, 4 and 6 of Table 5 being used. Equation (25) was cast in the form

\[
S_0 = X + \frac{Y}{[\text{H}^+]} + \frac{Z}{[\text{H}^+]^2}
\]  

(26)

in which \(X = S_1\), \(Y = K_1S_1\) and \(Z = K_1K_2S_1\). The equations


\[ \frac{X}{5.14 \times 10^{-3}} + \frac{Y}{26.4 \times 10^{-6}} + \frac{Z}{10} = 5.03 \times 10^{-2} \]
(line 2 of Table 5

\[ \frac{X}{6.90 \times 10^{-4}} + \frac{Y}{47.5 \times 10^{-8}} + \frac{Z}{10} = 14.7 \times 10^{-4} \]
(line 4 of Table 5

\[ \frac{X}{3.16 \times 10^{-2}} + \frac{Y}{9.97 \times 10^{-8}} + \frac{Z}{10} = 30.9 \times 10^{-4} \]
(line 6 of Table 5

were solved by use of determinants and led to the values:

\[ X = 3.77 \times 10^{-2} \]
\[ Y = 6.63 \times 10^{-7} \]
\[ Z = 6.34 \times 10^{-11} \]

from which

\[ S_1 = 3.77 \times 10^{-2} \]
\[ K_1 = 1.76 \times 10^{-3} \]
\[ pK_1 = 2.75 \]
\[ K_2 = 9.5 \times 10^{-5} \]
\[ pK_2 = 4.92 \]

These values for \( S_1 \) and \( K_1 \) check the values obtained by the simpler method above \( (S_1 = 3.90 \times 10^{-4} \text{ M}, K_1 = 2.95 \times 10^{-3}) \) fairly well. The value for \( K_2 \) would appear to be too small by at least one order of magnitude; calculation of the \( Z \) term in the course of solving the simultaneous equations involves taking a small difference of large numbers and the error inherent to the method may possibly be this large.
Calculation B-2. Correction to Calculation A by including the term \( \frac{K_2}{[H^+]^2} \). Modification of Equation (25) gives

\[
\log \frac{S_0}{S_1} - 1 \quad = \log K_1 + \log \frac{1}{[H^+]} + \frac{K_2}{[H^+]^2}
\]

(27)

Using the value of \( K_2 \) obtained in B-2 immediately above, the term \( \frac{K_2}{[H^+]^2} \) was calculated for each line of the data of Table 5. A plot made of \( \log \frac{S_0}{S_1} - 1 \) versus \( \log \frac{1}{[H^+]} + \frac{K_2}{[H^+]^2} \) appears in Figure 10, along with the plot of

\[
\log \frac{S_0}{S_1} - 1 \quad \text{versus} \quad \log \frac{1}{[H^+]^2}
\]

The difference between the two grows larger with increasing pH as expected as the ionization of \( H_2A^- \) to \( H_2A^{2-} \) increases. The correction is negligible at low pH and the value obtained for \( K_1 \) is the same as that obtained in Calculation A, \( pK_1 = 2.53 \).

Calculation B-3. Calculation of \( K_2 \) using known values of \( S_1 \) and \( K_1 \). Rearrangement of Equation (27) gives

\[
\log \frac{S_0}{S_1} - 1 - \frac{K_1}{[H^+]^2} \quad = \log K_1K_2 + \log \frac{1}{[H^+]^2}
\]

(28)

Using the values, \( S_1 = 3.77 \times 10^{-6} \) and \( K_2 = 9.5 \times 10^{-5} \), obtained in Calculation B-1, a plot of

\[
\log \frac{S_0}{S_1} - 1 - \frac{K_1}{[H^+]^2} \quad \text{versus} \quad \log \frac{1}{[H^+]^2}
\]

was made. The points were badly scattered and the best straight line yielded for intercept \( \log \frac{1}{[H^+]^2} = 0 + 2.54 \) from which
Figure 10. Determination of the first acid dissociation constant of Calcein from solubility measurements using a provisional value of the second dissociation constant.

1: \[ \log \left( \frac{S_0}{S_i} - 1 \right) \text{ versus } \log \left( \frac{1}{[H^+]} + \frac{K_2}{[H^+]^2} \right) \]

2: \[ \log \left( \frac{S_0}{S_i} - 1 \right) \text{ versus } \log \frac{1}{[H^+]} \]

Intercept \[ \log \left( \frac{1}{[H^+]} + \frac{K_2}{[H^+]^2} \right) = 0 \] = -2.53

or \[ \log \frac{1}{[H^+]} = 0 \] = -2.53.

\[ pK_1 = 2.53. \]
\[
\log K_1 + \log K_2 = +2.54; \log K_2 = 2.54 - \log K_1 = 2.54 + 0.25 = 5.1 \text{ and } pK_2 = 5.1. \text{ This agrees with the value of } 4.9 \text{ found in calculation 3-1 but the slope of the line in this plot was } -0.41 \text{ rather than the } 1.0 \text{ called for and the value is suspect.}
\]

**Summary**

The solubility of Calcein in a series of six solutions of pH varying from 1.85 to 3.50 has been determined; the analyses being made by a spectrophotometric method.

Treatment of the data on the basis that Calcein behaves as a monobasic acid over this range of pH gave a value for the intrinsic solubility of Calcein, \( S_1 \), of 25.0 mg. per 100 ml. \((3.50 \times 10^{-4} \text{ M})\) and a value of \( pK_1 \) of 2.5.

A more extensive treatment on the assumption that both of the first two ionization steps are involved, that is, that the species present over this pH range are \( H_2A, H_2A^- \) and \( H_2A^{2-} \), gave the values

\[
\begin{align*}
S_1 &= 24.1 \text{ mg.} / \text{100 ml.} \quad 3.77 \times 10^{-4} \text{ M} \\
K_1 &= 1.8 \times 10^{-4} \quad pK_1 = 2.7 \\
K_2 &= 9.5 \times 10^{-5} \quad pK_2 = 5
\end{align*}
\]

the agreement of the values of \( S_1 \) and \( K_1 \) being in satisfactory agreement with those obtained by the simpler method. The method involves a larger extrapolation in the computation of the second dissociation constant and the value of \( 4.9 \) is suspect.

Calcein is thus a fairly strong acid, the best value of \( pK_1 \) being 2.7. The second acid dissociation is considerably weaker, \( pK_2 = 5 \) as a provisional value.
C. Determination of Acid Dissociation Constants by Ultraviolet Spectrophotometry

The ultraviolet spectrum of Calcein at pK 1, 7, and 13 was reported by Wallach and co-workers (6). Actually the spectra reported are for a compound designated by Wallach and co-workers as "Compound A" but presumably this material was Calcein in more or less pure form. An effort was made by Wallach and co-workers to correlate changes in the spectra with pH but only very rough approximations were made of the values of the various acid dissociation constants.

With a highly purified Calcein available it became possible to make a detailed study of the ultraviolet absorption spectrum as a function of pH and to secure absorbance measurements over selected wavelengths and ranges of pH from which numerical values of the dissociation constants could be obtained.

Experimental work

Materials. The Calcein used was the highly purified material prepared earlier (7).

Buffers. The buffers were prepared the same way as described in the solubility section. The buffers covered the pH range of 1 to 13 at 0.25 pH unit intervals.

pH Measurements. A Beckman Expanded Scale pH meter was used to obtain all pH measurements. The correction factor described in the section on solubility measurements was applied.
Absorbance measurements. Both a Cary 14 Recording Spectrophotometer and a Beckman Model DU Spectrophotometer were used. The Cary 14 was used to obtain the continuous spectra and the Beckman DU was used to obtain absorbance measurements.

In work with the Cary 14 spectrophotometers, the buffers described above were used. To 50.00 ml. of each buffers was added 0.50 ml. of $4.0 \times 10^{-5}$ M Calcein giving a final concentration of Calcein of $4.0 \times 10^{-5}$ M. Buffers were used in the reference cell to eliminate errors caused by the absorption of the buffers although the buffers were chosen to have no absorption in the ultraviolet. Spectra were obtained over the wave length range 200 $\mu\mu$ to 600 $\mu\mu$.

In the work with the Beckman DU Spectrophotometer a glass flow-through cell was used. The Calcein was dissolved in 50.00 ml. of 0.1 N potassium hydroxide to which had been added about 200 mg. of ammonium acetate. The mixture was then titrated in 0.25 pH unit intervals with 0.2 N hydrochloric acid and the absorbance measured at each increment. Corrections for dilution were made in the calculations.

Results and discussion

The ultraviolet spectrum of Calcein at pH 1.09, 4.01, 5.46 and 6.90 were obtained at suitable concentrations; these are shown in Figure 11. Four absorption bands were present in the spectrum at pH 1.09, at 230 $\mu\mu$, at 280 $\mu\mu$, at 445 $\mu\mu$, and
Figure 11. Absorption spectrum of Calcein as a function of pH.

Concentration of Calcein: $4.0 \times 10^{-8}$ M

Curve 1: pH = 1.09  
Curve 2: pH = 4.01  
Curve 3: pH = 5.46  
Curve 4: pH = 6.90
at 468 μm. These bands underwent changes in position and absorbance with changing pH. The spectrum remained essentially constant at all values of pH greater than 7.

Absorption band at 230 μm. On increasing the pH above 1.09 this band decreased in intensity. Between pH 4 and 8 the absorption band was resolved into two peaks; these peaks were not widely separated, appearing for example at 234 μm and 237 μm at pH 4.5. At pH above 8 only a single peak was again present, at 243 μm. The change in absorbance was not great and it did not appear feasible to use this band for the determination of acid dissociation constants.

Absorption band at 280 μm. On increasing pH this band gradually increased in intensity and the wavelength of maximum absorption shifted to longer wavelength, 292 μm at pH 12.95. The change in absorbance was not great enough to provide useful data.

Absorption band at 445 μm. The wavelength of maximum absorption of this band shifted to longer wavelength with increasing pH and above pH 6 the band was merged with the band at 468 μm.

Absorption band at 468 μm. This absorption band underwent great change with pH, the wavelength of maximum absorption shifting from 468 μm at pH 1.09 to 500 μm at pH 12.95. The absorbance increased rapidly from 3.30 to 8.2; preliminary work indicated a point of inflection at pH 5.5.
This band was obviously associated with the functional groups involved in the third and fourth acid dissociation steps and more careful measurements of absorbance over this buffer region were made using the flow-through cell described above.

**Calculation of acid dissociation constants from absorbance data.** The plot of absorbance versus pH usually resembles a titration curve. The quantitative correlation of absorbance and acid dissociation constants is made by using the equation defining the latter

\[
HA = H^+ + A^-
\]

\[K_A = \frac{[H^+][A^-]}{[HA]} \quad (29)\]

from which

\[pH = pK_A - \log \frac{[HA]}{[A^-]} \quad (30)\]

and associating the change in absorbance with the changes in the relative amounts of the untitrated acid and salt. If the wavelength of maximum absorbance does not change with pH, the relative amounts of untitrated acid and salt are obtained by linear interpolation of the absorbance data

\[pH = pK_A - \log \frac{A_{HA} - A_n}{A_n - A_A^-} \quad (31)\]

\(A_{HA}\) and \(A_A^-\) being the absorbance of the untitrated acid and salt respectively, and \(A_n\) the absorbance of a solution in which the fraction, \(n\), of the acid has been neutralized. Values of \(A_{HA}\) and \(A_A^-\) are taken from the data at end values where the absorbance is no longer changing with pH. A plot of
pH versus $\log \frac{A_{HA^-} - A_n}{A_n - A_{A^-}}$ is linear; with $pK_A$ equal to pH when the log term is zero (point of inflection on the absorbance versus pH curve). If the wavelength of maximum absorption shifts with pH, meaning that the untitrated acid and the salt have different colors, then the absorbance is measured at two wavelengths, that of maximum absorption of the acid alone and that of maximum absorption of the salt. The two sets of data are handled separately, the absorbance of one decreasing with pH, the other increasing. The two curves will generally intersect at the point of inflection, often called the isobestic point.

As applied to Calcein, this approach proved more complicated than just presented. The wavelength of maximum absorption changed with pH, 470 μm at pH 4 to 500 μm at pH 8. The absorbance versus pH curve on the long wavelength side, at 500 μm, proved to be normal, Figure 12, that is, the expected S-shaped, titration curve was obtained, and the plot of pH versus $\log \frac{A_{HA^-} - A_n}{A_n - A_{A^-}}$. Figure 13, was linear, giving a midpoint value of 5.40.

The plot of absorbance versus pH at 470 μm, Figure 12, is not the expected S-shaped curve with negative slope crossing the curve of the absorbance at 500 μm at the mid-point; rather, the curve rises in the pH range 4 to 5.4, reaches a maximum at 5.5, and decreases slightly from pH 5.5 to 8.5.

The plot of absorbance versus pH at 450 μm, Figure 14, is
Figure 12. Absorbance of Calcein as a function of pH.

Concentration of Calcein: $4.0 \times 10^{-8}$ M

Curve 1. Absorbance measured at 470 $\mu$m.

Curve 2. Absorbance measured at 500 $\mu$m.
Figure 13. Absorption of Calcein. pH versus $\log \frac{A_{HA} - A_n}{A_n - A_A}$

Wavelength: 500 μm.
Figure 14. Absorbance of Calcein as a function of pH.

Concentration of Calcein: $4.0 \times 10^{-8}$ M.

Curve 1. Absorbance measured at 450 $\text{m}$.$\mu$.

Curve 2. Absorbance measured at 500 $\text{m}$.$\mu$. 
similar in general shape to that of the absorbance at 470 μm but the absorbance values are lower and the maximum falls at pH 4.55. A reasonable interpretation of these absorbance versus pH plots can be made on the assumption that the changes in absorption accompany two neutralization steps. Thus three species would be present over the buffer region under consideration, each with a characteristic absorption band:

- \( \text{H}_3\text{A}^- \) with maximum absorption at 450 μm,
- \( \text{H}_4\text{A}^- \) with maximum absorption at 470 μm,
- \( \text{H}_5\text{A}^- \) with maximum absorption at 500 μm

with some overlapping of the two steps.

The species \( \text{H}_2\text{A}^- \), the end product of the two neutralization steps, measured by the band at 500 μm, increases over the range pH 4 to 7; the midpoint pH, 5.40, is exactly the average of the third and fourth acid dissociation constants found by the potentiometric method:

\[
K = K_3K_4 \\
= 2.63 \times 10^{-5} \times 6.17 \times 10^{-7} \\
= 4.5 \times 10^{-6}
\]

\[
pK = \frac{1}{2}(pK_3 + pK_4) \\
= \frac{1}{2}(4.58 + 6.19) \\
= 5.39
\]

The species \( \text{H}_3\text{A}^- \), measured by the band at 470 μm, increases over the range pH 4 to 5.5 as it is formed by the neutralization of \( \text{H}_4\text{A}^- \); beyond the maximum at pH 5.5 it decreases as it is neutralized to \( \text{H}_2\text{A}^- \).

The species \( \text{H}_4\text{A}^- \), measured by the absorbance at 450 μm,
increases over the range pH 3 to 4.5 as it is formed by the neutralization of $\text{H}_5\text{A}^-$. The point of inflection at pH 4.05 is exactly the average of the values found by the potentiometric method for the second and third dissociation constants:

$$pK = \frac{1}{2}(pK_2 + pK_3) = \frac{1}{2}(3.53 + 4.58) = 4.05$$

The maximum occurs at pH 4.55, agreeing neatly with the potentiometric value of 4.58 for the third dissociation constant. The point of inflection on the long wavelength side occurs at pH 5.40, coinciding with the maximum in the 470 $\text{m} \mu$ curve and the point of inflection in the (rising) 500 $\text{m} \mu$ curve.

Thus, the entire picture is neatly explained by the presence of three absorbing species interrelated by two successive but overlapping acid dissociation steps.

**Summary**

The absorption spectrum of Calcein has been obtained at various values of pH from 1 to 13. Four absorption bands are present, at 230 $\text{m} \mu$, 280 $\text{m} \mu$, 445 $\text{m} \mu$ and 468 $\text{m} \mu$. Changes with the 230 $\text{m} \mu$ and 280 $\text{m} \mu$ bands were too small to be of use in assessing values for the acid dissociation constants. The two longer wavelength bands vary significantly in position and intensity over the pH range 4 to 8.5, the 445 $\text{m} \mu$ band merging with the 468 $\text{m} \mu$ band at about pH 6, the maximum of the combined band lying at 500 $\text{m} \mu$ at pH 8.5. The changes in these bands thus accompany the third and fourth neutralization steps.
The absorbance of Calcein in solutions in which the pH was monitored over the pH range 1 to 15 was measured at the wavelengths 450 mµ, 470 mµ and 500 mµ, the wavelengths associated with the two longer wavelength absorption bands. The plot of absorbance at 500 mµ versus pH was the normal S-shaped "titration" curve; the midpoint value, pH = pK = 5.40, is exactly the average of the values of the third and fourth dissociation constants (as negative logarithms: \( pK_A = 1/2(pK_1 + pK_2) = 5.39 \)) found by the potentiometric method.

The plots of absorbance at 470 mµ and 450 mµ versus pH proved more complex owing to the merging of the two absorption bands, a result of the overlapping of the third and fourth neutralization steps. Three species are present over the range of pH 3.5 to 7, each with its own absorption band: \( H_4A^-\) (absorption maximum at 450 mµ), \( H_3A^-\) (absorption maximum at 468 mµ) and \( H_2A^-\) (absorption maximum at 500 mµ).

Determination of Acid Dissociation Constants

By Fluorescence Spectroscopy

Introduction

The fluorescence of Calcein is, of course, the heart of the use of this reagent in analytical chemistry. This fluorescence is pH dependent, the intensity of the fluorescence of highly acid solutions, pH 1 to 3, being
essentially zero, that of neutral solutions, pH 7 to 9, being highly fluorescent, and that of highly alkaline solutions, pH 11 to 14, being non-fluorescent.

The detailed behavior of this fluorescence as a function of pH was studied using both excitation and emission spectra, correlating the changes in these spectra with the acid dissociation constants already measured, and using these changes to obtain values for the fifth and sixth acid dissociation constants.

Experimental work

Materials. Calcein. The Calcein used was the highly purified material prepared in earlier research (7).

Buffers. Buffers covering the pH range 1 to 13 were prepared according to the procedure outlined in the section on solubility measurements. Buffers were made for every 0.25 pH unit intervals over the pH range.

Apparatus. pH measurements were made with a Beckman Expanded Scale pH Meter. Fluorescence excitation and emission spectra were obtained using an Aminco-Bowman Spectrophotofluorimeter attached to a Moseley X-Y Recorder.
Excitation and emission measurements. To 49.50 ml. of buffer was added 0.50 ml. of $4 \times 10^{-4}$ M Calcein, the final concentration of Calcein in each solution being $4 \times 10^{-3}$ M. Excitation measurements were made from 200 $\mu$m to 600 $\mu$m with the emission monochromator set at 510 $\mu$m. These same Calcein-buffer solutions were used to obtain emission spectra from 350 $\mu$m to 600 $\mu$m with the monochromator of the excitation beam set at 480 $\mu$m. The pH of the solutions was checked prior to making the fluorescence measurements.

Results and discussion

Both fluorescence excitation and emission spectra were obtained on solutions of various pH from 1 to 13.

Fluorescence excitation spectra. The excitation spectra were obtained with the emission spectrometer set at 510 $\mu$m, the wavelength of maximum fluorescence. Typical excitation spectra those for solutions of pH 1.34, 7.29 and 10.17 are shown in Figure 15. Some eight maxima occur in the excitation spectrum at low pH; these are listed in Table 6. The position of these excitation maxima shift somewhat with pH and some merge with adjoining peaks. Thus, not all could be used for excitation measurements as a function of pH. The excitation bands at 237 $\mu$m, 305 $\mu$m and 484 $\mu$m proved most useful. Plots of the relative intensity of fluorescence as
Figure 15. Fluorescence excitation spectrum of Calcein as a function of pH.
Fluorescence monochromator set at 510 μm throughout.
Curve 1: pH = 1.34
Curve 2: pH = 7.29
Curve 3: pH = 10.17
a function of pH for excitation wavelengths of 237 m\(\mu\), 247 m\(\mu\), 305 m\(\mu\), 297 m\(\mu\), 484 m\(\mu\) and 494 m\(\mu\) are shown in Figures 16 and 17.

Table 6. Absorbance maxima observed in the excitation spectrum of Calcein with shift on increasing pH

<table>
<thead>
<tr>
<th>Wavelength of Maximum</th>
<th>Shifting To</th>
</tr>
</thead>
<tbody>
<tr>
<td>237 m(\mu)</td>
<td>247 m(\mu)</td>
</tr>
<tr>
<td>267 m(\mu)</td>
<td>264 m(\mu)</td>
</tr>
<tr>
<td>282 m(\mu)</td>
<td>288 m(\mu)</td>
</tr>
<tr>
<td>305 m(\mu)</td>
<td>297 m(\mu)</td>
</tr>
<tr>
<td>309 m(\mu)</td>
<td>327 m(\mu)</td>
</tr>
<tr>
<td>443 m(\mu)</td>
<td>455 m(\mu)</td>
</tr>
<tr>
<td>468 m(\mu)</td>
<td>473 m(\mu)</td>
</tr>
<tr>
<td>484 m(\mu)</td>
<td>494 m(\mu)</td>
</tr>
</tbody>
</table>

Fluorescence emission spectra. Fluorescence emission spectra of Calcein were obtained with the monochromator in the excitation beam set at 480 m\(\mu\). The fluorescence emission spectra were scanned (by the monochromator in the emission beam) over the range 350 m\(\mu\) to 600 m\(\mu\). Only one maximum was observed, this maximum shifting to longer wavelengths with increasing pH:

<table>
<thead>
<tr>
<th>pH</th>
<th>Wavelength of Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.34</td>
<td>510 m(\mu)</td>
</tr>
<tr>
<td>13.28</td>
<td>526 m(\mu)</td>
</tr>
</tbody>
</table>

Typical fluorescence emission spectra are shown in Figure 18 for the pH values 1.34, 4.90, 7.93 and 11.83. The relative fluorescence emission as a function of pH at two settings of the emission monochromator, 510 m\(\mu\) and 526 m\(\mu\) are shown in
Figure 16. Fluorescence of Calcein at various values of pH when excited by light of selected wavelengths. Fluorescence emission monochromator set at 510 mμ for all measurements.

Wavelength of exciting light.

Curve 1: 237 mμ
Curve 2: 247 mμ
Curve 3: 305 mμ and 297 mμ
Figure 17. Fluorescence of Calcein at various values of pH when excited by light of selected wavelengths. Fluorescence emission monochromator set at 510 μm for all measurements.

Wavelength of exciting light.

Curve 1: 484 μm
Curve 2: 495 μm
Figure 18. Fluorescence emission spectrum of Calcein as a function of pH.

Excitation monochromator set at 480 μm for all measurements.

Curve 1: pH = 1.34
Curve 2: pH = 4.90
Curve 3: pH = 7.93
Curve 4: pH = 11.86
RELATIVE FLUORESCENCE

WAVELENGTH μm

550

450

20

40

80

80
Figure 19. There is only one fluorescence emission band irrespective of the wavelength of the exciting light.

Calculation of acid dissociation constants from fluorescence measurements. The method of evaluating acid dissociation constants from fluorescence data follows the patterns used for absorbance data, beginning with the mathematical equation defining the acid dissociation constant:

\[
HA = H^+ + A^- \\
K_A = \frac{[H^+][A^-]}{[HA^-]} \quad (32)
\]

\[
pH = pK - \log \frac{[HA]}{[A^-]} \quad (33)
\]

The concentration terms are evaluated in terms of relative fluorescence by linear interpolation of the relative fluorescence of end-products (made at appropriate values of pH at which only one species is present). Equation (33) becomes

\[
pH = pK - \log \left( \frac{F_{HA} - F_n}{F_n - F_A^-} \right) \quad (34)
\]

in which \( F \) is the relative fluorescence, HA and A\(^-\) designate the acid form and salt form respectively, and \( n \) designates a mixture of the two forms in which the fraction \( n \) of the acid has been converted to the salt.

As applied to Calcein, it becomes apparent from Figures 16 and 17 that the pH range over which the fluorescence changes is 4 to 12. The third, fourth, fifth and sixth acid dissociation steps are thus the ones involved (see page 54
Figure 19. Fluorescence of Calcein as a function of pH.

Concentration of Calcein = $4.0 \times 10^{-6}$ M.

Excitation wavelength = 380 $\mu$m.

Emission monochromator set at:

Curve 1: 510 $\mu$m

Curve 2: 526 $\mu$m
for the mathematical equations defining the respective dissociation constants). The maximum in the fluorescence emission spectrum as a function of pH is broad and is centered more or less at pH 8, the pH of the end-point in the titration of Calcein with sodium hydroxide corresponding to the neutralization of the first four replaceable hydrogen atoms. Close inspection of Figures 17 and 18 reveals that on the low side of the maximum no point of inflection occurs but that on the high pH side three points of inflection are present, the middle one being located in each of the five curves presented at pH 10.7.

The potentiometric method yielded values for the third and fourth acid dissociation constants, $pK_3 = 4.58$ and $pK_4 = 6.19$. Considerable overlap occurred in the third and fourth stages of titration but it was not so serious as to obscure the individual character of the two steps and this showed clearly in the absorbance measurements, Figures 12 and 14 making possible independent measurements and checking evaluation of $K_3$ and $K_4$. This separation of the third and fourth steps is completely absent in the fluorescence data. This is undoubtedly a consequence of the fact that the wavelength of the maximum in fluorescence is independent of the wavelength of the exciting light.

A mathematical analysis of the relative fluorescence over the pH range 4 to 7 at each of the exciting wavelengths
studied was made using Equation (34). The results are given in Table 7. The value averages to \( \text{pH} = \text{pK}_A = 5.43 \). The value agrees well with the average of the values for \( K_3 \) and \( K_4 \) obtained by the potentiometric method (as negative logarithms \( \text{pK}_A = 1/2(\text{pK}_3 + \text{pK}_4) = 5.39 \)) and is close also to that obtained by absorption spectrophotometry (preceding section).

Table 7. Acid dissociation constants of Calcein as determined by fluorescence measurements

<table>
<thead>
<tr>
<th>Excitation Wavelength(^a)</th>
<th>( \text{pK} )</th>
<th>Slope</th>
<th>( \text{pK} )</th>
<th>Slope</th>
<th>( \text{pK} )</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>237( \text{m\mu} )</td>
<td>5.44</td>
<td>1.57</td>
<td>9.97</td>
<td>0.52</td>
<td>11.65</td>
<td>1.34</td>
</tr>
<tr>
<td>247( \text{m\mu} )</td>
<td>5.44</td>
<td>1.54</td>
<td>9.87</td>
<td>1.03</td>
<td>11.68</td>
<td>1.10</td>
</tr>
<tr>
<td>305( \text{m\mu} )</td>
<td>5.46</td>
<td>1.42</td>
<td>9.88</td>
<td>0.97</td>
<td>11.66</td>
<td>1.15</td>
</tr>
<tr>
<td>297( \text{m\mu} )</td>
<td>5.41</td>
<td>1.32</td>
<td>9.89</td>
<td>1.08</td>
<td>11.67</td>
<td>1.10</td>
</tr>
<tr>
<td>484( \text{m\mu} )</td>
<td>5.42</td>
<td>1.45</td>
<td>9.88</td>
<td>1.28</td>
<td>11.67</td>
<td>1.08</td>
</tr>
<tr>
<td>495( \text{m\mu} )</td>
<td>5.44</td>
<td>1.17</td>
<td>9.88</td>
<td>0.92</td>
<td>11.71</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Fluorescence \( b\) Wavelength

| 510\( \text{m\mu} \) | 5.41 | 1.36 | 9.87 | 1.07 | 11.68 | 1.13 |
| 526\( \text{m\mu} \) | 5.44 | 1.33 | 9.86 | 1.05 | 11.71 | 1.05 |

\( ^a\) Fluorescence emission wavelength: 510 \( \text{m\mu} \)

\( ^b\) Fluorescence excitation wavelength: 480 \( \text{m\mu} \)
The shoulder appearing on the high pH side of the plots of relative fluorescence as a function of pH are of particular interest. They provide evidence that two replaceable hydrogen atoms are involved and afford a method for evaluating them.

The data on relative fluorescence as a function of pH at various excitation wavelengths, Figures 17 and 18, were handled in three separate treatments in accord with Equation (34): (1) on the low pH side of the maximum; (2) over the K₅ buffer range, pH 8.6 to 10.5; and (3) over the K₆ buffer range, pH 10.9 to 12.2. Plots of pH versus log \( \frac{F_{HA}-F_n}{F_n-F_A} \) for the K₅ region are shown in Figure 20 and the results at all excitation wavelengths and fluorescence wavelengths summarized in Table 7. The mid-point values give pK₅ = 9.83 (average of measurements at 8 wavelengths); pK₆ = 11.64 (average of measurements at 8 wavelengths). The middle one of the three points of inflection on the high pH branch of the curves of relative fluorescence versus pH, referred to above, falling at pH 10.7 is really an end-point pH, the first end-point in the titration of two monobasic acids and as such should be related to the two acid dissociation constants by the relation developed in Part III, Section A, page 56, Equation (12)

\[
pH_{\text{end-point}} = \frac{1}{2}(pK_4 + pK_5) \\
= \frac{1}{2}(6.19 + 9.88) \\
= 8.03
\]

The agreement could be better but considering the diverse
Figure 20. Fluorescence of Calcein. pH versus $\log \frac{F_{HA} - F_n}{F_n - F_A}$

Curve 1: 247 m$\mu$
Curve 2: 305 m$\mu$
nature of the methods on which the constants are based may be considered satisfactory.

Summary

The fluorescence excitation and fluorescence emission of Calcein have been studied as a function of pH over the range 1 to 13.

Three major bands and five minor bands appear in the excitation spectrum, all of which undergo minor changes with pH in position and effectiveness for excitation. Six of the bands were used in obtaining data from which acid dissociation constants could be calculated.

One band only appears in the fluorescence emission spectrum and the maximum of this band shifts somewhat with pH.

The fluorescence of Calcein rises abruptly between pH 4 and 7 and falls equally abruptly between pH 9 and 12. The increase is uniform and mathematical treatment of the data yields for the effective acid dissociation constant the value \( pK_4 = 5.40 \); this value is exactly the average of the two dissociation constants found by the potentiometric method (as negative logarithms: \( pK_A = \frac{1}{2}(pK_3 + pK_4) = \frac{1}{2}(4.58 + 6.19) = 5.39 \)).

The relative fluorescence over the range of pH 9 to 12 is characterized by a shoulder separating the buffer regions of the fifth and sixth acid dissociation steps. On analysis
the data yields for the constants for these steps

\[ K_\text{s} = 1.3 \times 10^{-10} \quad \text{pK}_\text{s} = 9.9 \]

\[ K_\text{x} = 2.3 \times 10^{-12} \quad \text{pK}_\text{x} = 11.6 \]

Calculation of the pH at which the end-point in the potentiometric titration should have occurred for acids having pK4 = 6.19 and pK5 = 9.88 gives 8.03. The pH found was 8.30, a satisfactory agreement considering the diverse nature of the methods used to obtain the respective constants.
PART IV. NATURE AND PROPERTIES OF THE VARIOUS ANIONIC FORMS OF CALCEIN

Given values for the acid dissociation constants of Calcein as determined in Part III above

\[
\begin{align*}
K_1 &= 2.9 \times 10^{-3} & \quad pK_1 &= 2.5 \\
K_2 &= 3.4 \times 10^{-4} & \quad pK_2 &= 3.5 \\
K_3 &= 2.6 \times 10^{-5} & \quad pK_3 &= 4.6 \\
K_4 &= 6.4 \times 10^{-7} & \quad pK_4 &= 6.2 \\
K_5 &= 1.3 \times 10^{-10} & \quad pK_5 &= 9.9 \\
K_6 &= 2.3 \times 10^{-12} & \quad pK_6 &= 11.6
\end{align*}
\]

the problem arises of assigning each of these acid functions to a specific group in the molecule.

The third and fourth dissociation steps are associated with the strong absorption in the ultraviolet region, absorption maxima at 230 \(\text{m}\)  and 280 \(\text{m}\), as well as with bands in the visible region, maxima at 445 \(\text{m}\)  and 480 \(\text{m}\). The absorption in the ultraviolet is in the region of absorption of phenyl compounds, the wavelengths of the absorption maxima of phenol and benzoic acid being 201.5 \(\text{m}\)  and 270 \(\text{m}\), 230 \(\text{m}\)  and 270 \(\text{m}\)  respectively (13). The absorption of aliphatic carboxylic acids lies below 200 \(\text{m}\) (13). The phenolic groups of Calcein must then be supplying the third and fourth replaceable hydrogen atoms, the amino and carboxyl groups being separated from the phenyl ring by aliphatic, non-conjugated linkages.
Once the assignment of the third and fourth dissociation steps to the phenolic group is made, it becomes necessary to explain why only two of the carboxyl groups are titratable, why these two are much stronger acids than normal carboxylic acids, and why the two phenolic groups are much stronger than normal. All three questions are answered on the basis that both methyleneiminodiacetic acid groups of Calcein are present as zwitter ions. The transfer of the hydrogen atoms from the carboxyl groups to the amino groups in effect neutralizes two of the carboxyl groups and places a positive charge on the nitrogen atoms which by electrical repulsion make the neighboring acid groups stronger acids. Thus the structure assigned to the undissociated Calcein, $H_6A$, is the structure XIV.

The first and second replaceable hydrogen atoms, according to this view, come from the two free carboxyl groups, and the anion, $H_4A^-2$, has Structure XV, this neutralization having taken place without change in the absorption spectrum or fluorescence.

In the third and fourth dissociation steps the phenolic groups are neutralized. This is accompanied by a change in the ultraviolet and visible spectrum and the material becomes fluorescent. Fluorescence is generally associated with rigid, planar molecules and the third and fourth acid dissociation steps must be accompanied by some internal rearrange-
\[
\text{XV} \\
H_6A \\
\text{Non-fluorescent}
\]

\[
\text{XV} \\
H_4A^{-2} \\
\text{Non-fluorescent}
\]
ment which reduces the amount of free rotation within the molecule which dissipates the energy of the excited state and prevents fluorescence. Hydrogen bonding of the ammonium hydrogen atoms between the nitrogen atoms and the phenolate oxygen atoms could do this. The structure, then, for the anion, $\text{H}_2\text{A}^-$, is Structure XVI.

In the fifth and sixth neutralization steps, the ammonium hydrogen atoms are neutralized. No change in absorption accompanies these steps but fluorescence falls to zero. Because the absorption is not changed, the molecule is being excited as before but the energy is being transferred to a non-radiative process, quite possibly by increasing the amount of free rotation (of the methyleneimino-diacetic acid groups) and intramolecular collisions as the hydrogen bonding is reduced by removal of the hydrogen atoms by neutralization. Structure XVII is thus written for anion, $\text{A}^-$.  

The argument advanced above, that the positive charge on the nitrogen atom increases the acid strength of neighboring groups, is hardly new. It is used to explain the acid behavior of ethylenediaminetetraacetic acid for which the successive acid dissociation constants are (as negative logarithms) $pK_1 = 1.99$, $pK_2 = 2.67$, $pK_3 = 6.16$, and $pK_4 = 10.26$ (14). It was employed also by Schwarzenbach, Anderegg and Sallmann (8) to explain the acidic behavior of
Fluorescent

Non-fluorescent
the very first of the phenolic compounds into which the 
methyleneciminoaacetic acid group were introduced: phenol-
2-methyleneciminoacetic acid (H₂Phim; structure XVIII) and 
phenol-5-methyl-2,6-bis(methyleneiminoacetic acid) (H₅Ph-
diim; structure XIX). To explain the increased acidity of 
the phenol group in these compounds, Schwarzenbach and co­
workers (8) postulated a hydrogen bond between the pheno­
late and ammonium groups.

A comparison of the acid behaviors of the Schwarzenbach 
compounds and Calcein is of interest:

<table>
<thead>
<tr>
<th></th>
<th>H₅Phim</th>
<th>H₅Phdiim</th>
<th>Calcein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK₁</td>
<td>2.2 (C)</td>
<td>2.0 (C)</td>
<td>2.53 (C)</td>
</tr>
<tr>
<td>pK₂</td>
<td>8.17 (P)</td>
<td>2.9 (C)</td>
<td>3.53 (C)</td>
</tr>
<tr>
<td>pK₃</td>
<td>11.79 (A)</td>
<td>6.65 (P)</td>
<td>4.58 (P)</td>
</tr>
<tr>
<td>pK₄</td>
<td>9.74 (A)</td>
<td></td>
<td>6.19 (P)</td>
</tr>
<tr>
<td>pK₅</td>
<td>11.4 (A)</td>
<td></td>
<td>9.88 (A)</td>
</tr>
<tr>
<td>pK₆</td>
<td></td>
<td></td>
<td>11.64 (A)</td>
</tr>
</tbody>
</table>

C representing a carboxyl group, P a phenolic group and A 
an ammonium ion.

What is most surprising in all this is the strength of 
the phenolic groups in H₅Phdiim and Calcein. The acid 
dissociation constants of simple phenols lie in the range 
10⁻² to 10⁻¹⁰, but those of H₅Phdiim and Calcein are 100 to 
10,000 times stronger as acids.
\[
\text{XVIII} \\
H_3\text{Phim}
\]

\[
\text{XIX} \\
H_5\text{Phdiim}
\]
PART V. DETERMINATION OF THE FORMATION CONSTANTS
OF THE CALCIUM COMPOUNDS OF CALCEIN

Formation Constants

Given a pure, metal-free Calcein, application in analytical chemistry as a reagent for the direct fluorometric determination of the calcium could be worked out on a purely empirical basis. The original and the numerous subsequent applications of Calcein as a metallofluorochromic indicator were made on just such a basis. It was observed by Diehl and Ellingboe (1) that at high pH the Calcein alone did not fluoresce but in the presence of calcium it did. That was all that was needed for the development of an indicator, and although the situation was far from satisfying from a purely intellectual standpoint, it sufficed. Indeed, had work been done on the nature of the calcium derivative using the impure reagent, it very well could have been wrong and the result added to the all too long list of follies which plague the literature from someone making exact measurements on impure materials. In fact, this is exactly what has happened with Calcein.

A calibration curve is a plot of absorbance, or relative fluorescence, or some other property versus concentration. Ideally, a calibration curve is a straight line but this is not necessary in analytical work if conditions can be reproduced from standards to unknowns. Done carefully and over a broad enough range, the calibration curve can be used for
determining stoichiometry but it is then generally referred to as a spectrophotometric or fluorometric titration.

The calibration plot of relative fluorescence of Calcein versus concentration of calcium using early preparations of Calcein was a curve bending upwards. The curve did not begin at the origin. That the curve varied from one lot to another of the reagent and that no sensible stoichiometry could be derived from it should have been a tip off that the reagent was seriously contaminated. In any event, two reports were ultimately published dealing with the stoichiometry of the calcium-Calcein reaction: Wallach and Steck (9) and Bozhevol'nov and Kreingol'd (10). The findings were in conflict and it is probable that the reagents of both workers contained considerable calcium or other metals.

Wallach and Steck (9) reported that calcium and Calcein first combined in the ratio of 1 to 1, forming a non-fluorescent compound, and that with greater amounts of calcium, a second calcium atom entered the molecule, the 2 to 1 compound being fluorescent. They reported for the formation constant of the 2 to 1 compound the value \(10^{8.83}\).

Bozhevol'nov and Kreingol'd on the other hand, found only a 1 to 1 compound and that it was fluorescent and that the formation constant was \(10^{8.8}\).

Having now a pure metal-free Calcein from the earlier work (7), it became possible to settle the stoichiometry of
the calcium-Calcein reaction and do a more satisfying job than a mere empirical development of an analytical method. In fact, it quickly became evident that a basic study was in order, for the calibration plot was not the earlier S-shaped curve with initial upward sweep but three straight lines, Figure 21.

Experimental work

Reagents used. Calcein. The Calcein used was that prepared in earlier work (7).

Apparatus. A Beckman Expanded Scale pH Meter was used for all pH determinations. pH corrections were applied as mentioned previously.

A Turner Model 110 Fluorimeter was used for fluorescence measurements.

Fluorometric titration. The fluorometric titration of Calcein in the presence of varying amounts of calcium were carried out in the following manner. To 5.00 ml. of $1 \times 10^{-4}$ M Calcein was added $1 \times 10^{-4}$ M calcium in varying amounts to give solutions ranging from 0 to 4 moles of calcium per mole of Calcein. The solutions were then diluted to 50.00 ml. with 0.1 N potassium hydroxide, the pH measured (pH = 13.1) and the fluorescence of the various solutions measured using a Turner Model 110 Fluorimeter.

Potentiometric titration. Three potentiometric titrations were carried out.
The sodium hydroxide was standardized by triplicate titrations of primary standard potassium acid phthalate. The hydrochloric acid was then titrated against the standardized sodium hydroxide to determine its concentration.

All titrations were carried out using deionized water which had been boiled to expel the dissolved carbon dioxide. No further precautions were made to keep carbon dioxide out of the solution but the titrations were carried out quickly to minimize error due to carbon dioxide.

The first potentiometric titration was carried out as followed. To 0.1365 g. (2.13 x 10^{-4} moles) of Calcein and 0.0313 g. (2.13 x 10^{-4} moles) of calcium chloride dihydrate was added 15.88 ml. of 0.1074 N sodium hydroxide, the theoretical amount equivalent to eight replaceable hydrogen atoms or two equivalents of Calcein. This would correspond to the point at 200 percent titration in the direct titration of Calcein alone. Then 100 ml. of deionized water was added and the solution allowed to stir until all the Calcein had dissolved. The resulting solution was then titrated with 0.1106 N hydrochloric acid.

The same procedure was carried out twice more with different concentrations of calcium. The second titration was done using 0.1365 g. (2.13 x 10^{-4} moles) of Calcein and 0.0626 g. (4.26 x 10^{-4} moles) of calcium chloride di-
hydrate and 15.83 ml. of 0.1074 N sodium hydroxide. The 
third titration was done using 0.1365 g. (2.13 x 10^{-2} 
moles) of Calcein and 0.313 grams (2.13 x 10^{-3} moles) of 
calcium chloride dihydrate and 15.88 ml. of 0.1074 sodium 
hydroxide.

The three titrations were then the titrations of one 
to one, two to one, and ten to one calcium to Calcein.

Results and discussion
The data on the variation of the fluorescence of the 
purified, metal-free Calcein with increasing amounts of 
calcium are plotted in Figure 21. Two breaks are clearly 
discernible and these appear at the points where one and 
two moles of calcium have been added per mole of Calcein. 
Two compounds are present and both are fluorescent. More­
over, the formation of the first compound is essentially 
complete before the second compound forms.

Three titrations with sodium hydroxide of Calcein in 
the presence of calcium were carried out, the mole ratio of 
calcium to Calcein being: 1.00 to 1.00, Figure 22; 2.00 to 
1.00, Figure 23; and 10.0 to 1.00, Figure 24. For convenience 
these curves, together with that for the titration of Calcein 
alone are given in Figure 25. Because of the insolubility 
of Calcein at low pH and the slowness with which it 
dissolves during a titration, these potentiometric titra­
tions were actually carried out by adding a measured volume
Figure 21. Fluorescence of Calcein as a function of amount calcium added.

Concentration of Calcein: $1 \times 10^{-8}$ M.

MOLES CALCIUM PER MOLE CALCEIN

RELATIVE FLUORESCENCE

100

50

MOLES CALCIUM PER MOLE CALCEIN
Figure 22. Potentiometric titration of Calcein in the presence of an equimolar amount of calcium. Calcein taken: 0.1365 g. (2.13 x 10^-4 mole). Calcium chloride dihydrate added: 0.0313 g. (2.13 x 10^-4 mole). Concentration of sodium hydroxide: 0.1014 N.
\[ \text{a--MOLES OF BASE ADDED} \]

\[ \text{PERCENT TITRATION} \]
Figure 23. Potentiometric titration of Calcein in the presence of two moles of calcium.

Calcein taken: 0.1365 g. ($2.13 \times 10^{-4}$ mole).

Calcium chloride dihydrate added: 0.0626 g. ($4.26 \times 10^{-4}$ mole).

Concentration of sodium hydroxide: 0.1074 N.
Figure 24. Potentiometric titration of Calcein in the presence of ten moles of calcium.

Calcein taken: 0.1365 g. (2.13 x 10^{-4} mole).

Calcium chloride dihydrate added: 0.313 g. (2.13 x 10^{-3} mole).

Concentration of sodium hydroxide: 0.1074 N.
α-Moles of Base Added

PERCENT TITRATION

pH

3 6 9

50 100 150
Figure 25. Potentiometric titration curves of Calcein alone and in the presence of varying amounts of calcium. Data taken from Figures 21, 22, 23 and 24.

1: Calcein.
2: Calcein plus one equivalent of calcium.
3: Calcein plus two equivalents of calcium.
4: Calcein plus ten equivalents of calcium.
a - MOLES OF BASE ADDED

PERCENT TITRATION
of standard sodium hydroxide and back titrating with standard hydrochloric acid.

The titration curves of Calcein in the presence of calcium are identical almost to the point where four moles of base have been added per mole of Calcein \( (a = 4.0) \).

Up to this point, then, which corresponds to the neutralization of the two free carboxyl groups and the two phenol groups, calcium plays no part in the neutralization process. At this point calcium unites with Calcein displacing the hydrogen atoms of the ammonium groups. Or, putting it another way, union with calcium makes the ammonium ion much stronger acids, acid dissociation constants of the order of \( 10^{-7} \) (in contrast to \( K_5 = 1.32 \times 10^{-8} \) and \( K_6 = 2.29 \times 10^{-12} \)). These acids are then titrated (from \( a = 4.0 \) to \( a = 6.0 \)) with reasonably sharp end-points. For the mole ratio of 1.00 to 1.00 (Figure 22), the end-point occurs at \( a = 5.0 \), indicating that one calcium entered the molecule with the liberation of one proton.

With excess calcium, mole ratio 10.0 to 1.00 (Figure 24), the curve is essentially that of a dibasic acid and the end-point occurs at \( a = 6.0 \), indicating that two calcium atoms have entered the molecule with the liberation of two protons.

The data obtained at various stages of these titrations is given in Table 8. The treatment of this data to
evaluate the formation constants of the calcium derivatives follows.

Table 8. Titration with sodium hydroxide of Calcein in the presence of calcium, pH at various stages of the titration

<table>
<thead>
<tr>
<th>Mole Ratio</th>
<th>pH at C₀</th>
<th>pH at C₁</th>
<th>pH at C₂</th>
<th>C₀ at C₁</th>
<th>C₀ at C₂</th>
<th>C₀ at C₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>4.5</td>
<td>5.0</td>
<td>5.5</td>
<td>4.5</td>
<td>5.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Calcein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00:1.00</td>
<td>6.72</td>
<td>10.52</td>
<td>1.06x10⁻³</td>
<td>---</td>
<td>1.05x10⁻³</td>
<td>2.13x10⁻³</td>
</tr>
<tr>
<td>2.00:1.00</td>
<td>6.60</td>
<td>6.79</td>
<td>6.96</td>
<td>---</td>
<td>1.05x10⁻³</td>
<td>2.13x10⁻³</td>
</tr>
<tr>
<td>10.00:1.00</td>
<td>6.05</td>
<td>6.27</td>
<td>6.71</td>
<td>1.06x10⁻²</td>
<td>1.03x10⁻²</td>
<td>1.00x10⁻²</td>
</tr>
</tbody>
</table>

Initial concentration of Calcein as corrected for the dilution resulting from the addition of titrants

Calculation of formation constants of the one to one compounds from titration data

Method A. The titration of Calcein in the presence of an equimolar amount of calcium is shown in Figure 22. The titration curve is identical with that of Calcein alone almost to the point where four replaceable hydrogen atoms have been titrated; see Figure 26 in which the titration curves have been placed on the same graph for convenience in comparison. Between a = 4.0 and 5.0 the mixture behaves as a much stronger acid than does Calcein alone and only one hydrogen atom is involved for there is a sharp end-point at
\( a = 5.0 \). The fluorometric titration, Figure 21, indicates that the first calcium-Calcein compound formed is a one-to-one compound. Accordingly, the reactions over the region \( a = 4.0 \) to \( a = 5.0 \) and the correspondingly mass action constants are

\[
H_2A^{-4} = HA^{-5} + H^+ \quad K_s = \frac{[H^+][HA^{-5}]}{[H_2A^{-4}]} \tag{6}
\]

\[
HA^{-5} + M^{++} = MHA^{-3} \quad K_{MHA^{-3}} = \frac{[MHA^{-3}]}{[M^{+2}][HA^{-5}]} \tag{35}
\]

Solving Equation (6) for \( HA^{-5} \) and inserting this into Equation (35) gives

\[
K_{MHA^{-3}} = \frac{[MHA^{-3}][H^+]}{K_s[H_2A^{-4}][M^{+2}]} \tag{37}
\]

At the mid-point of this titration (at \( a = 4.50 \) moles of base added), the pH is 6.72 ([H^+] = 1.9 \times 10^{-7}). Considering the mid-point, the following assumptions are made:

1. that \( H_2A^{-4} \) is equal to one-half of the initial concentration (corrected for dilution caused by the alkali added), designated \([H_2A^{-4}]\); (2) reaction between the \( HA^{-5} \) formed and \( M^{+2} \) is complete and no \( HA^{-5} \) remains in solution, meaning that \([M^{+2}] = [MHA^{-3}] = \) one-half of the initial concentration of the metal ion, corrected for dilution. Because equimolar amounts of Calcein and calcium were taken,

\[
[M^{+2}] = [MHA^{-3}] = 1/2 \ [M^{+2}]_o = 1/2 \ [H_2A^{-4}]_o
\]
Equation (37) then becomes

\[ K'_{\text{MHA}^-} = \frac{[\text{H}^+][\text{HA}^-] + K_{\text{MHA}^-}M^{+2}}{[\text{H}_2\text{A}^-]} \]  

\[ K = 1.9 \times 10^{-7} \text{ (from Part II)} \]

\[ [\text{H}_2\text{A}^-] = 5.3 \times 10^{-2} \text{ (from the data of the titration)} \]

\[ [\text{H}^+]_{\text{midpoint}} = 1.9 \times 10^{-7} \text{ (from the data of the titration)} \]

\[ K'_{\text{MHA}^-} = \frac{1.9 \times 10^{-7}}{1.32 \times 10^{-10} \times 5.3 \times 10^{-2}} = 2.7 \times 10^8 \]

**Method B.** A somewhat different approach to this same problem, that of Schwarzenbach, Anderegg and Sallmann (8), leads to the same result but gives an evaluation of the error resulting from the assumptions made in Method A above.

For the reaction which takes place on neutralization in the presence of excess metal ion

\[ \text{H}_2\text{A}^- = \text{HA}^- + \text{MHA}^- \]

a mass action constant, called the provisional acid dissociation constant is written

\[ K' = \frac{[\text{H}^+][\text{HA}^-] + [\text{MHA}^-]}{[\text{H}_2\text{A}^-]} \]  

(38)

The value for this apparent dissociation constant is taken as the mid-point pH. Combining Equations (38) and (35) gives

\[ K' = K_5 \left[ \frac{[\text{H}_2\text{A}^-]}{[\text{HA}^-]} \right] + \frac{K_{\text{MHA}^-}M^{+2}M^{+2}}{[\text{H}_2\text{A}^-]} \]  

(39)
For the titration of Calcein in the presence of an equimolar amount of calcium, \( \text{pH}_{\text{mid-point}} = 6.70 \) and \( M^{+2} \) equals one-half the initial concentration of metal (same assumption as before)

\[
K^M_{\text{MHA}^{-3}} = \frac{1.9 \times 10^{-7} - 1.32 \times 10^{-10}}{1.32 \times 10^{-10} \times 5.3 \times 10^{-8}} = 2.7 \times 10^6
\]

This is the same result as before. The \( K_S \) term in the numerator makes no contribution.

The data for the other two titrations may be similarly used: for the calcium-Calcein ratio of two to one

\[
\text{pH}_{\text{mid-point}} = 6.60
\]

\[
(M^{+2}) = \frac{3}{4}(M^{++}) = 7.95 \times 10^{-4}
\]

\[
K^M_{\text{MHA}^{-3}} = \frac{25 \times 10^{-8} - 1.32 \times 10^{-10}}{1.32 \times 10^{-10} \times 7.95 \times 10^{-4}} = 2.4 \times 10^6
\]

and for the calcium to Calcein ratio of ten to one

\[
\text{pH}_{\text{mid-point}} = 6.05
\]

\[
(M^{+2}) = 0.95(M^{+2}) = 5.03 \times 10^{-3}
\]

\[
K^M_{\text{MHA}^{-3}} = \frac{89 \times 10^{-8} - 1.32 \times 10^{-10}}{1.32 \times 10^{-10} \times 5.03 \times 10^{-3}} = 1.3 \times 10^3
\]

No provision is made in this treatment for any of the second calcium compound which may be formed; such formation certainly must be considerable in the 10 to 1 titration and probably appreciable in the 2 to 1 titration. Accordingly, the value
$K_{\text{MHA}^-} = 2.7 \times 10^3$ is adopted for the formation constant of the monocalcium derivative of Calcein.

**Calculation of the formation constant of the two to one compound from titration data**

A formation constant for the compound $Cu_2$Calcein was calculated following a procedure similar to that used in Method A above for the one to one compound. The system was treated as a dibasic acid inasmuch as the titration curve of the mixture of ten moles of calcium to one of Calcein between $a = 4.0$ and $a = 6.0$ resembles that of a dibasic acid. The operation begins with the following chemical equations and definitions:

1. $K_a = \frac{[H^+][HA^-]}{[H_2A^-]}$ (6)
2. $K_b = \frac{[H^+][A^-]}{[HA^-]}$ (7)
3. $K_{H_2A^-} = 2[H^+] + A^-$
4. $K_5K_3 = \frac{[H_2A^-]}{[HA^-]}$
5. $HA^- + M^{++} = MHA^-^3$
6. $\frac{K_{MHA^-^3}}{K_{HA^-^3}} = \frac{[HA^-^3]}{[HA^-][M^{++}]}$ (35)
7. $MHA^-^3 + M^{++} = M_2A^-^2 + H^+$
8. $\frac{K_{MHA^-^3}}{K_{M_2A^-^2}} = \frac{[M_2A^-^2]}{[MHA^-^3][M^{++}]}$ (41)
9. $A^- + M^{++} = MA^-^4$
10. $\frac{K_{MA^-^4}}{[A^-][M^{++}]}$ (42)
\[ \text{MA}^- + M^{++} = M_2A^{-2} \quad \text{K}_{M_2A^{-2}} = \frac{[M_2A^{-2}]}{[\text{MA}^-][M^{++}]} \quad (43) \]

\[ A^{-2} + 2 M^{++} = M_2A^{-2} \quad \text{K}_{M_2A^{-2}} = \frac{[M_2A^{-2}]}{[A^{-2}][M^{++}]^{-2}} \quad (44) \]

The overall formation constant, \( K_{M_2A^{-2}} \), may be evaluated by introducing Equation (35) into Equation (44) giving

\[ K_{M_2A^{-2}} = \frac{[H^+]^2[M_2A^{-2}]}{[K_5K_a][H_2A^{-2}][M^{++}]^2} \quad (45) \]

and using data at the mid-point \( (a = 5.0) \), at which point the \( \text{pH} \) is 6.27 and the acid has been half neutralized to its salt, \( A^{-2} \). In the presence of the excess metal ion, \( A^{-2} \) is completely converted to \( M_2A^{-2} \), so that

\[ [H_2A^{-2}] = [M_2A^{-2}] \]

Thus,

\[ K_{M_2A^{-2}} = \frac{[H^+]^2_{\text{mid-point}}}{K_5K_a [M^{++}]} \quad (46) \]

The concentration of the metal ion is the initial concentration, 10 moles per mole of Calcein minus the 2.0 moles which has reacted or 0.8 \% corrected for dilution by the alkali added (0.80) \( (1.03 \times 10^{-2}) \) or \( 0.824 \times 10^{-2} \). Then,

\[ K_{M_2A^{-2}} = \frac{(5.36 \times 10^{-7})^2}{(1.32 \times 10^{-10})(2.29 \times 10^{-12})(8.24 \times 10^{-3})^2} \]

\[ = 1.4 \times 10^{11} \]
Application of the Schenkron method as used in Method B above for the one to one compound leads to

\[ \frac{K_{M^2A^-2}}{K_{M^2A^-2}} = \frac{K'' - K_0K_s}{[K_{M^2A^-2}][M^{3+}]^2} \quad (47) \]

in which the apparent dissociation constant, \( K'' \), is the square of the mid-point pH. This gives the same value for \( K_{M^2A^-2} \) as just obtained.

Of the various reactions and definitions given above, Equation (41) is of particular interest in that it furnishes the link between the one to one and the two to one compounds. The constant may be evaluated from the two formations constants now known as follows:

Starting with:

\[ K_{M^2A^-3} = \frac{\gamma_{S_{M^2A^-3}}}{[HA^-][M^{3+}]} = 2.7 \times 10^9 \quad (48) \]

and substituting terms derived from Equations (35) and (7) gives

\[ \frac{K_{X_{M^2A^-2}}}{K_{M^2A^-3}} = \frac{[K_{X_{M^2A^-2}}][H^+]^3}{[K_{M^2A^-3}][H^+][A^-][M^{3+}]} \]

from which

\[ K_{X_{M^2A^-2}} = \frac{K_{X_{M^2A^-2}}}{K_{M^2A^-3}} \frac{K_{M^2A^-3}}{K_{M^2A^-3}} \]

\[ = (2.29 \times 10^{-12})(1.4 \times 10^{11}) \]

\[ = 1.2 \times 10^{-7} \quad (pK_{X_{M^2A^-2}} = 6.92) \]
Summary

The fluorescence of Calcein of high purity at pH 13 with varying amounts of calcium (calibration curve or "fluorometric titration") consists of three lines with intersections at the points corresponding to one and two moles of calcium added per mole of Calcein. Both the one to one and the two to one compounds are fluorescent.

Three titrations with alkali of Calcein in the presence of calcium in the mole ratios of calcium to Calcein of 1:1, 2:1 and 10:1 were carried out. Up to four moles of alkali were added per mole of Calcein; all three curves are essentially identical with that of Calcein alone. Thus, binding of calcium to Calcein does not occur at pH values prevailing during the titration of the two free carboxyl groups and the two phenolic groups.

In the presence of calcium the ammonium ions of Calcein (zwitter ion structure) become much stronger acids. In a solution containing one calcium atom per mole of Calcein the end-point occurs at five moles of alkali added; in the presence of a large excess of calcium the end-point occurs at six moles. Thus one calcium is added with the liberation of one proton and then a second is added with the liberation of another.
That is to say, the one to one compound in the presence of a metal acts as a weak monobasic acid, $pK_{\text{ION}}^{+} = 6.92$. The pH at the mid-point in the titration of this acid, $a = 5.5$, should be 6.92. The pH observed, Figure 24, is 6.71. The calcium thus appears to form a bond between the phenolate and the nitrogen atoms. Inasmuch as the union with calcium occurs stepwise with the displacement of one proton in each step, it appears that this takes place at one of the methyleneiminodiacetic acid sites, and then at the others, a picture consonant with the symmetrical 4',5'-structure shown in Part II for Calcein. The compounds designated above as $\text{CaH}_{\text{A}}^{-3}$ and $\text{Ca}_{\text{A}}^{-2}$, that is, the one to one and the two to one compounds are, respectively, Structures XX and XXI.

Although only one proton was displaced on the union of each calcium atom with Calcein, two charges are neutralized. This is shown formally in Structures XX and XXI by the three bonds to the calcium atoms. The covalent chemistry of calcium, that for example in its reaction with ethylenediamine-tetraacetic acid (EDTA), shows that calcium is hexacovalent, the EDTA in $\text{Ca(EDTA)}^{-2}$ being hexadentate. It is probable that with Calcein the second carboxylate group of each of the methyleneiminodiacetic acid groups is also bound to the calcium atoms but no proof of this is offered here and to the reader is left the privilege of supplying carboxylate and or water molecules to fill out the coordination positions as he chooses.
CaHA$^3$

One-to-one compound

Cu$_2$A$^{-2}$

Two-to-one compound
The pertinent formation constants as deduced from the data of the titrations are:

\[ \text{HA}^{-5} + \text{Ca}^{+2} = \text{CaHA}^{-3} \quad K_{\text{CaHA}^{-3}}^{\text{Ca}} = 2.7 \times 10^5 \]

\[ \text{A}^{-3} + 2\text{Ca}^{+2} = \text{Ca}_2\text{A}^{-2} \quad K_{\text{Ca}_2\text{A}^{-2}}^{\text{Ca}} = 1.4 \times 10^{11} \]

\[ \text{CaHA}^{-3} + \text{Ca}^{+2} = \text{Ca}_2\text{A}^{-2} + \text{H}^{+} \quad K_{\text{Ca}_2\text{A}^{-2}}^{\text{Ca},\text{H}} = 1.2 \times 10^{-7} \]
PART VI. SUMMARY

The metallofluorochromic indicator called Calcein, devised at Iowa State University in 1956 for the EDTA titration of calcium in the presence of magnesium, has been subjected to further study. Although satisfactory and widely used as an indicator Calcein proved unsatisfactory as a reagent for the direct fluorometric determination of calcium. The difficulties were traced to major impurities of metals and a satisfactory method of synthesizing the reagent was devised in the early phase of the present investigation (Hefley, M. S. Thesis, 1965).

The present work, making use of this pure, metal-free Calcein, has been divided into three major parts: a determination of the structure of the compound; an investigation into the behavior of the compound as an acid as reflected in the titration, light absorption, and fluorescence characteristics; and a study of the calcium derivatives.

The determination of the structure is based largely on nuclear magnetic resonance spectroscopy. Calcein is derived from fluorescein by the introduction of two methyleneiminodiacetic acid groups (the chelating groups which give to the molecule the property of combining with metals). The NMR investigation necessarily involved supplementary studies on fluorescein and certain of its derivatives.
The NMR spectra of fluorescein, Calcein, six substituted fluoresceins and two substituted Calceins have been obtained and interpreted in terms of the known and unknown structures of the various molecules.

One portion of the NMR spectrum of each of the ten compounds studied is essentially identical in character, number, position and proton integration of peaks observed and is attributed to four protons on successive carbons in a benzene ring. Thus, no substitution into the phthalate ring of the Calcein compounds occurred during their synthesis.

Another portion of the NMR spectra of Calcein, 2',7'-dichlorocalcein and 4',5'-dimethylcalcein was essentially identical in character and position of the peaks in the region expected for aliphatic protons. Integration showed the presence of the eight methylene hydrogen atoms corresponding to those of the four acetic acid groups present, and four methylene hydrogen atoms of a second kind, as expected, for the two methylene groups linking the amino acids to the rings.

In a third region of the spectra appeared a group of peaks attributed to the protons of the resorcinol rings. Particular attention was paid to this part of the spectra of fluorescein, 2',7'-dichlorofluorescein, 4',5'-dibromo-fluorescein, 4',5'-diiodofluorescein, 4',5'-dimethylfluorescein, Calcein, 2',7'-dichlorocalcein, and 4',5'-dimethyl-
calcein. The similarities and differences in the number, position and character of the peaks and the integration over each for the number of protons led to a consistent and complete assignment of the various peaks. The simple spectrum of calcein in this region (one singlet peak integrating to four protons) made it clearly evident that the molecule is highly symmetrical, with one methyleneiminodiacetic acid group present in each resorcinol ring and in the positions between the ring oxygen atom and the phenol groups. That is, calcein is fluorescein-4',5'-bis(methyleneiminodiacetic acid) or more formally 3',6'-dihydroxy-4',5'-bis[N,N'-di(carboxymethyl)aminomethyl]fluoran. The NMR spectra predicted for the other eight structures possible for calcein are all more complex than the simple spectrum found.

The NMR spectrum shows that all six of the replaceable hydrogen atoms in the molecule of calcein exchange rapidly with the solvent used, dimethylsulfoxide.

The two methylenedicarboxylic acid groups in the calcein derived from 2',7'-dichlorofluorescein was shown by NMR spectroscopy to occupy the 4'- and 5'-positions.

The calcein derived from 4',5'-dimethylfluorescein appears from the NMR spectrum to be the symmetrically substituted compound 4',5'-dimethylfluorescein-1',2'-bis-(methyleneiminodiacetic acid) but work is required on this compound.
Four lines of attack were required for the complete elucidation of the properties of Calcein. No less than six replaceable hydrogen atoms are present in the molecule. Only the last two of the replaceable hydrogen atoms are too weak to be titrated in the normal fashion. The general nature of the problem has been outlined by titration with alkali; the first acid function has been determined by solubility measurements; the ultraviolet absorption has been investigated as a function of pH; and the fluorescence excitation and emission spectra have been investigated as a function of pH. The results of all four lines of investigation have been blended into an internally consistent picture detailing the behavior of Calcein with changing acidity.

The potentiometric titration of Calcein with sodium hydroxide yields a titration curve with one break, at pH = 8.30, corresponding to the replacement of four hydrogen atoms.

Because of the low solubility of Calcein, better data for the purposes of calculating acid dissociation constants is obtained by adding a measured excess of standard sodium hydroxide and back titrating with standard hydrochloric acid.

Because of the low solubility a value for the first acid dissociation constant could not be obtained from the titration data.
The titration curve shows no minor breaks or inflections up to the end-point indicating that the values of the four dissociation constants are more or less uniformly separated. At any particular pH then, only two steps of the titration overlap and the calculation of the successive constants is simplified.

The fourth step of the neutralization is that of a monobasic acid and a value of $K_4$ is obtained directly. The third dissociation constant was obtained from the fourth dissociation constant and titration data. Similarly the second was obtained from the third. A value for the second was also obtained from a value for the first constant and titration data.

The values obtained for the second, third and fourth acid dissociation constants are: $K_2 = 3.4 \times 10^{-4}$ (pK$_2$ = 3.5); $K_3 = 2.6 \times 10^{-5}$ (pK$_3$ = 4.6); $K_4 = 6.4 \times 10^{-7}$ (pK$_4$ = 6.2).

The solubility of Calcein in a series of six solutions of pH varying from 1.85 to 3.50 has been determined; the analyses being made by a spectrophotometric method.

Treatment of the data on the basis that Calcein behaves as a monobasic acid over this range of pH gave a value for the intrinsic solubility of Calcein, $S_i$, of 25.0 mg. per 100 ml. (3.90 x 10$^{-4}$ M) and a value of pK$_1$ of 2.5.

A more extensive treatment on the assumption that both of the first two ionization steps are involved, that is, that
the species present over this pH range are $H_2A$, $H_3A^-$ and $H_4A^{2-}$, gave the values

\[
S_1 = 24.1 \text{ mg./100 ml.} \quad 3.77 \times 10^{-2} \text{ M} \\
K_1 = 1.8 \times 10^{-3} \quad \text{pK}_1 = 2.7 \\
K_2 = 9.5 \times 10^{-5} \quad \text{pK}_2 = 5
\]

the agreement of the values of $S_1$ and $K_1$ being in satisfactory agreement with those obtained by the simpler method. The method involves a larger extrapolation in the computation of the second dissociation constant and the value of 4.9 is suspect.

Calcein is thus a fairly strong acid, the best value of $\text{pK}_1$ being 2.7. The second acid dissociation is considerably weaker, $\text{pK}_2 = 5$ as a provisional value.

The absorption spectrum of Calcein has been obtained at various values of $\text{pH}$ from 1 to 15. Four absorption bands are present, at 230 mµ, 280 mµ, 445 mµ and 468 mµ. Changes with the 230 mµ and 280 mµ bands were too small to be of use in assessing values for the acid dissociation constants. The two longer wavelength bands vary significantly in position and intensity over the pH range 4 to 8.5, the 445 mµ band merging with the 468 mµ band at about pH 6, the maximum of the combined band lying at 500 mµ at pH 8.5. The changes in these bands thus accompany the third and fourth neutralization steps.

The absorbance of Calcein in solutions in which the pH was monitored over the pH range 1 to 15 was measured at the
wavelengths 450 mμ, 470 mμ and 500 mμ, the wavelengths associated with the two longer wavelength absorption bands. The plot of absorbance at 500 mμ versus pH was the normal S-shaped "titration" curve; the midpoint value, pH = pK = 5.40, is exactly the average of the values of the third and fourth dissociation constants (as negative logarithms: pK_A = 1/2(pK_1 + pK_2) = 5.39) found by the potentiometric method.

The plots of absorbance at 470 mμ and 450 mμ versus pH proved more complex owing to the merging of the two absorption bands, a result of the overlapping of the third and fourth neutralization steps. Three species are present over the range of pH 3.5 to 7, each with its own absorption band: H_4A^-2 (absorption maximum at 450 mμ), H_3A^-3 (absorption maximum at 468 mμ) and H_2A^-4 (absorption maximum at 500 mμ).

The fluorescence excitation and fluorescence emission of Galcein have been studied as a function of pH over the range 1 to 15.

Three major bands and five minor bands appear in the excitation spectrum, all of which undergo minor changes with pH in position and effectiveness for excitation. Six of the bands were used in obtaining data from which acid dissociation constants could be calculated.

One band only appears in the fluorescence emission spectrum and the maximum of this band shifts somewhat with pH.
The fluorescence of Calcine rises abruptly between pH 4 and 7 and falls equally abruptly between pH 9 and 12. The increase is uniform and mathematical treatment of the data yields for the effective acid dissociation constant the value $pK_e = 5.40$; this value is exactly the average of the two dissociation constants found by the potentiometric method (as negative logarithms: $pK_A = 1/2(pK_3 + pK_4) = 1/2(4.53 + 6.19) = 5.39$).

The relative fluorescence over the range of pH 9 to 12 is characterized by a shoulder separating the buffer regions of the fifth and sixth acid dissociation steps. On analysis the data yields for the constants for these steps

\[
K_5 = 1.3 \times 10^{-10} \quad pK_5 = 9.9
\]
\[
K_6 = 2.3 \times 10^{-12} \quad pK_6 = 11.6
\]

Calculation of the pH at which the end-point in the potentiometric titration should have occurred for acids having $pK_4 = 6.19$ and $pK_5 = 9.88$ gives 8.03. The pH found was 8.30, a satisfactory agreement considering the diverse nature of the methods used to obtain the respective constants.

Structures have been proposed for each of the six anions of Calcine and the changes in structure correlated with the acid dissociation constants, the ultraviolet absorption, and the fluorescence.

The fluorescence of Calcine of high purity at pH 13 with varying amounts of calcium (calibration curve or "fluorometric
titration") consists of three lines with intersections at the points corresponding to one and two moles of calcium added per mole of Calcein. Both the one to one and the two to one compounds are fluorescent.

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Summarizing, then: the structure of Calcein has been determined; the six acid dissociation constants have been evaluated and identified with specific acid functions within the molecule; the ultraviolet absorption and fluorescence behavior as a function of pH have been determined and correlated with the structure; and the nature of the calcium derivatives has been elucidated.
BIBLIOGRAPHY


2. Diehl, H., Calcein, Calmagite, and o,c’-Dihydroxyacetobenzene. Titrimetric Colorimetric and Fluorometric Reagents for Calcium and Magnesium, G. Frederick Smith Chemical Company, Columbus, Ohio, 1964.


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