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Structure, fluorescence, and chelating properties of Calcein

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Structure, fluorescence, and chelating properties of Calcein

by

Richard Markuszewski

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In Charge of Major Work

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For the Graduate College

Iowa State University
Ames, Iowa

1976

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I. INTRODUCTION

Calcein was first synthesized by Diehl and Ellingboe[31] and used as an indicator for the complexometric determination of calcium in the presence of magnesium. Calcein, prepared by a Mannich-type condensation of fluorescein, formaldehyde, and iminodiacetic acid, can be viewed as a fluorescein molecule substituted by two methyleneiminodiacetic acid groups, each of which is equivalent to one-half of the ethylenediaminetetraacetic acid molecule (EDTA). Thus, Calcein has the unique fluorescence and acid-base properties of fluorescein combined with the chelating and acid-base properties of EDTA.

As with fluorescein, the fluorescence of Calcein is a function of pH, and in addition, the fluorescence is greatly influenced by the presence of certain metal ions. Fluorescein exhibits a brilliant, green-yellow fluorescence starting at a pH of about 5, increasing up to a pH of about 8, and remaining constant at all pH above 8. The equally brilliant green-yellow fluorescence of Calcein, on the other hand, begins at pH about 4, reaches a maximum at pH 8, and then decreases, disappearing completely at pH 12. The fluorescence at high pH is instantaneously restored by the addition of calcium, barium, or strontium, owing to the formation of fluorescent compounds. Magnesium also forms a fluorescent compound with Calcein, but the fluorescence is

observed only around pH 10, because at higher pH magnesium is precipitated as the hydroxide. The fluorescence of Calcein in the neutral pH region is quenched by transition metal ions such as the cobaltous, nickel, ferric, and cupric ions. A few transition metal ions, cadmium, zinc, or mercury in particular, are exceptions in that they form fluorescent compounds with Calcein; in this respect they resemble the alkaline earth metals, but the fluorescence occurs at much lower pH, about 7.

The characteristic fluorescence of Calcein, which is uniquely dependent on the pH of the solution and on the nature of the metal ion present, serves as the basis for the many uses of Calcein discovered since its introduction in 1956. Calcein is the first of the so-called metallofluorochromic indicators, also known as metallofluorescent or metalfluorechromic indicators. The reversible appearance and disappearance of fluorescence in the presence or absence of calcium, respectively, makes Calcein an unsurpassed indicator for the complexometric determination of calcium, especially in the presence of magnesium.

After the initial use of Calcein for the determination of calcium in limestone, gypsum, and magnesium and sodium salts,[31] the field of applications widened to include the determination of calcium in such diverse materials as lithium salts,[105] silicates,[60] ferromanganese slags,[100] titanium dioxide,[94] cellulose,[141] and phosphate-containing samples.[162]

The use of Calcein for the chelometric determination of calcium was critically compared with that of other indicators.[38,51,53] Similar behavior of Calcein in the presence of other alkaline earth metal ions was used for the complexometric determination of strontium and barium.[83] Magnesium was also determined, but at a lower pH to prevent the precipitation of magnesium hydroxide.[149]

The application of Calcein as indicator for the microdetermination of calcium in biological materials such as blood serum, urine, and milk found immediate and wide-spread use. The number of publications is large, and only a few of the earlier ones will be listed in the bibliography of this work.[2,4,12,16,62,77,122,140] The interest generated by Calcein among clinical and biochemical workers is described briefly by Diehl.[28] An excellent review of the use of Calcein for the determination of calcium in materials of clinical interest is given by Siest et al.[129]

The quenching of the fluorescence of Calcein by copper(II) makes Calcein an ideal indicator for the indirect complexometric determination of many metal ions with ethylenediaminetetraacetic acid (EDTA). It is especially useful for those metals which normally form complexes with EDTA that are too colored and thus cannot be determined by using the common metallochromic indicators. The usual procedure is to add excess EDTA to complex the metal ion and

then to titrate the excess EDTA with copper(II) ions in the presence of Calcein to the disappearance of the fluorescence. Such methods were applied, for example, for the determination of aluminum, titanium, and nickel;[63] cobalt, iron, and copper;[155,157] titanium, nickel, chromium, cobalt, and iron;[158] copper and manganese;[148] and aluminum in the presence of iron.[33]

Calcein was also used for the indirect determination of anions. Sulfate was determined[36] indirectly by the complexometric titration of excess barium. Bromide, iodide, and cyanide were determined[147] by fluorescence quenching caused by silver ions in argentometric titrations. Thiocyanate, bromide, and iodide were also determined[138] by argentometric titrations in which Calcein was used as an adsorption indicator, changing its color from yellow-orange to pink. Cyanide was determined[118] by leaching of copper from the non-fluorescent Calcein-copper compound and measuring the regenerated fluorescence.

Calcein as a metallofluorochromic indicator in complexometric titrations can be used alone or in a mixture with other indicators which enhance the end-point by screening unwanted color or residual fluorescence caused by impurities. Tucker[143] proposed the addition of thymolphthalein for a more satisfying color change. Svoboda et al.[136] used a mixture of Calcein with phenolphthalein complexone for complexometric

titrations in the presence of large amounts of sodium ions, which are known to enhance the background fluorescence of Calcein, probably because of some interaction of sodium with the Calcein, and make the end-point less sharp. This increased fluorescence of Calcein in the presence of sodium ions has been well established.[32,85] For that reason, use of potassium hydroxide rather than sodium hydroxide is recommended for fluorometric studies involving Calcein. Kirkbright and Stephen[76] used acridine, another fluorescent dye but with a fluorescence that is different from that of Calcein, to screen out unwanted background fluorescence.

More recently, Hoyle and Diehl[67] prepared the di-potassium pentacalcium dicalcein salt, named Statocalcein, to be used as an indicator in complexometric titrations of calcium. They showed that a 0.02 per cent solution of Statocalcein is remarkably stable over long periods of time and functions as a satisfactory indicator, even after prolonged storage.

The use of Calcein for the direct fluorometric determination of calcium, strontium, and barium was first proposed by Körbl and Vydra,[83] in 1957, and shown to be feasible by Wallach et al.[151] in 1959. But it was not until 1963 that calcium was determined in this manner by Kepner and Hercules[75] and by Wallach and Steck.[150] The direct fluorometric determination was also suggested for aluminum at a

low pH; cobalt, copper, and nickel could be determined fluorometrically from the amount of fluorescence quenching; and zinc could be determined from the increase of fluorescence owing to the displacement of the cobalt in the nonfluorescent Calcein-cobalt compound by zinc to form the highly fluorescent Calcein-zinc compound.[150] Recently, Hefley and Jaselskis[59] used Calcein for the direct fluorometric determination of cadmium.

An interesting application of Calcein is its use as a fluorescent stain for mineralized tissue;[135] tissue bearing calcium becomes fluorescent indicating the calcium-bearing regions. The method was used as a marking dye for the growth of bone and dentine,[133,134] tooth and alveolar bone,[72] bone formation and bone resorption.[104] Von Jürgensonn[146] used Calcein for the detection of calcium in histochemical investigations.

Yet another use of Calcein is as spray reagent for organosulfur compounds separated on thin-layer chromatographic plates. The reagent is basically a compound of Calcein with palladium chloride, which is nonfluorescent. When sprayed onto chromatographic spots containing organosulfur compounds, the sulfur combines with the palladium, releasing Calcein from the nonfluorescent Calcium-palladium compound to form a fluorescent spot in an alkaline medium containing calcium. The method was used to determine

organosulfur compounds,[48] organophosphorus pesticides,[18] and phenylthiohydantoins of amino acids[68] after separation by thin-layer chromatography.

Such wide acceptance and such diverse uses of Calcein have generated a sizeable literature. The number of publications dealing with the chemistry and applications of Calcein and related compounds is approaching 200. The most recent monograph in the English language was published by Diehl[27] in 1964, followed by a review article in 1967.[28] A chapter in a book on fluorescence analysis by White and Argauer[154] includes a sizeable discussion of metallofluorochromic indicators.

With the increasing literature on Calcein came the commonly associated proliferation of other names. When Calcein was subsequently synthesized in Europe in 1957, it was given the name fluorescein complexone[83] by analogy to the o-cresolphthalein complexone prepared by a similar synthesis.[1] Calcein is available commercially from a Czech firm as the sodium salt under the name of fluorexon.[83] Much of the literature in the Russian language refers to Calcein either as fluorescein complexone or fluorexon. The name Calcein W was given by Wilkins and Hibbs[160] to a preparation of Calcein that was treated so as not to contain any fluorescein impurities. The dipotassium pentacalcium dicalcein salt, prepared by Hoyle and Diehl[67] to provide

an indicator that is stable in solution, was given the name Statocalcein. The name fluorescein chelator appears in a book on fluorescence assay.[144] In the applications of Calcein as a dye for labeling mineralized tissue, Suzuki and Matthews[135] introduced the name DCAF, an abbreviation for bis[di(carboxymethyl)aminomethyl]fluorescein, for Calcein; the acronym was appropriated by other workers in that field. The different names in use for the compound Calcein create a regrettable situation and are a major inconvenience in searches of the literature.

An even more unfortunate turn of events occurred in the assignment of a structure for Calcein. When Diehl and Ellingboe first synthesized Calcein, in 1956, they admitted that the compound was not sufficiently pure for a determination of structure. Czech workers[84,85] claimed the preparation of a compound of higher purity but made no attempt to determine the structure. In another publication,[81] they speculated as to the structure, favoring substitution in the 4',5'- positions (Structure I) and the 2',7'- positions (Structure II). Chemical Abstracts assigned to Calcein, fluorexon, and fluorescein complexon the formal name 2',7'-bis[[bis(carboxymethyl)amino]methyl]fluorescein (Structure II), although other assignments appear at various places in the indexes.

Wallach *et al.*[151] were the first to propose a definitive structure for Calcein. They claimed that the major component

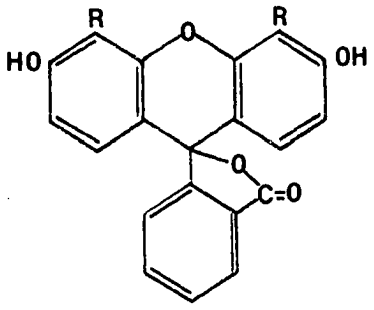
Figure 1. Structures of Calcein

Structure I. 3',6'-Dihydroxy-4',5'-bis[N,N'-bis-(carboxymethyl)aminomethyl]fluoran.
Name and structure of Hefley[58]

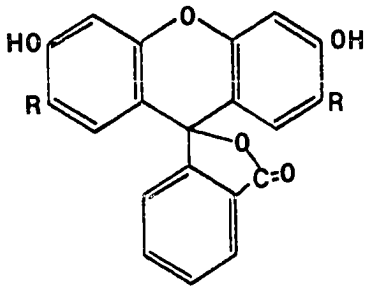
Structure II. 2',7'-Bis[[bis(carboxymethyl)amino]-methyl]fluorescein.
Name and structure of Chemical Abstracts.

Structure III. 3',6'-Dihydroxy-2',4'-bis[N,N'-di(carboxymethyl)aminomethyl]fluoran.
Name and structure of Wallach *et al.*[151]

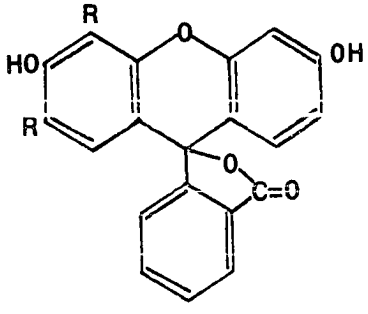
Structure IV. 4',5'-Bis[[bis(carboxymethyl)amino]-methyl]fluorescein.
Name and structure of this work


R
R
HO
OH
O
O
C=O

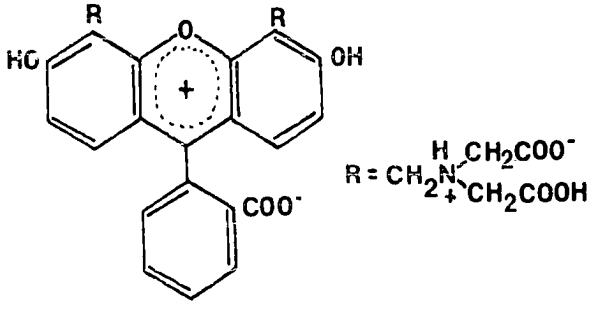
I.


O
HO
OH
R
R
O
C=O

II.


R
O
HO
OH
R
O
C=O

III.


R
R
O
HO
OH
+
COO⁻
H CH2COO⁻
R = CH2 N
+ CH2COOH

IV.

of the impure Calcein is fluorescein substituted unsymmetrically in the 2',4'- positions by two methyleneiminodiacetic acid groups and is thus 3',6'-dihydroxy-2',4'-bis[N,N'-di(carboxymethyl)aminomethyl]fluoran (Structure III). They presented evidence based on the acid-base properties, changes in the absorption and fluorescence spectra as a function of pH, and the infrared absorption spectrum. The evidence is not convincing, particularly in the infrared work. The structure postulated is unusual for derivatives of fluorescein, it is highly unsymmetrical and highly crowded sterically. Russian workers[19,20,21,87], unfortunately, accepted this structure without question and based the interpretation of their work on this structure. Suzuki and Matthews[135] also accepted Structure III, although in their qualitative work on bone marking, the structure is not critical.

Hefley in 1967[58] presented indisputable proof, based largely on NMR spectroscopy, that in Calcein the two methyleneiminodiacetic acid groups occupy the symmetrical 4',5'- positions of the fluorescein molecule (Structure I), and is thus properly named 3',6'-dihydroxy-4',5'-bis[N,N'-bis(carboxymethyl)aminomethyl]fluoran. She also determined the acid dissociation constants of each of the six replaceable hydrogen atoms in Calcein and calculated the formation constants of the 1:1 and 1:2 Calcein-calcium compounds. She

made the erroneous assumption that the molecule possesses the closed lactone structure and that the lactone ring remains unopened throughout the entire pH region, even at pH 12-13. It is highly improbable that an easily hydrolyzable 5-membered lactone ring can exist at this high pH. This faulty assumption was based to a large degree on the improper understanding of the structure and the acid-base and fluorescence properties of fluorescein itself, current in the literature. Hefley followed the assignment of Orndorf and Hemmer[106] of the lactone structure (Structure V, page 19) to yellow fluorescein, reasoning by analogy that Calcein, also yellow in color, has the lactone structure. In view of the symmetric nature of Calcein, even at high pH, as borne out by Hefley's NMR work, the need to preserve this symmetry was an understandable guide in leaving the lactone ring unopened. However, even with an open form structure in solutions of high pH, a symmetrical structure for Calcein can be easily conceived, one involving a zwitter ion structure and a high degree of resonance. However, it is first necessary to clarify the more basic chemistry of the parent fluorescein.

Hefley's determination of the six acid dissociation constants of Calcein was an elegant piece of work, combining data from solubility measurements, acid-base titrations, absorption spectrophotometry in the ultraviolet, and

fluorescence excitation and emission spectrophotometry. She noted the surprisingly strong acid nature of the third and fourth hydrogen atoms, which she assigned to the phenolic groups present in the molecule. Wallach et al.[151] were similarly impressed by the strength as acids of the phenolic hydrogen atoms of Calcein. For an explanation of the highly acidic nature of the phenolic hydrogen atoms of the phenolic groups, both workers adopted the explanation of Schwarzenbach et al.[124] which postulates a zwitter ion structure for iminodiacetic acid. Both failed to notice the similarity of the strength as acids of the third and fourth hydrogen atoms of Calcein to the two acidic hydrogen atoms of fluorescein, the parent molecule.

In the present work, the structure of fluorescein, both in the solid state and in solution, has been firmly established and a sound basis for the structure of Calcein provided, Structure IV. The chemistry of fluorescein, especially the acid-base properties, has been thoroughly studied and much of the discordant findings reported clarified. Once the chemistry of fluorescein was understood, that of Calcein fell into place. In addition, the interesting increase in the fluorescence of Calcein at low pH in the presence of aluminum, first observed by Wallach and Steck,[150] has been investigated; the nature of the union of aluminum with Calcein has been worked out, the

formation constant governing the equilibrium estimated, and the associated changes in the fluorescence correlated with the changes of the species present with pH.

This dissertation thus consists of two parts, the first dealing with fluorescein, the second with Calcein. The overall goal, of course, was to place the chemistry of fluorescein and Calcein on a firm foundation so that applications of the compounds to analytical chemistry could be made with assurance and understanding. Basically, the problems were ones of structure, both in the solid and in aqueous solution of varying pH. A variety of tools have been brought to bear: acid-base behavior, solubility as a function of pH, fluorescence excitation and emission spectrophotometry as a function of pH, infrared, NMR, and mass spectroscopy, and X-ray diffraction. Drawing the evidence from these various techniques into a coherent and consistent package has been both challenging and fruitful.

II. FLUORESCEIN

A. The Structure of Fluorescein, History

Although fluorescein was first prepared, by Baeyer[6], over one hundred years ago, an unequivocal assignment of structures for the solid forms of the compound and for the species in solution is still lacking. Not that there has been any lack of interest in the compound. The number of publications dealing with fluorescein, both with chemistry and with uses, is so large as to almost preclude an exhaustive search of the literature. The uses of fluorescein and its derivatives are many: in cosmetics; in the dyeing of cloth; in food as a coloring agent; in geology as tracers of water flow and markers of contamination; in the biological sciences as staining agents; in medicine as antiseptics; in analytical chemistry as adsorption and fluorescent indicators; and most recently, in the preparation of tunable dye lasers. The vast amount of such work would lead naturally to the assumption that the chemistry of fluorescein, its structure, its physical properties, and its acid-base behavior, would be well known. Such, however, is not the case.

The considerable doubt, which has continued to the present time, as to the structure of fluorescein has arisen in part because there are three solid forms of the compound,

only two of which were known prior to the work being reported in this thesis. At one time as many as five solid forms, exclusive of the new colorless form, were thought to exist; this particular bit of confusion resulted from a misidentification of mixtures of the red and yellow solid forms and from errors in ascertaining the amount of water associated with the solid materials. Moreover, commercial preparations of fluorescein are badly contaminated with heavy metals, which alter the chemistry and physical properties sufficiently that any work on fluorescein not specifically stated to have been purified must be considered suspect. Common among the metal contaminants are mercury, aluminum, zinc, and iron (Hefley[57]); these metals are derived from the catalysts used during manufacture to effect the fusion of resorcinol and phthalic anhydride and are carried into the final product by some hitherto unexplored chelating property of the fluorescein molecule.

Much of the literature on the chemistry of fluorescein, unfortunately, is sadly deficient in detail. Vital information on the identity and purity of materials used in individual investigations has often been omitted and other important matters have been reported sparsely or not at all. Even such important items as the solvent used to prepare solutions, the concentration and pH of solutions and the nature of the drying before use, have been glossed over.

Frequently no mention is made even as to whether fluorescein itself or the disodium salt, commercially called "uranine", was used; indeed, many authors consider the two to be identical.

Finally, no modern review of the chemistry of fluorescein has been made, one making full use of the concepts of aromaticity and resonance and employing the information which can be obtained from the recent developments in ultraviolet, infrared, mass and NMR spectrometry.

The principal structures which have been proposed for the various forms of fluorescein are shown in the accompanying figures (Figures 2 and 3). These structures are not presented in the order in which they were proposed chronologically, rather Structures V through IX are the ones advanced as a result of the present study, and others are numbered more or less as they appear successively in the literature. Thus, in the following survey of the historical development of the chemistry of fluorescein, Structure V is mentioned first but Structure XI next.

It is interesting to note at the start that the two earliest workers with fluorescein are counted among the great organic chemists of all time: Adolf Baeyer, in 1871[6] and again in 1876[7] and in 1882[8]; and Emil Fischer in 1874.[41] The early papers of Baeyer and Fischer, and of Meyer,[97] were devoted to establishing the composition of fluorescein and

Figure 2. Structures of fluorescein

Structure V. Lactone form: colorless solid

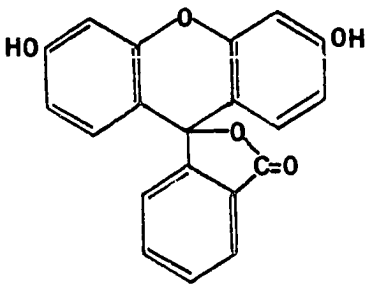
Structure VI. Zwitter ion form: yellow solid and neutral form in aqueous solution (pH 3-4)

Structure VII. Para-quinone—carboxylic acid form: red solid. Resonance forms a and b

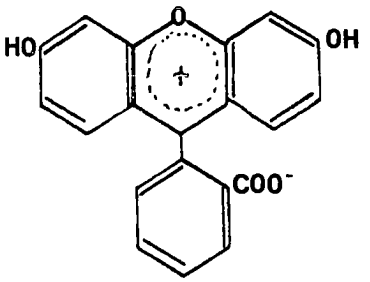
Structure XI. Structure of Kehrmann and Dengler,[74] 1908

Structure XII. Ortho-quinone form

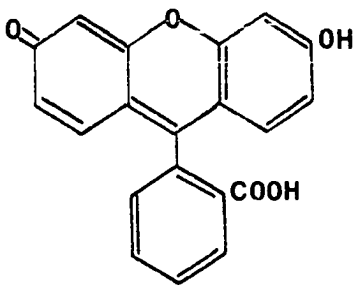
Structure XIII. Structure of Fieser and Fieser[40]



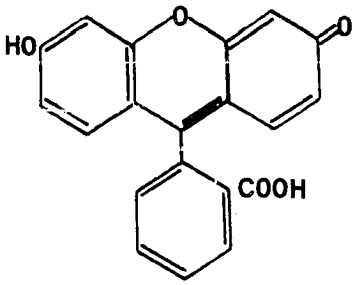
V.



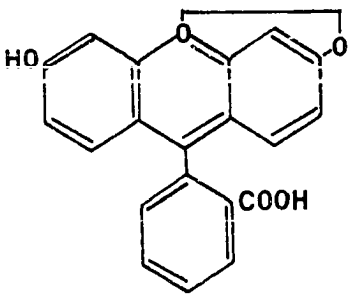
VI.



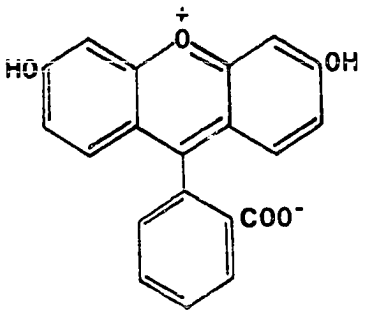
VIIa.



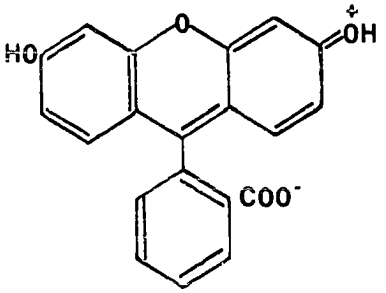
VIIb.



XI.



XII.



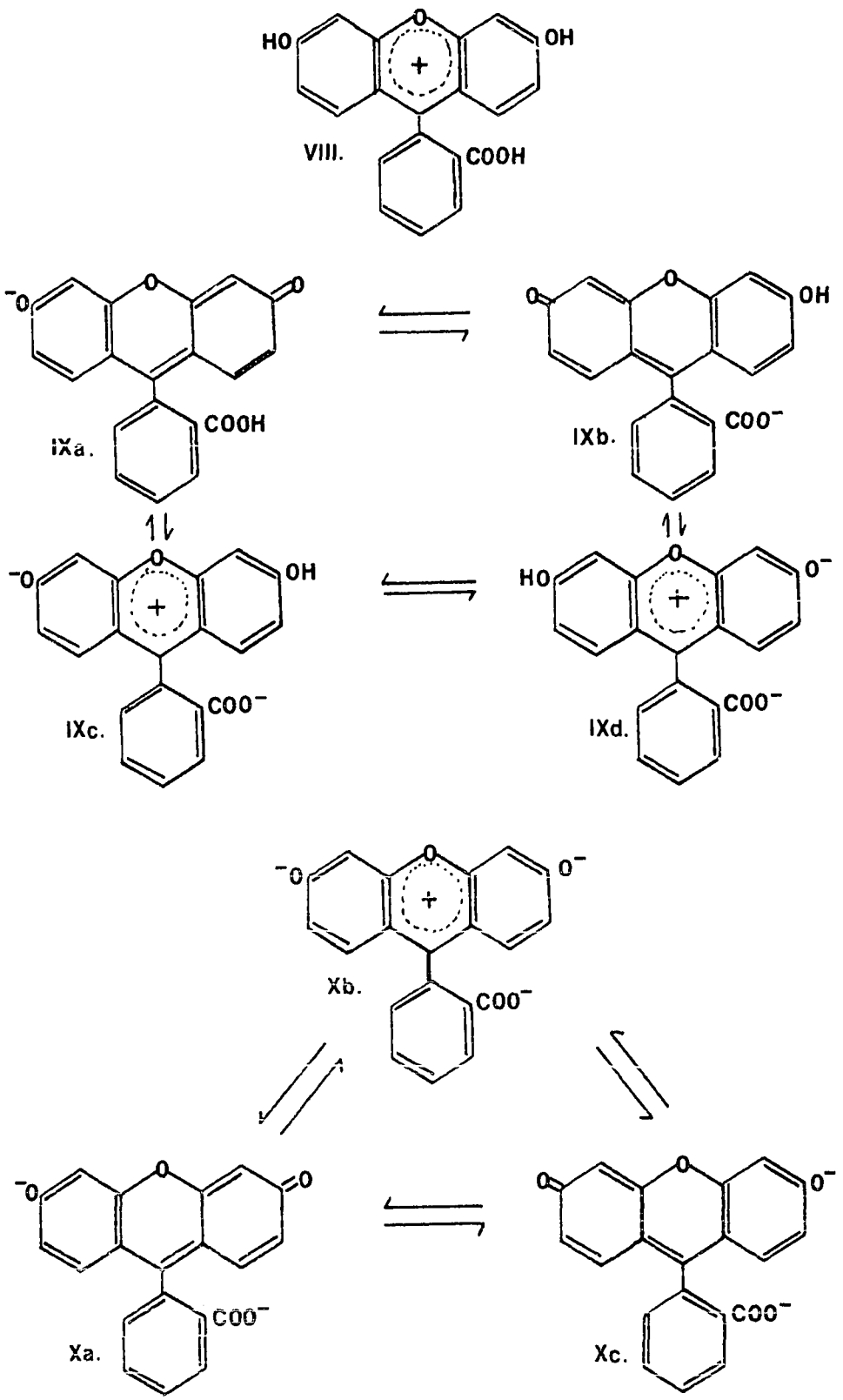
XIII.

Figure 3. Structures of ionic forms of fluorescein (H_2Fl)

Structure VIII. Monocation (H_3Fl^+), pH $<\sim 3$

Structure IX. Monoanion (HFl^-), pH ~ 4-7.
Resonance forms a and b of Zanker and Peter[163]

Structure X. Dianion (Fl^{2-}), pH $>\sim 7$.
Resonance structures a and c predominant.
Resonance structure b transient intermediate


HO
OH
VIII.
COOH
IXa.
COOH
IXb.
OH
COO⁻
IXc.
OH
COO⁻
IXd.
HO
O⁻
COO⁻
Xb.
COO⁻
Xa.
COO⁻
Xc.
O⁻
COO⁻

related compounds but also produced the first concepts of the structure.

Baeyer, when he first prepared fluorescein, by fusing together phthalic anhydride and resorcinol, obtained a "yellow-red" compound which was soluble in ethyl alcohol and was reprecipitated as a yellow solid by adding water; recrystallization from ethyl alcohol yielded a dark red material. To the yellow powder, obtained by adding water to an aqueous-alcohol solution or by acidifying an aqueous alkaline solution at room temperature, Baeyer assigned the formula $C_{20}H_{12}O_5 \cdot H_2O$; to the dark red crystalline powder, obtained by recrystallization from ethyl alcohol, he assigned the formula $C_{20}H_{12}O_5$. By analogy with the chemistry of phenolphthalein, he[8] assigned the closed lactone form, Structure V, to the yellow compound. Bernthsen[15] accepted the lactone structure for fluorescein and assigned to the salts and esters a quinonoid structure (VII). Kehrmann and Dengler[74] in 1908 were apparently the first to question this assignment. The argument was simply that such a lactone should be colorless in analogy to phenolphthalein and compounds related to it. Kehrmann and Dengler even predicted the existence of a colorless form of fluorescein, one having the lactone structure, but then suggested for the known form Structure XI. The unusual strain in the bonding in this structure made the structure unacceptable to others.

A quinone structure for fluorescein, with a free carboxylic acid group (lactone ring opened), Structure VII, was first proposed by Kropp and Decker.[88] Such quinonoid compounds are colored.

Other workers, too, were uncomfortable with the lactone structure for fluorescein, among them Pope and Howard[108,109] and Fischer and Hepp,[43] who prepared the diacetyl ester and dimethyl ether derivatives of fluorescein, both of which proved colorless.

This early confusion was compounded by Liebig[91] who claimed to have prepared five different forms of yellow fluorescein, the variations being produced by changing the solvent used for recrystallization. The differences observed were attributed largely to differences in the nature and number of molecules of solvent of crystallization.

The work of Orndorff and Hemmer,[106] at Cornell University early in the 1920's, did much to clarify the chemistry of fluorescein. Orndorff and Hemmer firmly established that fluorescein exists in only two solid forms, one yellow and one red, the two forms being anhydrous, isomeric, and interconvertible, and having the compositions $C_{20}H_{12}O_5$. Orndorff and Hemmer found that the red form can be prepared cleanly by precipitating from hot alkaline solutions by acidifying with hydrochloric acid, formic acid, or acetic acid. Yellow fluorescein can be prepared cleanly by

precipitation from alkaline solution at room temperature by dilute hydrochloric acid or acetic acid. Red fluorescein can be prepared also by: 1) precipitation in the presence of ethyl alcohol; by 2) heating a suspension of yellow fluorescein in water to boiling; and 3) by heating yellow fluorescein to 250-260°C. The latter conversion, heating the yellow solid, proceeds without loss of weight; thus the presence of solvent of crystallization and also decomposition were ruled out.

Orndorff and Hemmer found that on exposure to dry ammonia gas, red fluorescein gained weight but yellow fluorescein did not. Reasoning that a carboxyl group must be present to explain such absorption, they assigned the para-quinone structure, Structure VII, to the red form and the lactone structure, Structure V, to the yellow form. The phenol groups present in the lactone structure, they reasoned, were too weakly acidic to absorb ammonia. Later, attempts by Freytag[49] to repeat this absorption of ammonia experiment failed; the absorption of ammonia proved to be neither selective nor stoichiometric and this part of the Orndorff and Hemmer work must now be considered faulty. Their assignments, however, are the ones commonly found in the textbooks at the present time.

Orndorff and Hemmer, themselves, supplied further argument against the lactone structure for fluorescein when they

prepared eosin, a tetrabromo derivative of fluorescein, in a colorless form; to this colorless form they assigned the lactone structure, and to the red form the para-quinone-carboxylic acid structure.

To the present time the textbooks have continued to equivocate. Wawzonek,[152] for example, gives the Orndorff and Hemmer assignments but then lists numerous examples of colorless derivatives of fluorescein, assigning to them the lactone structure and further stating that the lactone ring is opened by the action of solvent or heat. Fieser and Fieser[39] also contend that a lactone structure does not account for the color of fluorescein and give for the structures the para-quinone and ortho-quinone structures, Structure VII and XII, respectively; but they do this without mentioning that both red and yellow solids exist. Structure XII is apparently the first in which the hydrogen atom of the carboxyl group has been moved to the resorcinol part of the molecule and a positive charge placed on this part of the molecule to balance the negative charge left on the carboxyl group. In a later text by Fieser and Fieser,[40] still another form, Structure XIII, is shown for the para-quinone structure. In a major organic text by Karrer,[73] the description of fluorescein is sketchy and Structures VII and XII are given without mention of the existence of yellow and red forms of the solid. Förster,[47] in a monograph on

the fluorescence of organic compounds, assigns the lactone form to the "neutral" molecule but makes no mention of color. Still more recently, Hendrickson, Cram and Hammond[61] present only one structure, XII, without specifying color.

Correlation of the structure of fluorescein with acid-base behavior and color or absorption spectrum began with Orndorff and Hemmer[106] who observed that the same absorption spectrum of fluorescein in solution is obtained whether the solution be prepared from red or yellow fluorescein. They concluded that in all solvents only the quinone form, Structure VII, exists. Furthermore, they felt strongly that yellow and red fluorescein are only physical modifications of the same chemical compound. Ramart-Lucas,[112,113] from a study of the ultraviolet spectra, arrived also at the conclusion that the same absorption spectrum was obtained starting with either the red or yellow solid. Her conclusion, however, was that both the lactone form (colorless and non-fluorescent) and the quinone form (colored and fluorescent) were present in the solution, with the quinone form predominating and the relative amounts of the two forms dependent on the solvent and concentration.

The Japanese workers, Nagase, Ohno, and Goto,[101,102] confused the picture by claiming to have prepared an additional solid form, orange in color, by precipitation from alkaline solution with acetic acid at pH greater than

5. The orange form apparently contained one molecule of water which was not removed at 110°C. The solubility of the three forms decreased in the order yellow, orange, red. The authors assigned to the yellow solid the lactone structure (V), to the orange, the para-quinone structure (VII), and to the red the ortho-quinone structure (XII). The assignments were made on the basis of the behavior on the absorption and release of ammonia and hydrogen chloride, X-ray diffraction patterns, and comparison with ethyl ether and ester derivatives. On the basis of polarographic and absorption data, they concluded further that all three forms give identical solutions. The actual species in solution depends on the pH, the ortho-quinone structure (XII) being present below pH 2, the lactone structure (V) present between pH 3 and 4, and the para-quinone structure (VII) above pH 5. No regard was apparently taken for acid-base behavior as exhibited in the charges carried at different stages of neutralization.

A potentiometric titration of fluorescein dissolved in alcohol with sodium hydroxide was carried out by Dolinsky and Jones.[34] The titration was performed as an assessment of purity (expressed as neutralization equivalent), but from the single inflection point in the titration curve, at two equivalents of base per mole of fluorescein, Dolinsky and Jones concluded that the ionization constants of the phenolic

and carboxylic acid groups were so similar that the groups are not differentiated by potentiometric titration (in alcohol). By implication, Dolinsky and Jones apparently assumed the structure to be a quinone. Dolinsky and Jones did not state the color of the solid fluorescein used; because it was prepared by precipitation from alkaline solution with acetic acid, it was probably the yellow form.

Hefley[57] in 1965 titrated red and yellow fluorescein dissolved in both water and in ethanol. The solubility of the solid forms in water was so low that she concluded that the potentiometric titration data cannot be used to estimate the dissociation constants as acids. The titration curves in ethanol were identical, indicating conversion on dissolution to a single species. Hefley proposed that in ethanol, the red form is converted to the yellow form; this is in direct contradiction to Orndorff and Hemmer[106] and to her own observation that the red form is the one precipitated on concentrating an ethanol solution. The faulty interpretation probably came about because the solutions (of both forms) are yellow.

The first infrared spectrum of fluorescein was obtained by Davies and Jones,[25] who compared the spectra of phenol, phenolphthalein, benzoic acid and its salts, and fluorescein. They concluded that fluorescein exists in the "classical structure", that is, the lactone form (Structure V). They

failed to mention whether the fluorescein studied was the red or yellow form and how the solutions were prepared which were used to form deposits on the plates of sodium chloride and silver chloride.

A second infrared spectrometric study of fluorescein was conducted by Sklyar and Mikhailov.[131] The spectra of both the yellow and the red solids was obtained from petrolatum suspensions. The absorption bands characteristic of both the lactone grouping and the carboxylic acid groups were absent from both spectra. Although admitting that they found differences, in the 1600-1700 cm^{-1} region, between the spectra of the yellow and red solids, Sklyar and Mikhailov assigned to both forms the zwitter ion structure (Structure XII) explaining away the differences as being caused by the amorphous character of yellow fluorescein.

The first serious attempt to correlate structure and the extent of neutralization of fluorescein was the work in 1958 of Zanker and Peter[163] who speak of the various "protonated forms" of fluorescein: the neutral molecule, the divalent anion (known since Baeyer's time as the fluorescent species), the monovalent anion (the range of existence of which had never been clearly defined), and the monovalent cation (present in strongly acid solutions and phosphorescent). From the absorption spectra over the

ultraviolet and visible region, Zanker and Peter obtained the first values for the acid dissociation constants: $pK_1 = 1.95$, $pK_2 = 5.05$, and $pK_3 = 7.00$. The spectra were obtained in mixtures of dioxane and water and perhaps the most important result of the work was their observation that fluorescein in dioxane was almost colorless and that the amount of yellow material present increased in proportion to the amount of water added. Zanker and Peter regarded the neutral form as existing in two forms, the colorless lactone (Structure V) and the yellow para-quinone (free carboxylic acid, Structure VII), the equilibrium between them depending on the nature of the solvent. As a transient intermediate in the conversion of the monovalent cation to the neutral species they postulated the zwitter ion structure (XII). No consideration was given the red solid form beyond a mention in the introductory paragraph of the paper of its existence and a concluding speculation that the conversion of the yellow solid to the red at high temperature involved the splitting of the lactone ring and the migration of the proton.

The dual behavior of fluorescein, that is, of acting as a cation and as an anion depending on pH, had already been anticipated, by Scharf[121] from electrophoresis studies, and even much earlier by Holmes[66] who found the isoelectric point to be at pH about 3. From paper electrophoresis

experiments on solutions of fluorescein buffered at various pH, Scharf found that at pH below 3.1 fluorescein migrated toward the cathode, indicating the presence of a cationic species; at pH greater than 3.7, fluorescein migrated toward the anode, indicating the presence of the normally observed anionic form. This reversal of migration direction went through an isoelectric point at pH 3.1-3.7, at which no migration was observed.

Other properties of fluorescein in the pH region of the isoelectric point were observed by Tezak and Tezak[139] who measured the relative intensity of fluorescence of fluorescein both as a function of pH and of concentration. The minimum in the intensity of fluorescence and presumably also a minimum in solubility, was found to occur at pH 3. Although the paper deals primarily with the precipitation process, no measurements of solubility were made. The results were interpreted in terms of the prototropic forms and acid dissociation constants of Zanker and Peter.[163]

Preliminary to an extensive investigation of the effects of light on fluorescein, Lindqvist[93] determined the dissociation constants of fluorescein by the spectrophotometric method. The studies were made on fluorescein purified by chromatography on alumina plus talcum and precipitated by acetic acid (and thus presumably in the yellow form). The values obtained for the dissociation constants were:

pK_1 = 2.2; pK_2 = 4.4, and pK_3 = 6.7. These values were obtained on water solutions; they are in rough agreement with the values of Zanker and Peter which were obtained on dioxane-water mixtures. Lindqvist also found fluorescein colorless in dry, organic, nonpolar solvents such as dioxane. By drying the dioxane thoroughly he obtained completely colorless solutions. Contrary to Scharf, Lindqvist found that the equilibrium between the colorless and quinonoid forms was established instantaneously. Lindqvist regarded, and interpreted his extensive studies of the effects of flash photolysis, in terms of the same five aqueous species and structures postulated by Zanker and Peter,[163] that is; for the monovalent cation, Structure VIII; for the neutral species a mixture of the colorless lactone (Structure V) and para-quinone (VII); for the monovalent anion, Structure IXa,b; and for the bivalent anion, Structure Xa,c.

Rozwadowski[117] studied the absorption and emission spectra of chromatographically pure red fluorescein as a function of pH. Utilizing the same species and structures of fluorescein of Zanker and Peter,[163] Rozwadowski interpreted the spectra and the decay times of the fluorescence in terms of the species and structures postulated by Zanker and Peter and the acid dissociation constants of the excited states, which he estimated. Some of the interpretation appears confusing, especially the implications that various forms coexist simultaneously over the entire pH range.

The simultaneous existence of several ionic forms of fluorescein over a wide range of pH was also assumed by Kantardzhyan[71] and by Kantardzhyan and co-workers to explain findings on the fluorescence spectra in aqueous-dioxane solutions of fluorescein and uranine.[5,54] There appears to be some assumption that perhaps solutions prepared from uranine differ from those prepared from fluorescein, and the studies involved the additions of large amounts of acids and bases. The Zanker and Peter structures are used in the explanations. Two additional forms of fluorescein were postulated, on the basis of changes in the visible absorption and fluorescence spectra of highly alkaline solutions. One form, blue in color, appeared after a few hours and was related to the disappearance of fluorescence; after prolonged storage (two or more days), the solution became colorless. However, acidification of these solutions restored the previous forms. This restoration is in contradiction to previous observations[107] on the decolorization of fluorescein in strong alkali, findings which were attributed to the rupture of the oxygen bridge in the xanthene ring followed by other disruptive irreversible changes.

The above review has been confined to those papers which deal principally with structure. The fluorescence of fluorescein has, of course, attracted a greal deal of attention and there is a considerable number of papers

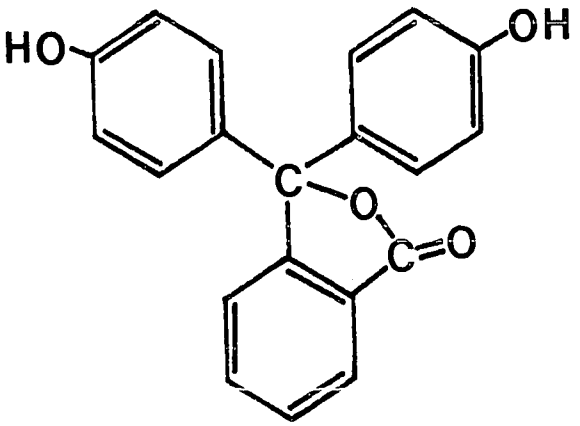
dealing with the fluorescence, varying from mere descriptions and applications of the fluorescence to involved theoretical treatments; the three or four of these which bear on structure are mentioned above. Of the papers concerned strictly with structure, excluding the early papers of Baeyer and others in which the basic features of the composition and structure were established, there are two papers which could be termed "landmark investigations". These are the papers of Orndorff and Hemmer[106] published in 1927, and Zanker and Peter,[163] published in 1958.

Orndorff and Hemmer cleared away the faulty experimental observations and resulting misconceptions on composition and structure, and largely on the basis of the absorption of ammonia gas, an experiment now known to be faulty, postulated for the yellow solid form of fluorescein the lactone structure (Structure V) and for the red solid the para-quinone structure (VII). These are the assignments found in the textbooks of organic chemistry to this day, although some authors have expressed misgivings. The Zanker and Peter work is outstanding because it recognized clearly 1) the existence and importance in solutions of cationic, neutral and anionic forms of the fluorescein, forms which depend on the degree of neutralization (proportion of replaceable hydrogen present), and 2) that a colorless form of fluorescein exists in nonpolar solvents such as dioxane

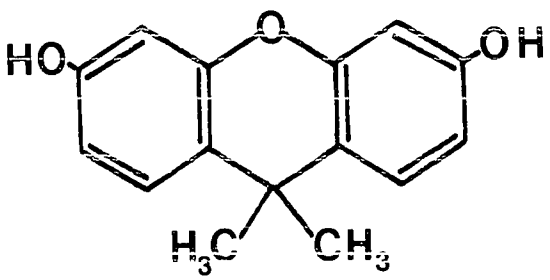
and that this colorless form is the lactone. All of the recent workers have used the Zanker and Peter concepts and although these concepts are not entirely correct they have been useful in explaining fluorescence phenomena (Tezak and Tezak,[139] Lindqvist,[93] Rozwadowski,[117] Kantardzhyan,[71] Avetisyan et al.,[5] and Grigoryan et al.[54]). The Zanker and Peter study was based principally on absorption in the ultraviolet as a function of pH and on behavior and spectra in dioxane and dioxane-water. The two papers on the infrared spectra of fluorescein (Davies and Jones[25] and Sklyar and Mikhailov[131]) were sadly incomplete but this is quite understandable as they were done at a time when the use of infrared in organic structure work was just developing.

In the present dissertation, a reexamination is made of the structures of fluorescein in the red and yellow solids and in solution in water of varying pH. Use is made of 1) fluorescence as a function of pH, 2) solubility as a function of pH, 3) potentiometric titration, and 4) infrared spectroscopy. New assignments are made for the structures of the red and yellow solids and for the neutral species and the cation in solution; new values are advanced for the acid dissociation constants; and the nonfluorescence of the singly-charged anion has been established.

For the understanding of the structures of fluorescein, it is convenient to compare the structures of the closely



XIV. Phenolphthalein



XV. 3,6-Dihydroxy-9,9-dimethyl-xanthene

related compounds phenolphthalein and 3,6-dihydroxy-9,9-dimethylxanthene. Phenolphthalein (Structure XIV) is often used as an example of a colorless compound with a lactone ring; it is nonfluorescent, owing presumably to the absence of the oxygen bridge present in fluorescein which holds the molecule rigidly in planar configuration. The 3,6-dihydroxy-9,9-dimethylxanthene molecule (Structure XV) is also similar to that of fluorescein except that the carbon atom in the 9- position carries two methyl groups; it is colorless and only moderately fluorescent in alkaline solution.

B. Materials

1. Introduction

As mentioned in Part II.A above, much of the conflicting information on fluorescein has resulted from failure to purify, properly or at all, the material studied and from failure to identify which form of fluorescein was used, whether the yellow or red solid or the disodium salt (uranine). Purification of the commercial material is not exactly easy. The heavy metals present are not eliminated by simple recrystallization or dissolutions in alkali and precipitation with acid. Nor are organic impurities present easy to eliminate (Hanousek[55]). Chromatography has been used, on alumina plus talcum, by Lindqvist,[93] and on alkaline alumina by Diehl.[30] The chemistry of fluorescein on alumina

is surprisingly complex; large volumes are required, and the procedure is tedious and uncertain. The best purification procedure is to convert the fluorescein to diacetylfluorescein, recrystallize the latter from benzene, and to hydrolyze the diacetylfluorescein back to fluorescein. This was the procedure followed in the present work.

Although both the yellow and the red solid forms of fluorescein had been known for some time previously, it was Orndorff and Hemmer[106] who, in 1927, clarified the earlier confusions about the yellow, red, orange, anhydrous, hydrated, and other odd forms of fluorescein previously reported, and who developed clean-cut methods for synthesizing the only true individual species, the yellow and red solids.

The yellow solid is formed when an alkaline solution of fluorescein is acidified carefully, best with acetic acid, at room temperature. The red solid is formed when a hot alkaline solution of fluorescein is acidified with a strong acid such as hydrochloric acid. These are the procedures followed in the present work.

The yellow solid is converted to the red form by heating the dry solid or by heating a strongly acidified suspension in water. The red form is also formed when fluorescein is crystallized from ethyl alcohol.

Even in the early days, the structures of the yellow and red solid forms of fluorescein were subjects of debate, and that an additional colorless form should exist was predicted as early as 1908, by Kehrmann and Dengler.[74] In 1914, Fischer and Hoffmann[44] prepared the colorless form of fluorescein, but it proved to be an addition product containing solvent, three molecules of quinoline per molecule of fluorescein in one case, three molecules of pyridine in another. These colorless addition products were stable for a few days but then became progressively more yellow as the quinoline or pyridine was lost to the atmosphere. Although it seems to have been known for some time that solutions of fluorescein in organic, nonpolar solvents are almost always colorless, such evidence was not considered by Orndorff and Hemmer.[106] A new era in the thinking about the structure of fluorescein began in 1958 when Zanker and Peter[163] reported that fluorescein is almost colorless in dry dioxane and went on to study the increase in the yellow color with successive additions of water to the dioxane solution. In the present work, the colorless form of fluorescein has been isolated from dioxane solution by the freeze-drying technique. Because freeze-drying is carried out in the absence of moisture from the air, the solvent was removed without converting the fluorescein to a colored form.

2. Preparation of materials

a. Diacetylfluorescein The crude fluorescein (200 g) was mixed with 100 g of anhydrous sodium acetate and added to 1 l. of acetic anhydride. The mixture was refluxed for approximately 4 hours. After completion of the reaction, the hot mixture was poured into approximately 5 l. of deionized water. The tan-colored, curdy precipitate which formed was allowed to settle overnight. It was then filtered and washed with approximately 50 ml of ethyl alcohol.

Attempts to purify the diacetylfluorescein from hot ethyl alcohol as described by Dolinsky and Jones[34] and by Hefley[57] were unsuccessful. Treatment of the precipitate with 4 gallons of hot 95 per cent ethyl alcohol produced an alcoholic solution which was deep yellow-brown in color but the major portion of the diacetylfluorescein remained undissolved as a fine, sandy powder of ivory color. The yield of the ivory powder was 173 g; the m.p. was 199-204°C. From the yellow-brown alcohol solution, 68 g of the same ivory colored material was recovered by the addition of a large amount of deionized water, followed by acidification with concentrated hydrochloric acid. The m.p. of this material was 199-202°C. The two batches were combined and purified by recrystallization from hot benzene. After dissolution in hot benzene, the yellowish solution was allowed to evaporate partially on a hot plate, and upon

cooling, a precipitate was formed which was lighter in color than the starting material. The recrystallization was repeated two more times and 177 g of an off-white powdery product was obtained; this material melted at 203.5-205.5°C. The literature values are 199.5°C,[34] 200°C,[106] 200-202°C,[101] and 205-206°C.[92]

b. Yellow fluorescein The pure diacetylfluorescein was hydrolyzed by the method of Dolinsky and Jones.[34] The compound was suspended in 95 per cent ethyl alcohol containing the appropriate amount of sodium hydroxide and the mixture was refluxed for approximately 20 minutes. The hot mixture was then filtered, and the filtrate was diluted with deionized water, cooled to room temperature, and treated dropwise with glacial acetic acid to produce a yellow precipitate. The product was filtered, washed with copious amounts of deionized water, and dried at 110-120°C for 2 hours. The compound did not melt below 300°C, but between 260 and 280°C it was completely converted to the red form. The equivalent weight was determined by potentiometric titration with 0.1 N sodium hydroxide. The average of three determinations (167.5, 165.1, 166.0) was 166.2; the theoretical equivalent weight for fluorescein, two replaceable hydrogen atoms, is 166.15.

c. Red fluorescein The red form of fluorescein was prepared by Mr. Richard C. Miller from the purified diacetylfluorescein by pouring the hot alkaline solution, obtained

from the hydrolysis of diacetylfluorescein, into hot dilute hydrochloric acid. The red precipitate was filtered, washed, and dried. The purity of the red precipitate, as determined by potentiometric titration, was 99.96 per cent.[49] Again, the material did not melt below 300°C.

Red fluorescein was also prepared by heating yellow fluorescein in a sublimation apparatus at about 250°C. After several days, the fluorescein did not appear to sublime, but the original yellow powder was converted into red crystals of seemingly regular shape. Inspection under the microscope, however, revealed that the material was highly twinned, so much so that the crystals were not suitable for a single-crystal X-ray investigation. No more work was done on the red fluorescein prepared in this fashion.

d. Colorless fluorescein The colorless form of fluorescein was prepared by freeze-drying a solution of fluorescein in dioxane. Approximately 5 g of fluorescein (red form) was added to approximately 300 ml of dioxane. Upon slight heating, the compound dissolved completely producing a yellowish solution. After freeze-drying, the residue was almost colorless with only a slight hint of yellowish coloration.

The melting point of the colorless fluorescein could not be determined with certainty. At a temperature above 120°C, the compound started to acquire more and more yellow

color until by about 175°C it was deep yellow. It began to soften at 181°C and then melted, but not too sharply, at 182-185°C to an orange-brown liquid. At 186°C, the liquid began to form small islands of a solid material, until it completely solidified by 200°C into nice orange-red crystals. Upon continued heating, the orange crystals began to display red centers at about 270°C, and by 285°C they were converted into red crystals which did not change further up to 300°C.

The equivalent weight of the colorless fluorescein, determined by potentiometric titration with 0.1 N sodium hydroxide, was 191.2, indicating the probable presence of dioxane of crystallization. The theoretical equivalent weight of fluorescein containing one-half molecule of dioxane is 188.2. The number of dioxane molecules per molecule of fluorescein is thus calculated as 0.57.

The loss in weight upon drying was determined. The colorless solid, 0.3311 g, was placed in a vacuum oven and kept at room temperature for 2½ days. The weight changed to 0.3254 g, indicating a weight loss of 1.7%. Duplicate titrations of the dried material produced equivalent weights of 184.2 and 188.4, corresponding to 0.41 and 0.50 molecules of dioxane, respectively, per molecule of fluorescein. The amount of fluorescein was determined in one of the titrated samples (the one with the equivalent weight of 184.2) by diluting the sample to 1 l. with 0.1 N

sodium hydroxide, taking a 1 ml aliquot, diluting it to 50 ml with 0.1 N potassium hydroxide, and measuring the fluorescence. A solution prepared similarly by using pure red fluorescein served as the standard. The amount of fluorescein found in the sample weighing 0.1234 g was 0.1063 g, or 85.5 per cent fluorescein. The theoretical amount calculated for a sample corresponding to 1 molecule of fluorescein plus 0.5 molecules of dioxane is 88.3 per cent fluorescein.

e. 4',5'-Dimethylfluorescein 4',5'-Dimethylfluorescein was synthesized by Hefley[58] from 2-methylresorcinol and phthalic anhydride. Initial potentiometric titrations with alkali indicated that the compound was impure. The compound was subsequently purified by conversion to the diacetyl derivative by the method of Shriner et al.[128] The dimethylfluorescein was refluxed in pyridine in the presence of acetic anhydride, and the mixture was cooled, diluted with deionized water, and treated with hydrochloric acid to precipitate the dimethylfluorescein diacetate. The solid was filtered, washed with 2 per cent cold hydrochloric acid and recrystallized twice from ethyl alcohol. The m.p. was 241.5-242.5°C.

The diacetyl derivative was then hydrolyzed by refluxing with potassium hydroxide in ethyl alcohol, the mixture was filtered while hot, and the filtrate was diluted with deionized water and treated with 1:1 hydrochloric acid to

precipitate the reddish orange 4',5'-dimethylfluorescein. The compound did not melt up to 300°C. Above 325°C charring began, and the compound was completely carbonized by 350°C. The equivalent weight obtained by potentiometric titration with 0.1 N sodium hydroxide was 181.8. The theoretical equivalent weight for 4',5'-dimethylfluorescein with 2 replaceable hydrogen atoms is 181.15. Thus the purity was 99.1 per cent.

f. 3,6-Dihydroxy-9,9-dimethylxanthene 3,6-Dihydroxy-9,9-dimethylxanthene was synthesized by following essentially the procedure of Hanousek[55,56] which involves the condensation of resorcinol and acetone in the presence of anhydrous zinc chloride. The product was colored light brown and, as evidenced by erratic melting points ranging from anywhere around 165°C to about 240°C, rather impure. Repeated recrystallizations from ethyl alcohol and treatments with activated charcoal did not improve the melting point range to the reported 260°C, nor did they remove the light brown color present in the preparation.

The compound was purified by making the acetylated derivative and recrystallizing. The diacetyl derivative was prepared by refluxing the compound with acetic anhydride in pyridine and pouring the mixture into ice water to obtain the precipitate. The solid was filtered, washed with 2 per cent cold hydrochloric acid, and recrystallized several times

from hot ethyl alcohol. The final product was yellowish-to-light brown with a sharp melting point at 153-155°C; the literature value is 151.5-152.5°C.[55] The diacetyl derivative was subsequently hydrolyzed by refluxing in alcoholic sodium hydroxide for about 30 minutes. After cooling, the mixture was diluted with water and acidified with dilute hydrochloric acid to produce a fine, almost colorless, needle-like precipitate with a slight ivory-to-light tan tinge. The melting point was 263-265°C; the literature value is 260[55] and 266°C.[56]

g. Phenolphthalein Commercial phenolphthalein (J. T. Baker, Co., N.F., Lot # 37,201) was used without any further purification or drying.

C. Structure of Solid Forms of Fluorescein

1. X-ray diffraction spectrometric study

a. Apparatus and procedure The X-ray diffraction powder patterns for the yellow, red, and colorless forms of fluorescein were obtained by Professor Donald L. Biggs of the Department of Earth Sciences at Iowa State University, Ames, Iowa, on a Geiger-Müller counter instrument, the Norelco Diffractometer Type 42266. The powdered samples were placed in a rectangular, flat-bed aluminum mounting and irradiated with X-rays from a copper target (Cu K_{α_1}

with λ = 1.54050 Å). The diffracted radiation was recorded as a series of peaks on a paper strip chart recorder.

b. Results and discussion X-ray diffraction powder patterns were obtained for the yellow, red, and colorless forms of fluorescein. The angles θ were measured for each pattern, and the interplanar spacings were derived from the equation

$$d = \frac{\lambda}{2 \sin \theta}$$

where d is the interplanar spacing in Angstrom units, λ is the wavelength of the incident radiation, and θ is half of the angle between the incident and diffracted rays. Tables from a National Bureau of Standards publication[103] were used for the conversion of θ into d values. The values were rounded off to three significant places.

The d values for the yellow, red, and colorless forms of fluorescein are listed in Table 1. It is well established that the pattern for each polymorphic form of a substance is specific and can be used as a means of identification.[78] The d values in the table are sufficiently different and distinctly unique to each form of fluorescein. Thus there is no question that the characteristic differences in color are associated with specific differences in the crystal structure and are caused neither by impurities nor by particle size. The claim that the yellow form of fluorescein is amorphous

Table 1. Interplanar d spacings of the yellow, red, and colorless forms of fluorescein from X-ray diffraction patterns (in Angstroms)

Yellow	Red	Colorless
7.31s	8.26s	7.57m
5.55vw	7.43s	7.49m
5.29m	6.60m	5.48w
4.74s	5.60s	5.04w
4.50m	5.29s	4.58m
4.29w	4.87s	4.39s
4.05s	4.79s	4.34s
3.83s	4.74s	3.84s
3.61vs	4.39m	3.66s
3.52m	4.09vw	3.49m
	3.86s	3.39w
	3.85s	3.37w
	3.68m	3.35w
	3.66m	3.18m
	3.48s	3.02w
	3.37vs	2.67w
	3.14vs	

Peak strength

vs - very strong
s - strong
m - medium
w - weak
vw - very weak

whereas the red form is crystalline[131] is also refuted. The X-ray diffraction patterns of all three forms are sharp, indicating a definite crystalline structure. For non-crystalline or amorphous substances, the pattern does not consist of sharp peaks but rather of one or a very few broad, diffuse halos.[78]

2. Mass spectrometric study

a. Apparatus and procedure Mass spectra were obtained for the yellow, red, and colorless forms of fluorescein, for the diacetyl derivative of fluorescein, for 4',5'-dimethylfluorescein, and for dioxane on the Atlas CH4 Mass Spectrometer.

The solid samples were introduced by a conventional vacuum lock system, the liquid dioxane sample by volatilization at 150°C. The source temperature was increased to a level that provided suitable vaporization. The ionization energy was varied between 16 and 70 eV, the accelerating voltage was 3000 V, and the collector type was SEV (1.6). The m/e peaks were recorded as five-element galvanometer tracings on light-sensitive paper.

b. Results and discussion The mass spectra of yellow and red fluorescein are identical both in the number and location of the m/e peaks and in their relative intensity. Thus it is apparent that application of heat for vaporization of the sample converted the yellow fluorescein

to the red form, an event that is not unexpected inasmuch as heating converts yellow fluorescein into red fluorescein. This conversion of the solid has been observed by Orndorff and Hemmer[106] and has been repeated in this work.

In the mass spectra the parent peak, P, at 332 m/e agrees with the molecular weight of 332.30 for fluorescein, $C_{20}H_{12}O_5$. A distinct P − 1 peak at 331 m/e is also observed. The spectrum is characterized by the following m/e peaks: 304 weak; 288 and 287 very strong; 272 and 271 strong; and 259 and 258 medium-to-weak. There are evidently two different pathways for the degradation of fluorescein, consistent with the m/e peaks in the pattern. In the less favorable pathway, the peak at 304 m/e corresponds to a loss of CO (P − 28); subsequent decarboxylation gives rise to the peaks around 259 m/e (P − 28 − 45). In the more favorable pathway, the very strong peaks at 288 and 287 m/e correspond to decarboxylation (P − 44,45), and then the subsequent loss of O or OH results in the peaks at 272 and 271 m/e. The degradation patterns are characteristic of compounds containing phenolic, ketone, carboxylic, and ether functions.[96,130] The pattern at lower m/e values becomes too complex for analysis; there are prominent peaks at 202 and at 143 m/e, but no fragmentation sequence was found to explain them.

For the colorless form of fluorescein, the mass spectrum is similar to that of red and yellow fluorescein, except that

many of the peaks are weaker. The parent peak is absent and becomes visible only after application of 200 units of heat (as compared with 85 and 95 units for yellow and red fluorescein, respectively) and subsequent ionization at 70 eV. Such weak appearance of the parent peak is typical of 5-membered lactone rings with the γ carbon substituted,[130] and is consistent with the assignment of the lactone structure for the colorless fluorescein. After vaporization and ionization, the degradation pattern becomes identical with that of yellow and red fluorescein. A notable exception is a medium-to-weak peak at 237 m/e. This peak is not present in the spectra of yellow and red fluorescein and is probably characteristic of the lactone structure only.

Another difference in the mass spectrum of colorless fluorescein is the distinct peak at 88 m/e, followed by one at 58 m/e, indicating the presence of dioxane, as expected. For confirmation, a mass spectrum of dioxane was obtained. The prominent parent peak at 88 m/e and the strong peak at 58 m/e (P − 30), corresponding to the loss of an OCH_2 group, are characteristic of that class of compounds. Thus the mass spectrum of colorless fluorescein is another proof that dioxane is present.

The mass spectrum of diacetylfluorescein is also instructive. The parent peak, P, at 416 m/e confirms the molecular weight of diacetylfluorescein, $C_{24}H_{16}O_7$. The

next prominent peak at 330 m/e corresponds to the loss of two CH_3CO groups (P – 2x43); a weaker peak at 373 m/e corresponds to the loss of only one CH_3CO group (P – 43). The loss of OR groups is typical of esters.[130] Subsequent peaks at 304, 288, 272, and 259 m/e indicate a degradation pattern similar to that of the parent compound, fluorescein itself. An additional peak at 314 m/e may be due to the loss of oxygen from the fragment corresponding to 330 m/e.

Thus the mass spectra are indicative of the easy convertability of yellow fluorescein into red fluorescein by application of heat. The mass spectrum of colorless fluorescein points to an initial lactone structure which, upon ionization, follows a degradation pattern essentially similar to that of the other two forms. The presence of dioxane in the colorless form of fluorescein was also confirmed. The mass spectrum of the diacetyl derivative is typical of an ester of that type, first splitting off the ester group and then following the fragmentation pathway of the parent compound.

The mass spectrum of 4',5'-dimethylfluorescein has more peaks than any of the other fluoresceins, but the assignment of a fragmentation pattern is rather straightforward. A very intense peak at 360 m/e is the parent peak P which confirms the molecular weight of 360 for a fluorescein substituted by two methyl groups, $C_{22}H_{16}O_5$. The loss of both

methyl groups gives rise to a moderate peak at 330 m/e (P − 2x15); subsequent decarboxylation results in the strong peak at 286 (330 − 44). In another pathway, the molecule is first decarboxylated, and then it loses one methyl group and then the other methyl group, accounting for the very strong peak at 316 (P − 44), 301 (316 − 15), and 286 m/e (301 − 15), respectively. A moderate peak at 272 m/e is the result of a CH_2 loss from the 286-m/e fragment (286 − 14). Subsequent loss of OH or CO gives rise to the very weak peaks at 255 (272 − 17) and 244 m/e (272 − 28), respectively. Starting with a degradation pattern at 226 m/e, the spectrum becomes too complex for analysis.

3. Infrared spectrophotometric study

a. Apparatus and procedure The infrared spectra of the yellow, red and colorless forms of fluorescein were obtained on solid films of the fluorescein using the Beckman IR8 Spectrophotometer.

The samples were prepared by suspending the solid in a minimum amount of ether, smearing the suspension over the surface of a sodium chloride plate, and allowing the solvent to evaporate rapidly. The residue was a thin film, the color of which resembled that of the original sample; thus, no significant conversion of one form of the fluorescein into another occurred. The film adhered strongly to the

plate, and this allowed the plate to be mounted in the spectrophotometer for the absorption measurements.

The potassium bromide pellet technique proved unsatisfactory as a method of preparing samples for infrared measurements. Often, the prepared pellets were mechanically too brittle to permit normal handling. On other occasions, blotches of moisture and discoloration were visible within the pellet. Potassium bromide is very hygroscopic; this has been noted previously and the effects on infrared spectra of the almost unavoidable absorption of moisture studied. Because the state of fluorescein is affected by the presence of water and the exclusion of moisture proved very difficult, the use of the potassium bromide pellet method was abandoned.

The nujol mulling technique and the use of other organic liquids was considered and abandoned, principally because the particular solid form assumed by fluorescein depends greatly on the liquid present or from which the solid is formed. In addition, the absorption band of the carbonyl group, the band of particular significance in this study, is known to be sensitive to the effects of solvents.[114]

The infrared spectrum of 3,6-dihydroxy-9,9-dimethylxanthene, a compound used for comparative purposes, was obtained on a potassium bromide pellet by using the Perkin-Elmer Model 237B Infrared Spectrophotometer.

b. Results and discussion The infrared spectra obtained from the solid films of the yellow, red, and colorless forms of fluorescein are marked in general by broad bands, low absorption, and not as much detail as found in spectra commonly obtained for compounds in solution. The disadvantages of solid film spectra are well-known.[114] Crystal orientation in preferred direction, variations with particle size, method of preparing the sample, molecular association, and polymorphism all affect the quality of the spectra. Additional difficulties are encountered because of adsorption effects and interactions with alkali halide matrices with compounds bearing carboxylic or phenolic groups. Despite the difficulties, sufficiently good spectra were obtained on films in the present work to permit assignments of structures to each of the three solid fluoresceins.

The infrared spectra of the yellow, red, and colorless forms of fluorescein are presented in Figures 4, 5, and 6, respectively, and the characteristic absorption bands are listed in Tables 2 and 3.

For convenience in study, the infrared absorption bands of fluorescein are best grouped into four regions: 1800-1600 cm^{-1} characteristic of the carbonyl group; 1600-1350 cm^{-1} characteristic of the aromatic skeletal carbon-carbon stretch; 1300-1100 cm^{-1} characteristic of the carbon-oxygen stretch;

Figure 4. Infrared spectrum of yellow fluorescein

Solid film

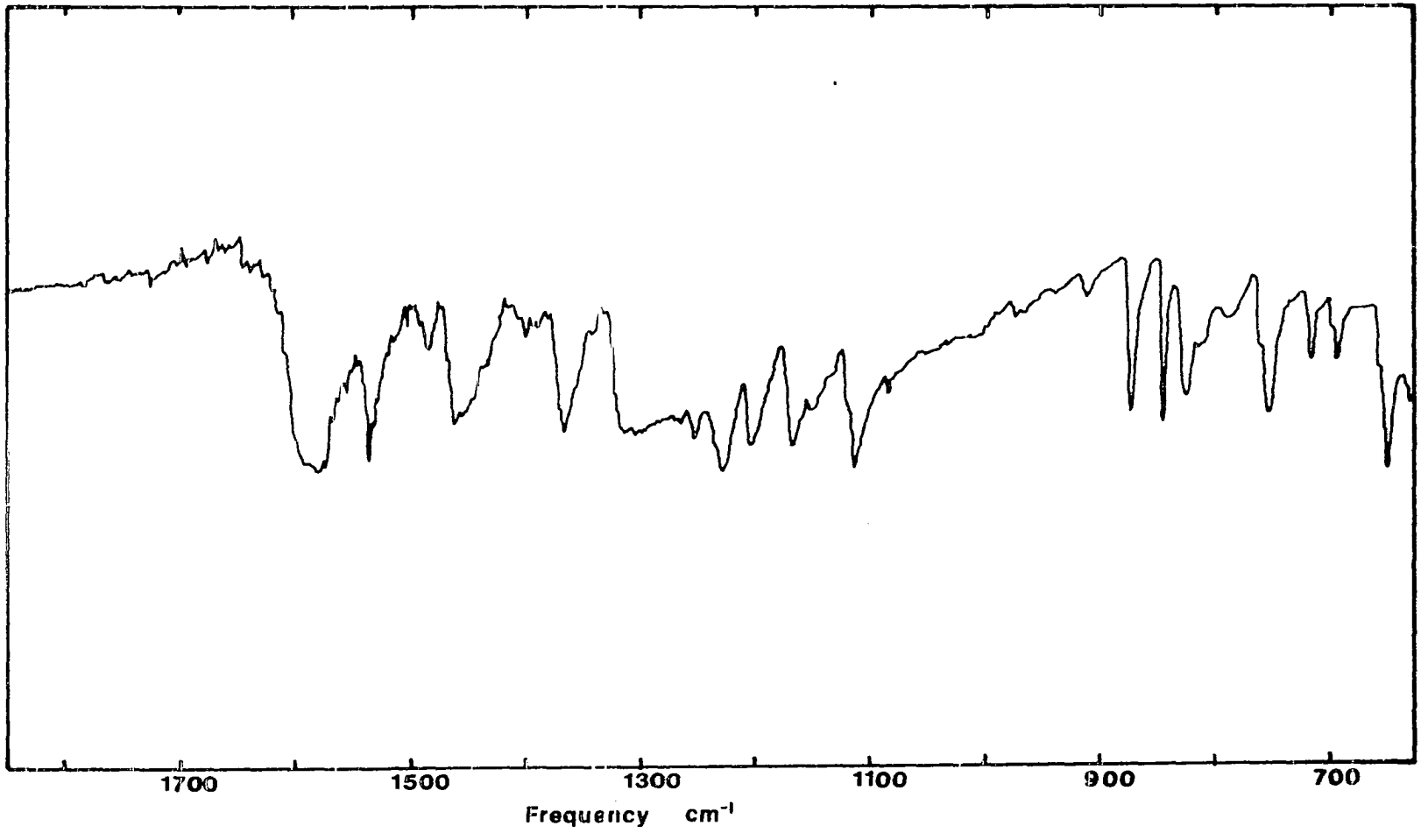

1700
1500
1300
1100
900
700
Frequency cm⁻¹

Figure 5. Infrared spectrum of red fluorescein

Solid film

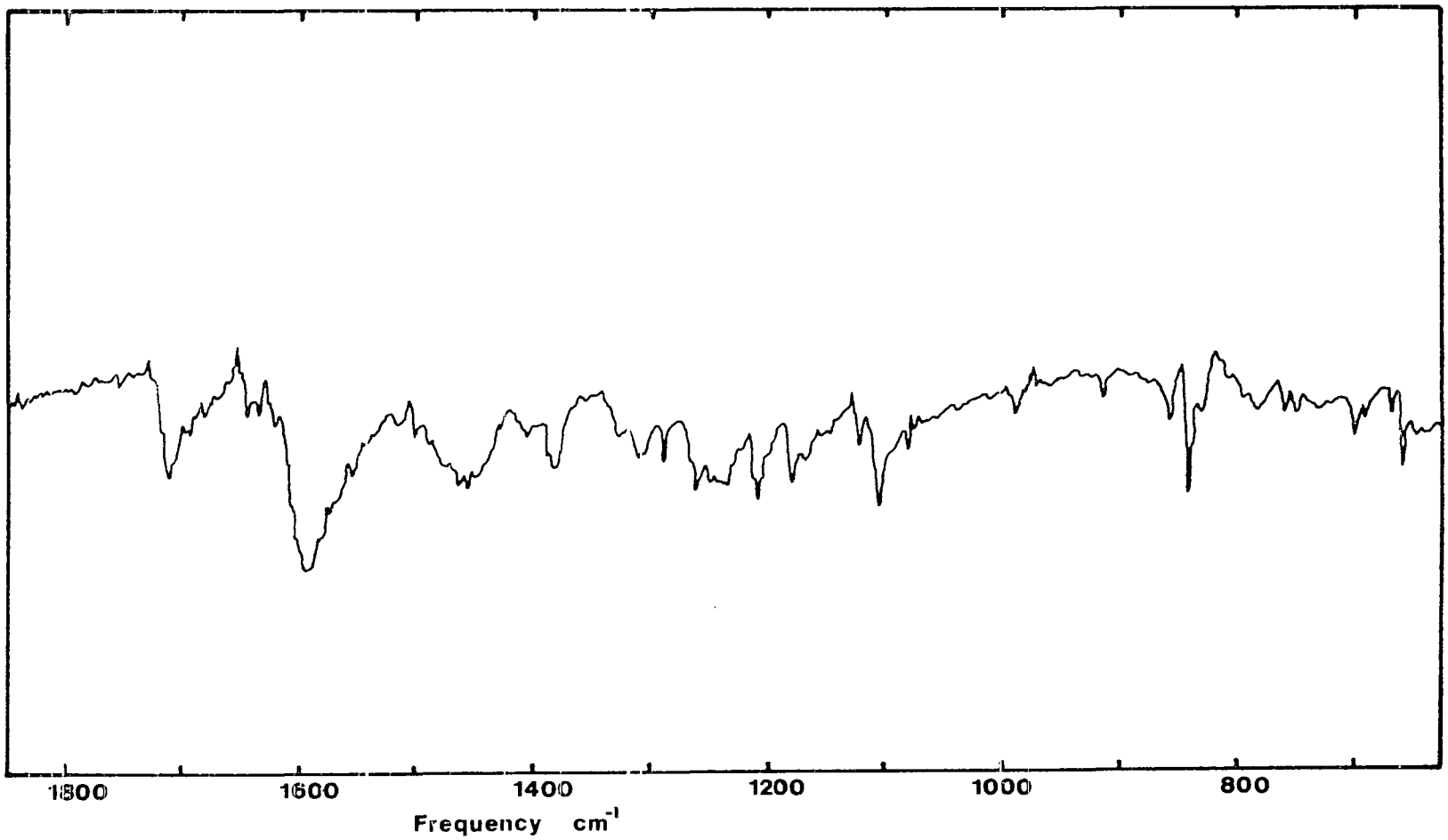

1800
1600
1400
1200
1000
800
Frequency cm-1

Figure 6. Infrared spectrum of colorless fluorescein

Solid film

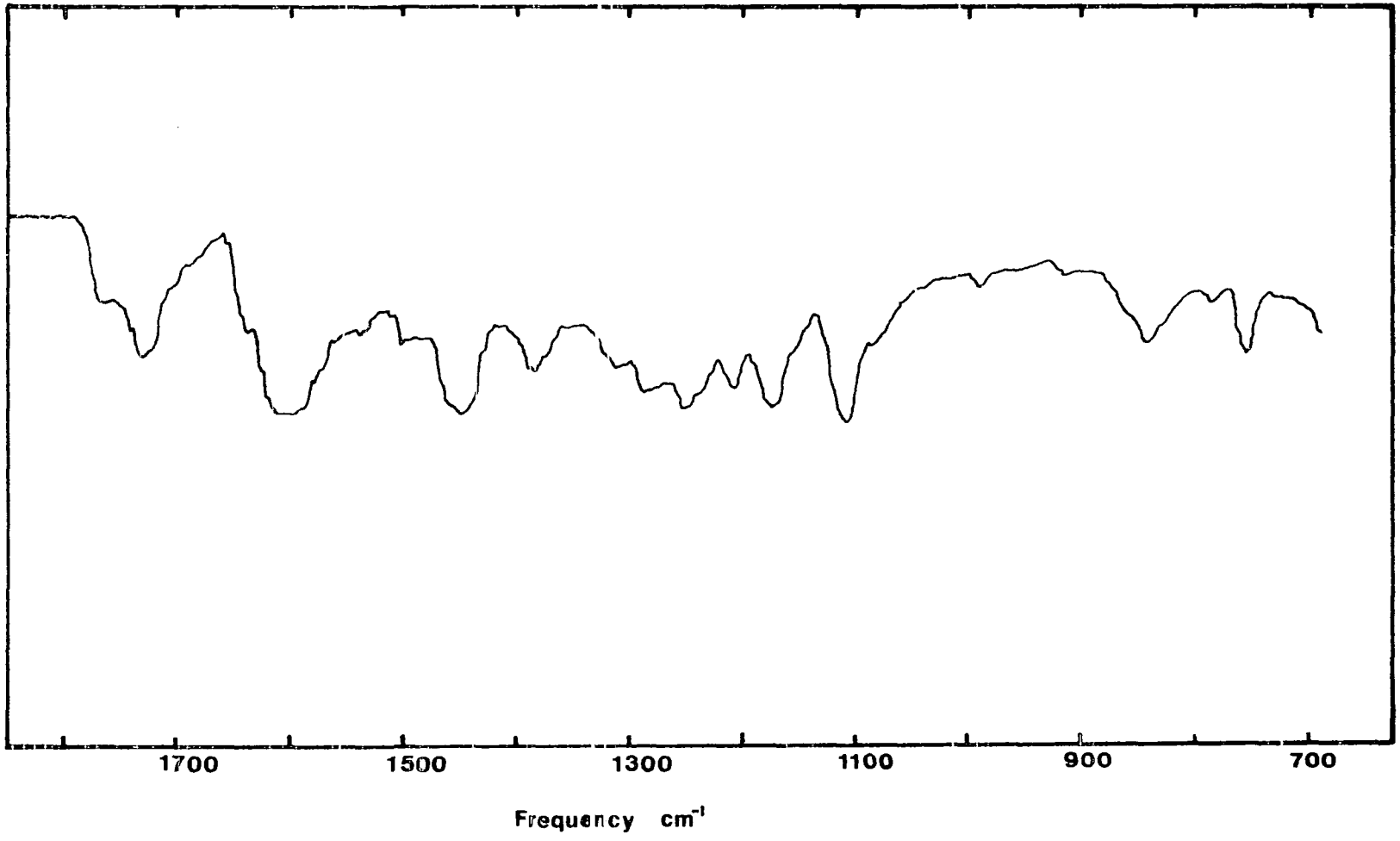

1700
1500
1300
1100
900
700
Frequency cm-1

Table 2. Major infrared absorption bands of fluorescein

Form	Carbonyl-type frequencies (cm^{-1})				
	Lactone	Carboxylic Acid	Carboxylate	Quinone	Others
Yellow	none	none	-[a]	-[b]	1536
Red	none	1711	-[b]	-[c]	none
Colorless	1730 plus 1770-1760 shoulder	none	-[b]	-[b]	none

Form	Aromatic skeletal carbon-carbon stretching frequencies (cm^{-1})		
Yellow	1595-1572[d]	1461-1450 plus 1430 shoulder[e]	1370
Red	1600-1590[f]	1465-1455	1382
Colorless	1610-1590	1460-1450	1385
Disodium salt[g]	1575[d,f]	1460[e]	1380
Disodium salt[h]	1580[d,f]	1460[e]	1398

[a]Expected but see note d.

[b]None expected and none observed.

[c]Expected but see note f.

[d]Contains asymmetric carboxylate stretching bands.

[e]Contains symmetric carboxylate stretching band.

[f]Contains conjugated carbonyl band.

[g]Data of Freytag, ref. 49.

[h]Data of Davies and Jones, ref. 25.

Table 3. Minor infrared absorption bands of fluorescein

Form	Carbon-oxygen stretching frequency region (cm^{-1})						
Yellow	1315 - 1280br	1255w	1229m	1202m	1159m	1112m	
Red	1310w	1290w	1262m	1234m	1210m	1180w	1108m
Colorless	1315vw	1285w	1250w	1232vw/sh	1206vw	1180-70w	1110m

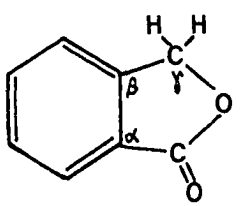
Form	Aromatic carbon-hydrogen bending frequency region (cm^{-1})							
Yellow	871m	--	845m	821 + 810sh	788w	752m	716w/sh	694w/sh
Red	none	858w	841m	--	780vw/br	--	720w	700w
Colorless	none	--	845m	--	785vw	754m	--	--

br - broad
w - weak
vw - very weak
m - medium
sh - shoulder

and 900-650 cm^{-1} characteristic of the aromatic carbon-hydrogen bending vibrations. In the last three of these regions, the infrared spectra of all three forms of fluorescein are very similar. In the carbonyl region, however, major differences occur.

In the region of carbonyl absorption, 1800-1600 cm^{-1}, no band at all is present in the spectrum of yellow fluorescein, one at 1711 cm^{-1} is present in that of red fluorescein, and one at 1730 cm^{-1} is present in that of colorless fluorescein. In the spectrum of yellow fluorescein, a band is present at 1536 cm^{-1} which does not appear in either the spectrum of red fluorescein or that of colorless fluorescein. The interpretations placed on these bands are: 1) the band at 1711 cm^{-1} indicates the presence of a free carboxylic acid group in red fluorescein; 2) the band at 1730 cm^{-1} indicates the presence of a lactone ring in colorless fluorescein; and 3) the band at 1536 cm^{-1} indicates the presence of an oxygen-containing pyrylium-type ring in yellow fluorescein. The structures assigned then are: 1) for the colorless form, the lactone structure, Structure V (page 19); 2) for the red form, the para-quinone–carboxylic acid structure, Structure VIIa,b (page 19); and 3) for the yellow form, the zwitter ion structure, Structure VI (page 19).

Structure V, assigned to the colorless form of fluorescein, contains a five-membered, γ-lactone ring. The absorption of a normal, saturated, five-membered lactone ring generally falls in the frequency region 1780-1760 cm^{-1},[35] and it is such a frequency which earlier workers, Davies and Jones[25] and Sklyar and Mikhailov,[131] searched for unsuccessfully in the spectra of yellow and red fluorescein. Phthalide (Structure XVI), generally considered as a model

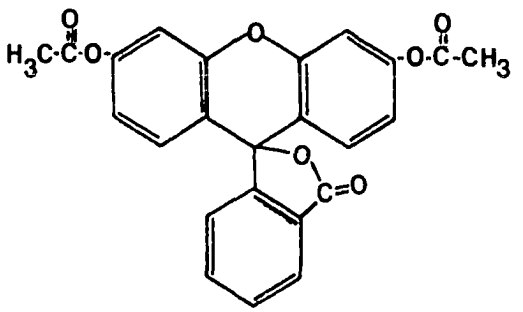


XVI. Phthalide

compound for the lactone ring, has a carbonyl absorption band at 1776 cm^{-1}.[111] However, substitution of the hydrogen atoms on the carbon atom in the γ-position results in a considerable variation in the frequency of the lactone band. Phthalides in which a hydroxyl or chloride group replaces one of the hydrogen atoms on the γ-carbon atom and a disubstituted phenyl ring replaces the other hydrogen atom have carbonyl frequencies ranging from 1802 cm^{-1} to 1757 cm^{-1}.[52] Other substituents can decrease the frequency considerably. In the related compound carrying a methyl group on the

γ-carbon atom and a hydroxyl group on the aromatic ring in position 7, the carbonyl frequency appears at 1727 cm^{-1}.[65] For several phthalides with various substituents for the hydrogen atoms at the γ-position, the carbonyl absorption bands range from 1745 cm^{-1} to as low as 1715 cm^{-1}.[153] In the closed, lactone form of fluorescein, the carbon atom in the γ-position can be considered as being substituted by two phenyl-like groups with hydroxyl groups in the para-positions; the carbonyl frequency can be expected to be decreased. A group of similar compounds, the phthaleins, for which the lactone structure is undisputed because they are colorless in the solid, neutral form, do have such lowered frequencies;[110] in phenolphthalein the band appears at 1725 cm^{-1}, in cresolphthalein at 1700 cm^{-1}, and thymol-phthalein at 1725 cm^{-1}. Davies and Jones[25] found the carbonyl band of phenolphthalein at 1740 cm^{-1}. Thus, the assignment of the band at 1730 cm^{-1} in the spectrum of colorless fluorescein to the carbonyl group of a lactone ring is most reasonable, despite the objection raised by Sklyar and Mikhailov[131] that such a frequency is too low for a five-membered lactone ring.

In their work, Sklyar and Mikhailov[131] assigned the unusually strong absorption band at 1760 cm^{-1} present in the spectrum of diacetylfluorescein (Structure XVII) to the lactone ring. On the basis of the above discussion, however,



XVII. Diacetylfluorescein

this assignment seems unwarranted. The position and intensity of the band in diacetylfluorescein indicates rather that the absorption is due to the acetate group. Aromatic esters absorb at 1760 cm^{-1}.[24] Rao[114] reports a band at 1761 cm^{-1} for acetate esters of the type RCOOAr. Thus 1730 and not 1760 cm^{-1} is the frequency associated with the lactone ring of Structure V of fluorescein.

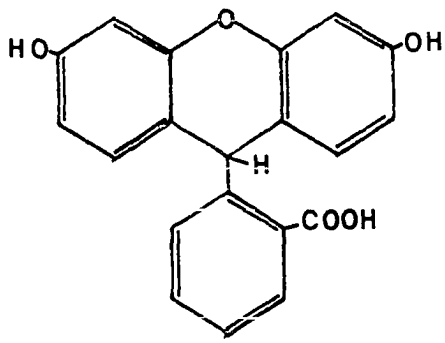
In the work of Davies and Jones,[25] the carbonyl band of fluorescein was observed at 1729 cm^{-1}. Although they did not mention which colored form of fluorescein was used in their study, it can be presumed that during the preparation of the sample by deposition of a solid film from a solution of an unspecified solvent they prepared the closed lactone form. Indeed, the rest of their spectrum is similar to that obtained in this work for the colorless form of fluorescein. Thus, it is not surprising that they assigned

the closed lactone form, "the classical formula" in their own words, as the structure of fluorescein (yellow or red).

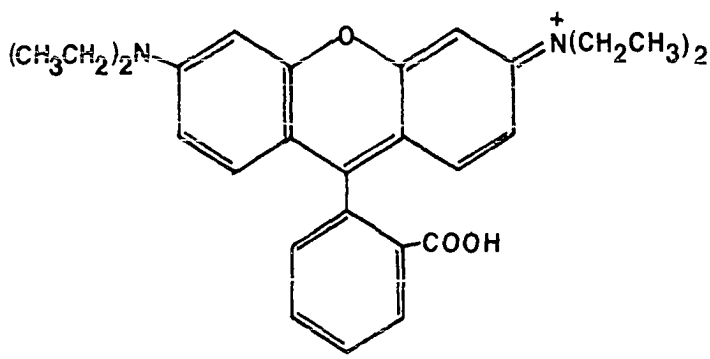
In the present work, there appears in the spectrum of colorless fluorescein, on the 1730-cm^{-1} band, a sizable shoulder at 1770-1760 cm^{-1}. Colorless fluorescein carries 0.5 molecules of dioxane, the solvent from which it was formed, per molecule of fluorescein and it would be convenient to assign this shoulder to dioxane. In the infrared spectrum of dioxane, there appears a very weak band at 1725-1720 cm^{-1} and much stronger bands at 868 and 888 cm^{-1}.[119] Rao[114] found bands in the region 1750-1700 cm^{-1} which were variable and which he attributed to impurities. In the spectrum of colorless fluorescein, obtained in this work, no bands appear at 868 and 888 cm^{-1}. The shoulder at 1770-1760 cm^{-1} thus presents a problem. A plausible explanation is that dioxane and fluorescein form an "adduct" or "addition compound" of such nature that the normal vibration of the carbonyl group of the lactone ring is distorted.

Structure VIIa,b (page 19), assigned to the red form of fluorescein, is the para-quinone structure carrying a carboxylic acid group. The band found at 1711 cm^{-1} in the spectrum of red fluorescein is typical of aromatic carboxylic acids. For benzoic acid the band appears at 1690 cm^{-1}, for fluorescin (the reduced form of fluorescein, Structure

XVIII) the band appears at 1706 cm^{-1},[49] and for Rhodamine B (a dye analogous to red fluorescein, Structure XIX) at 1715-1705 cm^{-1}.[110]



XVIII. Fluorescin



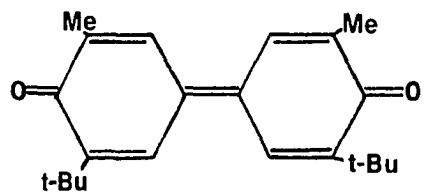
XIX. Rhodamine B

A valid criticism has been raised by Davies and Jones,[25] that the presence of a carboxylic acid function in the fluorescein molecule should result in absorption in the 3500-2500 cm^{-1} region. Besides the absorption of the carboxylic acid group, this region should also contain bands owing to the phenolic hydroxyl group and the aromatic carbon-hydrogen stretch band at 3100-3000 cm^{-1}.[130] However, no significant absorption bands are found in the entire region 3500-2000 cm^{-1} in the spectra of all three forms of fluorescein. The very weak and broad band at about 3200-3000 cm^{-1} observed in this work in the spectrum of red fluorescein is too small for inclusion in the discussion. This lack of absorption is attributed to the nature of infrared spectra obtained on solid films deposited on sodium chloride plates. The difficulties encountered in this method have been discussed previously.

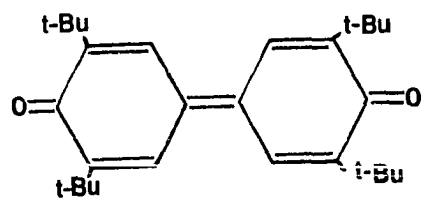
A more serious concern is the apparent lack of an absorption band corresponding to the quinone carbonyl group of red fluorescein, as required by Structure VIIa,b. The carbonyl absorption bands for quinone-type compounds are generally found at 1685-1626 cm^{-1}.[114] Yet no absorption band in this region appears in the spectrum of red fluorescein. The reason for this is that the frequency of the carbonyl group is decreased considerably whenever the carbonyl group is conjugated with double bonds.[14,24]

Conjugation of a carbonyl group with carbon-carbon double bonds and phenyl groups results in delocalization of the π electrons, thus causing the absorption to be shifted to lower frequencies.[130]

A decrease in the frequency of the carbonyl group absorption as a function of conjugation can be seen in many of the compounds tabulated by Szymanski.[137] In some of the compounds, such as XX and XXI, the carbonyl absorptions



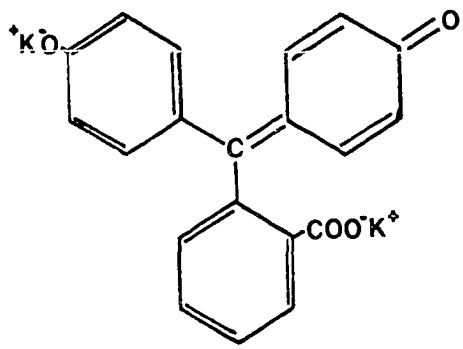
XX.



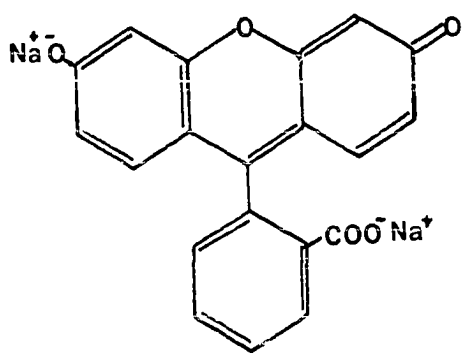
XXI.

appear at 1604 cm^{-1} and 1597 cm^{-1}, respectively. In structure VIIa,b, assigned to red fluorescein, a very high

degree of conjugation of the quinone results in a decrease of the frequency of the carbonyl absorption and merging with the broad aromatic absorption band at 1600-1590 cm^{-1}. In the infrared spectra of the dipotassium salt of phenolphthalein (Structure XXII) and the disodium salt of fluorescein (Structure XXIII), in both of which the same quinone



XXII. Dipotassium salt of phenolphthalein



XXIII. Disodium salt of fluorescein

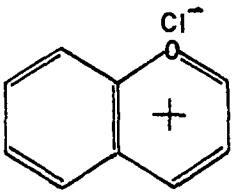
group is present as in Structure VIIa,b the absorption bands appear at 1572 and 1580 cm^{-1}, respectively,[25] again indicating a merging of the carbonyl absorption with the aromatic absorption band.

Structure VI (page 19), assigned to the yellow form of fluorescein, is a zwitter ion involving a carboxylate group. This assignment was made because in the spectrum of yellow fluorescein no absorption band appears in the region 1800-1600 cm^{-1} and thus neither lactone ring nor carboxylic acid group can be present in the molecule. The carboxylate anion is characterized by two absorption bands, a strong band at 1600-1560 cm^{-1}, corresponding to the asymmetric stretching vibrations, and a weaker band at 1430-1400 cm^{-1}, corresponding to the symmetric stretching vibrations. A major absorption band, that associated with the aromatic carbon-carbon stretch, is present in the spectrum of each of the three solid forms of fluorescein: in the yellow at 1595-1572 cm^{-1}, in the red at 1600-1590 cm^{-1}, in the colorless at 1610-1590 cm^{-1}. For yellow fluorescein this band appears at lower frequency than for the red and colorless forms; the shift to lower frequency can be attributed to a merging of this band with that of the asymmetric stretch of the carboxylate group. The merging of the asymmetric stretching band of the carboxylate group and the aromatic carbon-carbon stretch was also observed by Davies and Jones[25]

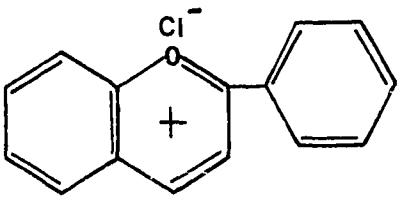
in the spectra of the disodium salt of fluorescein (Structure XXIII) and of the dipotassium salt of phenolphthalein (Structure XXII), in both of which the carboxylate anion is surely present. They attempted to explain this phenomenon in terms of a resonating oxygen system with centers in which the oxygen frequencies are coupled. The symmetric stretching vibration of the carboxylate group, at 1430-1400 cm^{-1}, is weak and of much less use for diagnostic work than the asymmetric stretching vibration; sometimes it is not seen at all, especially in complex molecules in which other strong absorption bands lie nearby. This may be the case for yellow fluorescein, although the band may simply be buried under the 1461-1450 cm^{-1} band, especially since there appears to be a weak shoulder at about 1430 cm^{-1}.

There is present in the spectrum of yellow fluorescein a sharp, well-defined band at 1536 cm^{-1}. This band had been observed previously[49,131] but no importance was attached to it. The band does not appear in the spectrum of either red fluorescein or colorless fluorescein. The structure proposed above for yellow fluorescein contains a central, six-membered, oxygen-containing ring carrying a positive charge. This ring structure is identical with that in a class of compounds known as the pyrylium salts. The pyrylium salts constitute an important family of compounds which include the anthocyanin dyes, the yellow, red, and blue coloring

matters of the flowers, fruits and plants. Prominent in the family are the benzopyrylium and flavylium salts:



XXIV. Benzopyrylium chloride



XXV. Flavylium chloride

The infrared spectra of pyrylium ring compounds are characterized by two absorption bands, one at 1650-1615 cm^{-1}, attributed[115,126] to a symmetric carbon-oxygen stretching vibration, and a second, attributed to an asymmetric stretching vibration, with a frequency stated by various authors to be 1540-1530 cm^{-1},[126] 1552 cm^{-1},[142] 1548-1520 cm^{-1},[9] and 1560-1530 cm^{-1}.[3] In the spectrum of yellow fluorescein, the symmetric stretching band is probably merged with the strong, broad band resulting from the aromatic carbon-carbon stretch at 1595-1572 cm^{-1}, but the

Figure 7. Infrared spectrum of 3,6-dihydroxy-9,9-dimethylxanthene

Potassium bromide pellet

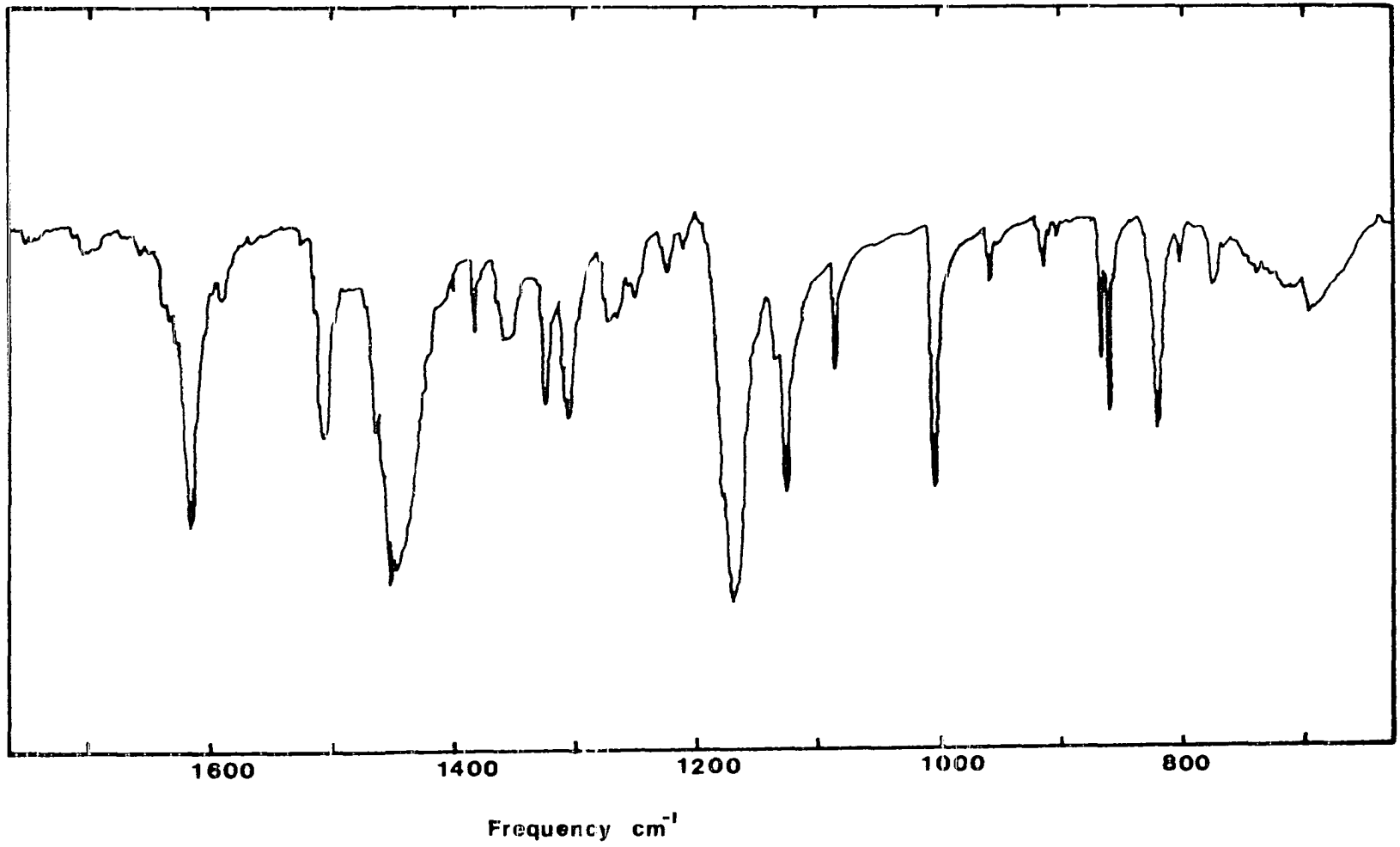

1600
1400
1200
1000
800
Frequency cm⁻¹

Table 4. Infrared absorption bands of 3,6-dihydroxy-9,9-dimethylxanthene

Band	Intensity	Assignment of vibration
1620	strong	Aromatic skeletal carbon-carbon stretch
1502	medium	Aromatic skeletal carbon-carbon stretch
1450	very strong	Asymmetric methyl bending plus aromatic skeletal carbon-carbon stretch
1379	very weak	Symmetric methyl bending
1350	weak	Unassigned, but probably associated with carbon-oxygen vibrations
1322	weak	
1300	weak	
1166	very strong	Carbon-oxygen stretch of phenolic group
1120	medium	Carbon-carbon in-plane strain plus methyl rocking deformation
1000	medium	Symmetric carbon-oxygen-carbon stretch
852	weak	Carbon-hydrogen out-of-plane bending, aromatic
813	weak	Carbon-hydrogen out-of-plane bending, aromatic

unique band at 1536 cm^{-1} can be used with certainty as support for the assignment of Structure VI (page 19) for yellow fluorescein.

Aside from the well-defined band at 1536 cm^{-1} in the spectrum of yellow fluorescein, the infrared spectra of all three solid forms of fluorescein are very similar at frequencies below 1600 cm^{-1}. The major bands at about 1600, 1450 and 1380 cm^{-1} in each spectrum are associated with skeletal aromatic carbon-carbon stretching vibrations and can be found in many aromatic compounds. For example, in the spectrum of 3,6-dihydroxy-9,9-dimethylxanthene, Structure XV (page 36), a compound which is very similar to the xanthene portion of the molecule of fluorescein, the bands at 1620 and 1450 cm^{-1} are among the most prominent (Figure 7 and Table 4). In the 1380-1370 cm^{-1} region, the bands are strong in the spectra of the three fluoresceins but weak in the spectrum of 3,6-dihydroxy-9,9-dimethylxanthene. In the spectra of phenolphthalein (Structure XIV, page 36) and of its dipotassium salt (Structure XXII, page 72), the bands appear at 1366 cm^{-1} and at 1367 cm^{-1}, respectively.[25] Possibly these bands can be attributed to vibrations in the phthalate portion of the fluorescein molecule.

The absorption bands in the spectra of the three forms of fluorescein in the region of the carbon-oxygen stretching frequencies (1300-1100 cm^{-1}) and aromatic out-of-plane

bending frequencies (900-650 cm^{-1}) associated with adjacent carbon-hydrogen bonds[130] are presented in Table 3. Again, no significant difference between the various forms can be observed. The presence of a moderate absorption band at 754 cm^{-1} in the spectrum of the colorless form and at 752 cm^{-1} in that of the yellow form and the absence of an absorption band in this region in that of red fluorescein, may be attributed to the similar symmetry of the colorless and yellow forms, whereas such symmetry is lacking in the red form owing to the quinone structure at one end of the molecule and the phenolic structure at the other. A strong absorption band at 750 cm^{-1} is found in the spectrum of xanthene,[110] which also has a symmetry similar to those of the colorless and yellow forms of fluorescein.

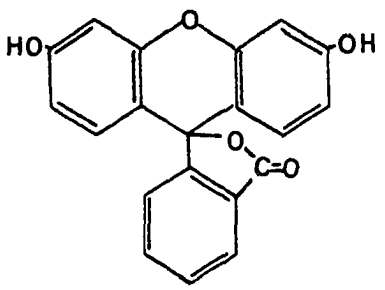
4. Assignment of structures

The isolation of the colorless form of fluorescein in the solid state (Section II.B.2, above) alters the pattern of thinking about the structures of the yellow and red solid forms of fluorescein and in particular directs attention to the incorrect assignment of Orndorff and Hemmer[106] of the lactone structure to the yellow solid.

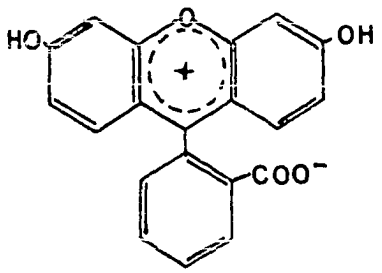
The X-ray diffraction powder patterns presented in Section II.C.1 show that crystallographically each of the yellow, red, and colorless solid forms of fluorescein is a

definite and individual species. This finding disposes effectively of the argument that the yellow solid is simply an amorphous form of fluorescein, the red form being crystalline.

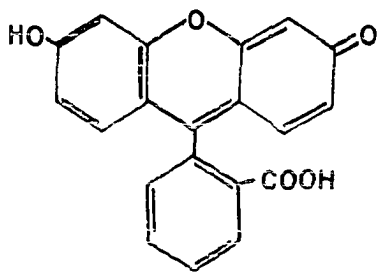
From a correlation of information secured from the studies presented above, mass spectrometry (Section II.C.2), and particularly infrared spectrophotometry (Section II.C.3), I propose for the three solid forms of fluorescein the structures:



V. Colorless form



VI. Yellow form



VII. Red form

a. Colorless fluorescein – the lactone structure

As early as 1908, it was predicted[74] that a colorless form of fluorescein should exist, one in which the lactone ring is present. Indeed in 1914, colorless forms were prepared,[44] in the form of "addition compounds" containing pyridine and quinoline. It is curious that in their classical paper, in 1927, in which they straightened up the chemistry of fluorescein, Orndorff and Hemmer[106] completely ignored the colorless solids and in the end assigned the lactone structure to the yellow solid. The consequences of this misassignment have been unfortunate. The Orndorff and Hemmer assignments are found in the textbooks to this day and numerous workers in the areas of fluorescence have gone astray in interpreting results on the reasonable assumption the texts were correct. The explanation of the Orndorff and Hemmer neglect of the colorless form is not difficult to find. In the presence of water, the colorless form changes into the yellow form with remarkable ease and speed; indeed, the colorless form is never observed except in anhydrous solvents.

The reasons for assigning the lactone structure to the colorless solid fluorescein are: 1) analogy to related chemistry, 2) evidence from the infrared spectrum, 3) and evidence from the mass spectrum. The related chemistry will be reviewed first, the infrared evidence (presented in the

preceding section) then summarized, and finally the deductions made from the mass spectrum correlated with the other evidence.

In the structure of the lactone form of fluorescein (Structure V), all three of the benzene rings are benzenoid in character, the carbon atom in the 9'-position is attached to the other parts of the molecule by four single bonds, no chromophore group is present in the molecule, and the compound is colorless. A related compound, phenolphthalein (Structure XIV, page 36), similar in all respects to fluorescein except that the bridging oxygen atom is absent, is colorless. Another similar but simpler molecule is 3,6-dihydroxy-9,9-dimethylxanthene (Structure XV, page 36), in which the carbon atom in the 9-position carries two methyl groups; it is also colorless. An apparent exception is fluorescin (Structure XVIII, page 69), the reduced form of fluorescein, which should be colorless but is pale yellow; fluorescin is very easily oxidized and the yellow color is undoubtedly caused by a contamination of fluorescein. The difference between the stability of the lactone ring of fluorescein and that of phenolphthalein can be explained by two arguments. First, the oxygen bridge in fluorescein makes the lactone ring more strained and therefore more easily opened than that in phenolphthalein. Second, the same oxygen bridge stabilizes the zwitter ion structure

(Structure VI) postulated for yellow fluorescein; this is discussed in the next subsection.

The infrared spectrum of colorless, solid fluorescein is marked by a prominent absorption band at 1730 cm^{-1}, not present in the spectra of the red and yellow solids. This band falls in the region, 1745-1715 cm^{-1}, in which a band appears in the spectra of substituted phthalides of phenolphthalein, and of derivatives of phenolphthalein, compounds all known to contain the lactone ring. The band results from absorption by the carbonyl group (carbon-oxygen stretching frequency) and differs in position from that of carbonyl groups in free carboxylic acids and in carboxylate anions. The details are given in Section II.C.3 above.

In the mass spectrum of the colorless form of fluorescein, the parent peak is absent at low-heat volatilization and becomes visible only after the input of considerable heat. In the mass spectra of the yellow and red forms of fluorescein, on the other hand, the parent peaks are easily observed, even at low heat. The weak appearance of the parent peak in the mass spectrum of the colorless fluorescein is characteristic of compounds with a five-membered lactone ring substituted at the γ-carbon atom. Aside from the weak parent peak, the mass spectrum of the colorless form of fluorescein also contains a medium-to-weak peak at 237 m/e which does not appear in the mass spectra of the yellow and

red forms of fluorescein. It also contains a peak at 88 m/e and one at 58 m/e, owing to the presence of dioxane. In all other respects, the mass spectrum of the colorless fluorescein is identical to that of yellow or red fluorescein.

b. Red fluorescein – the para-quinone structure The assignment of the para-quinone structure (Structure VII) to the red, solid form of fluorescein is not new. This structure was assigned to the red form by Orndorff and Hemmer[106] but the present work places the assignment on a firmer basis. The evidence is derived from: 1) the chemistry of related and analogous compounds; 2) the infrared spectrum; and 3) the mass spectrum.

The para-quinone structure contains a highly conjugated chomophore and exists in various resonance forms. Literally hundreds of compounds of similar structure are known and all are highly colored. Concentrated alkaline solutions of fluorescein and the solid disodium salt (Structure XXIII, page 72) have the same color as red, solid fluorescein; this is not surprising inasmuch as all have the same para-quinone structure. Alkaline solutions of phenolphthalein and the solid dipotassium salt (Structure XXII, page 72) are highly colored because of the same para-quinone chromophore.

The infrared spectrum of red, solid fluorescein is marked by a prominent absorption band at 1711 cm^{-1} in the region of absorption by carbonyl groups (1800-1600 cm^{-1}) and

more specifically in the region of the frequencies corresponding to a free (nondissociated) carboxylic acid group. The frequency of the carbonyl absorption band of the quinone function, normally observed at 1685-1626 cm^{-1}, is shifted to lower frequency because of the high conjugation of the quinone group and merged with the broad aromatic absorption band at 1600-1590 cm^{-1}. Such shifts to lower frequencies of highly conjugated systems are well-known. A detailed discussion of this is presented in Section II.C.3, above.

The mass spectra of red fluorescein and of yellow fluorescein are identical, indicating that during volatilization the application of heat the yellow form is converted to the red form. The mass spectra are characterized by peaks caused by decarboxylation, loss of carbon monoxide, and loss of oxygen and hydroxyl groups; these degradation patterns are consistent with a structure containing carboxylic acid, quinone, ether, and phenolic functions.

c. Yellow fluorescein – the zwitter ion structure

The zwitter ion structure I propose for the yellow, solid form of fluorescein (Structure VI) was postulated by Zanker and Peter[163] as a transient intermediate in the conversion of colorless fluorescein in solution in dioxane to the yellow form on the addition of water. Zanker and Peter were interested only in the forms of fluorescein in solution and considered an aqueous solution of fluorescein to consist of

a mixture of the lactone and para-quinone forms; their own evidence indicated really that in pure water only the colored form was present and Lindqvist showed that the equilibrium between the two is established very rapidly.[93]

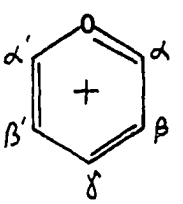
The proof that the yellow, solid form of fluorescein is the zwitter ion (Structure VI) is based on 1) the amphoteric nature as reflected in the exceptionally high melting point, the low solubility, and the unusual, highly acidic character of the hydrogen atoms, a matter which is discussed in Sections II.D.2 and II.D.3; 2) evidence from the infrared spectrum; 3) analogy to the pyrylium compounds. The zwitter ion structure offers neat explanations for hitherto unexplained phenomena observed in the chemistry of compounds related to fluorescein.

The infrared spectrum of the yellow, solid fluorescein is marked by three features which differentiate the yellow solid from the red and colorless fluoresceins. First, no absorption band appears in the region 1800-1600 cm^{-1} characteristic of the carbon-oxygen stretching vibration of the carbonyl group; the presence of either a free (non-dissociated) carboxylic acid group and of a lactone group is thus ruled out. Second, a prominent band appears at 1536 cm^{-1}, not present in the spectra of the red and colorless solid fluoresceins but present in a class of compounds known as the pyrylium salts; by analogy then, fluorescein

contains a six-membered, oxygen-containing ring carrying a positive charge, that is, yellow fluorescein is a zwitter ion. The positive charge on the ring must then be balanced by a negative charge on the carboxyl group, that is, the latter is present as a carboxylate anion, and the hydrogen atom then must be present as a second phenolic group and the two resorcinol rings of the fluorescein molecule are both benzenoid in character. Finally, the two absorption bands characteristic of the carboxylate group appear in the spectrum of yellow fluorescein, at 1596 cm^{-1} and at 1461-1450 cm^{-1}, but both bands are merged with or superimposed on major bands corresponding to aromatic carbon-carbon stretching vibrations; a detailed study, however, indicates that both bands are present. The complete treatment of the infrared spectrum of yellow fluorescein is presented in Section II.C.3.

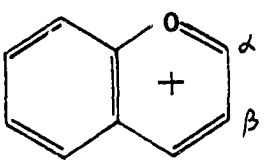
The zwitter ion structure postulated for yellow, solid fluorescein poses two problems of great interest: the position of the positive charge on the six-membered, oxygen-containing ring, and the source of the yellow color. Two oxonium structures are possible: one in which the positive charge is located on the bridging oxygen atom, a second in which it is located on the carbon atom in the 9- position, that is, the 9-carbon atom is a carbonium ion. The central, heterocyclic ring in the zwitter ion structure of fluorescein

is similar to that in the highly colored pyrylium salts. Benzopyrylium and flavylium salts, which include the naturally occurring anthocyanin dyes responsible for much of the color in the plant world, have been studied extensively. The literature in this field is enormous, a fair part of it being devoted to the study of the distribution of the positive charge on the atoms of the ring.



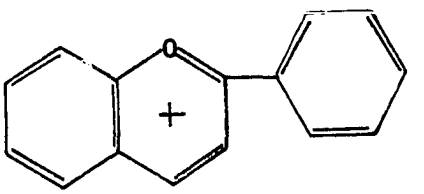
XXVI.

Pyrylium cation



XXVII.

Benzopyrylium cation



XXVIII.

Flavylium cation

An excellent review of the possible structures of the pyrylium salts is that of Hill.[64] The location of the positive charge on the ring was studied by Föhlisch and coworkers[45,46] by NMR, infrared, and ultraviolet spectrometry. In a review of the chemistry of the pyrylium salts, Balaban and coworkers[10] use the common notation of the positive charge on the oxygen atom; they emphasize, however, that this is only a matter of convenience because all the chemistry of the pyrylium salts is that of substances with the positive charge on either the α- or the γ- position. In another

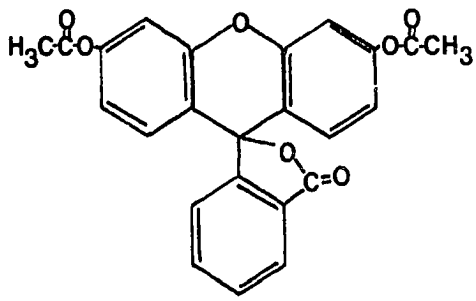
study, Shriner and Moffett[127] concluded that benzopyrylium salts are quite different from the common oxonium salts obtained from ethers or γ-pyrones; they are more similar to the carbonium-type salts derived from triphenylcarbinols. Recently, Martensson and Warren[95] performed molecular orbital calculations on the pyrylium ring and concluded that the positive charge cannot be assigned to any particular atom; rather, it exists in an aromatic-like distribution over the entire ring, although the α- and γ-carbon atoms definitely have partially increased concentrations of the positive charge, δ^+. A charge distribution of this type explains the unusual stability of the pyrylium ring in which the four electrons of the α-,α'-,β-,β'-carbon atoms and the unshared electron pair of the oxygen atom provide the six π electrons for an aromatic system. Reasoning by analogy then, the aromaticity and uniform charge distribution have been adopted in this work for fluorescein and incorporated into the zwitter ion structure for the yellow form.

The yellow color of the zwitter ion structure of fluorescein is a consequence of the positively charged pyrylium-type ring portion of the molecule. Such structures are always colored, and compounds which possess these structures are widespread in nature accounting for the colors of flowers and fruit. The colors are very pH dependent, changing from yellow, orange, or red on the acid side to

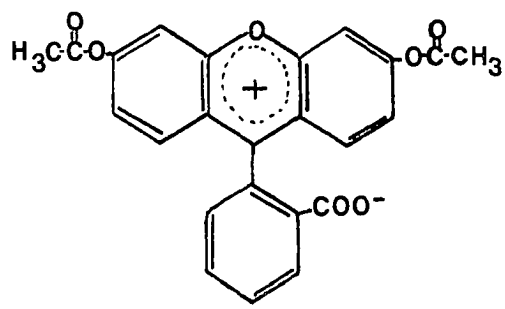
purple or blue on the alkaline side. That the yellow color is derived from the positive charge of the ring and not from the negative charge of the carboxylate group is seen from the behavior of fluorescein in strongly acidic solutions, in which the fluorescein cation has the structure of a positively charged ring and a free carboxylic acid group (Structure VIII, page 21). Such solutions are invariably yellow. In addition, the well-known carbonium ion structures of simple triphenylmethane compounds are also colored yellow to orange.

The zwitter ion structure of yellow, solid fluorescein is useful in explaining not only the color but other characteristics of fluorescein and its derivatives. A puzzling observation about diacetylfluorescein was made by Hefley[57] and confirmed during the course of the present work. During thin-layer chromatography on silica gel with a methanol-benzene mixture as a driving solvent, diacetylfluorescein yields two spots, no matter how pure the diacetylfluorescein. Incomplete acetylation of the original fluorescein and partial hydrolysis of the diacetylfluorescein were ruled out by repeating the acetylation and by repeated recrystallizations, and by the deliberate addition of free fluorescein as a tracer and proof of purification. It is now proposed that diacetylfluorescein, a colorless compound, can under certain conditions exist both as the lactone and

as the yellow zwitter ion form, Structures XVIIa and XVIIb,



XVIIa. Diacetylfluorescein colorless



XVIIb. Diacetylfluorescein yellow

respectively. Two spots would be expected on the chromatography plates. Such opening of the lactone ring would also account for the slight, yellowish discoloration of diacetylfluorescein caused by exposure to moist air. It explains also the yellow form of diacetylfluorescein prepared by Nagase and coworkers[101] by adding a few drops of sulfuric acid to the acetylation mixture during the preparation of diacetylfluorescein.

d. Structure of fluorescein upon dissolution It has been postulated by many workers that fluorescein dissolved in a particular solvent exists in only one form, regardless of which form of solid fluorescein is used to prepare the solution. It was easily shown that in dry, organic, non-polar solvents, like dioxane, fluorescein exists as the

colorless lactone structure. It remains now to be established which form exists in aqueous solution and how it is affected by changes in pH. The obvious choice, of course, is to assign the zwitter ion structure (Structure VI) of yellow fluorescein to the form of fluorescein in solution, since such solutions are yellow. The solutions become deeply red-brown only when alkaline and highly concentrated. Indeed, assignment of the zwitter ion structure to the form of fluorescein in solution provides not only a ready explanation for the color but also for the amphoteric nature of fluorescein, as revealed by solubility studies, and for the highly acidic nature of the hydrogen atoms, as shown by potentiometric titrations. These observations will be the subject of discussion of the next section.

D. The Prototropic Forms of Fluorescein (Fluorescein in Solution)

1. Introduction

It has been observed previously that in solution only one form of fluorescein exists, depending on the solvent, but independent of the form of fluorescein that is used as the starting material. However, the nature of the species in solution has not been satisfactorily explained, to a large extent because the zwitter ion characteristics of fluorescein have been sadly neglected.

In this section, the solubility, acid-base, and fluorescence properties of fluorescein are studied in aqueous solution in order to establish the nature of the neutral, cationic, and anionic species in solution. The predominance of each species as a function of pH is discussed and expressed in mathematical and graphical terms. The pertinent acid dissociation constants are evaluated by various methods and correlated with the appropriate equilibria in solution. The fluorescence properties are studied as a function of pH, and the nature of the fluorescent species is discussed.

The existence of the colorless lactone form in dioxane solution is demonstrated by NMR spectrometry. The species in dioxane solution is the same, regardless which form of fluorescein is used to prepare the solution.

As an aid in the discussion, the acid dissociation reactions of fluorescein, H_2Fl, that are possible in aqueous solution are summarized below, and the acid dissociation constants describing each reaction are defined by mathematical equations. Because fluorescein is amphoteric and dissolves in acid solution to form the cation H_3Fl^+, the equation describing this reaction is also included.

$$(1)\quad H_3Fl^+ = H_2Fl + H^+ \qquad K_{H_3Fl^+} = \frac{[H^+][H_2Fl]}{[H_3Fl^+]}$$

(2) $H_2Fl = HFl^- + H^+$ $\qquad K_{H_2Fl} = \frac{[H^+][HFl^-]}{[H_2Fl]}$

(3) $HFl^- = Fl^{2-} + H^+$ $\qquad K_{HFl^-} = \frac{[H^+][Fl^{2-}]}{[HFl^-]}$

All the acid dissociation constants determined in this work for fluorescein and those found in the literature are compiled in Table 5. Where enough information was available from the original work, the ionic strength at which the acid dissociation constant was determined is included.

The structures for the species H_3Fl^+, H_2Fl, HFl^-, and Fl^{2-} in solution and the associated acid-base equilibria are summarized again in Figure 8. Where resonance structures are possible, they are included and marked by a small letter after the Roman numeral assigned to the ionic form. The predominant resonance structures in solution are identified. In the text, referral to a structure of a species by a Roman numeral alone includes necessarily the likely resonance structures.

Table 5. Determination of the acid dissociation constants of fluorescein (H_2Fl)

pK Value	Reaction	Method	Reference
$pK_{H_3Fl^+}$	$H_3Fl^+ = H_2Fl + H^+$		
2.15,[a,b] 2.13[a,c]		solubility	This work
2.2		spectrophotometry	93
1.95		spectrophotometry	163
2.00[d]		polarography	11
pK_{H_2Fl}	$H_2Fl = HFl^- + H^+$		
4.74,[a,b] 4.71[a,c]		solubility	This work
4.75		potentiometric titration	This work
4.42[a,e]		fluorescence	This work
4.75,[b] 5.82[c,e,f]		potentiometric titration	49
5.05		spectrophotometry	163
4.4		spectrophotometry	93
4.75[d]		polarography	11
5.2[e]		paper electrophoresis + spectrophotometry	121

pK_{HFl^-}	$HFl^- = Fl^{2-} + H^+$	
6.55	potentiometric titration	This work
6.28[a]	fluorescence	This work
6.5,[b] 7.15[c,e,f]	potentiometric titration	49
7.0	spectrophotometry	163
6.7	spectrophotometry	93
7.0[d]	polarography	11
7.38	polarography	70
9.19[g]	polarography	50
6.2[e]	paper electrophoresis + spectrophotometry	121
6.05[e]	spectrophotometry	66

[a] Ionic strength $\mu = 0.1$.

[b] Starting material - yellow fluorescein.

[c] Starting material - red fluorescein.

[d] Ionic strength $\mu = 1.0$.

[e] Estimated.

[f] In 50 per cent ethanol.

[g] In 50 per cent methanol.

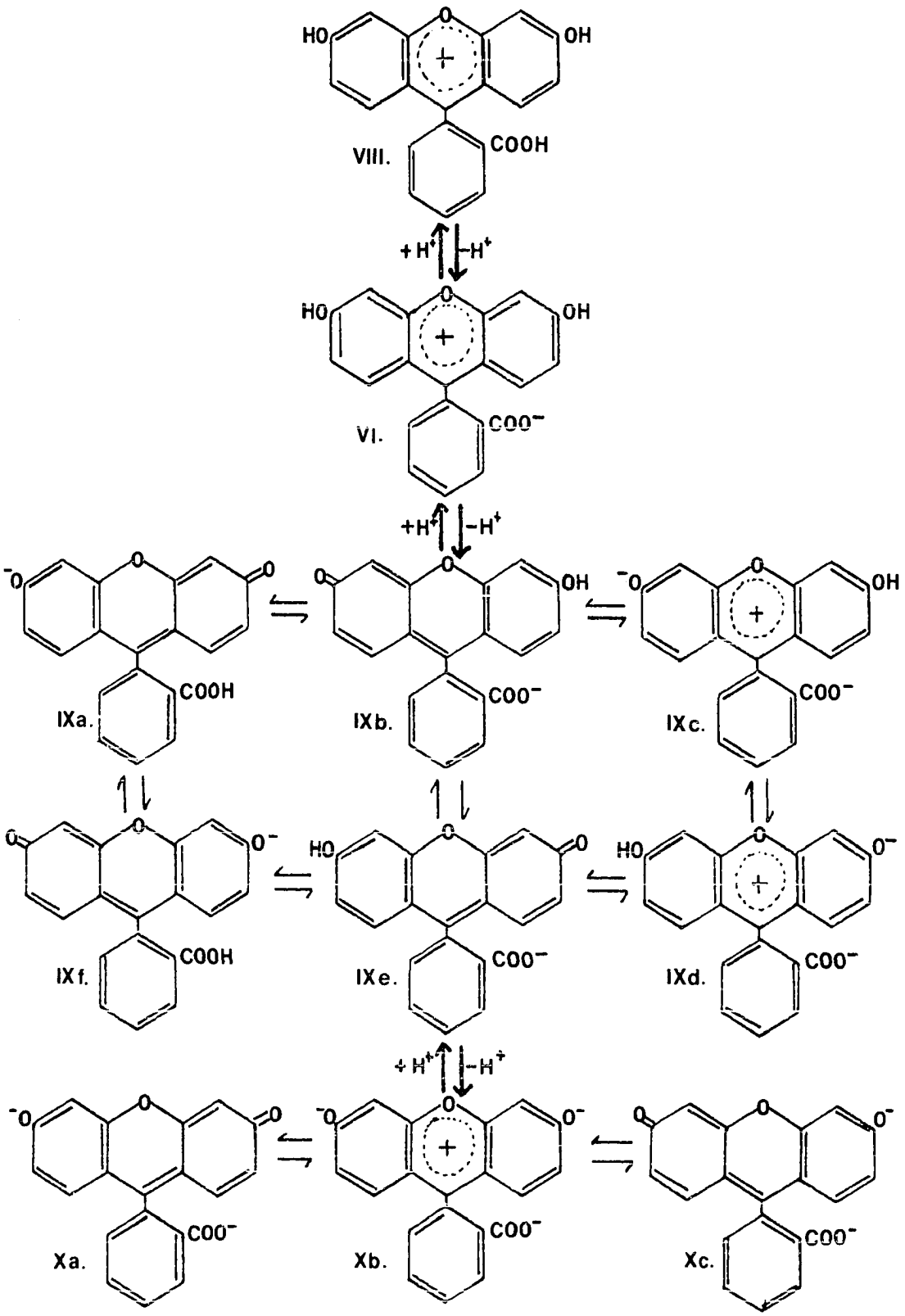
Figure 8. The prototropic forms of fluorescein (H_2Fl):acid-base equilibria of fluorescein in solution

Structure VIII. Monocation (H_3Fl^+), pH $<\sim 3$

Structure VI. Neutral molecule (H_2Fl), pH $\sim$ 3-4

Structure IX. Monoanion (HFl^-), pH $\sim$ 4-7.
Predominant resonance forms c and d

Structure X. Dianion (Fl^{2-}), pH $>\sim 7$.
Predominant resonance forms a and c


HO
OH
COOH
VIII.
+H+
-H+
HO
OH
COO-
VI.
+H+
-H+
COOH
IXa.
OH
COO-
IXb.
OH
COO-
IXc.
COOH
IXf.
HO
COO-
IXe.
HO
COO-
IXd.
+H+
-H+
COO-
Xa.
COO-
Xb.
COO-
Xc.

2. Solubility of red and yellow forms of fluorescein as a function of pH

a. Preparation of buffers Buffers were prepared in the pH region from 1 to 6 using 0.1 M solutions of hydrochloric acid, potassium acid phthalate (KHP), and potassium hydroxide. Dilutions were made with 0.1 M solution of potassium chloride to maintain the ionic strength constant. The pH of the buffer solutions was measured before and after saturation with fluorescein, using a Corning Model 10 pH meter with a Beckman glass electrode and a saturated calomel electrode as reference electrode. The pH meter was standardized against a standard buffer solution of pH 4.01, prepared from potassium acid phthalate according to NBS specifications, as outlined in Diehl,[29] p. 58.

b. Solubility measurements Approximately 25 ml of each buffer solution was placed in a 2-oz. plastic bottle provided with a screw cap. Into each bottle sufficient red or yellow fluorescein was added to ensure saturation of the resulting solution. Bottles and suspensions were then shaken on a mechanical shaker for 72 hours at room temperature, 23° ± 0.5°C. Each solution was subsequently filtered through a sintered glass crucible, and the final pH was measured. An aliquot of 5.00 ml of each solution was diluted to 100.0 ml with 0.1 M potassium hydroxide and the relative fluorescence of the solution measured.

c. Preparation of calibration curves Red and yellow forms of highly purified fluorescein were dried at 100°-110°C for three hours and 100.0 mg of each were diluted to exactly 1 l. with 0.1 M potassium hydroxide. Stock solutions containing 1.0 p.p.m. of fluorescein were prepared by diluting 1.00 ml of the above solutions to 100.0 ml with 0.1 M potassium hydroxide. Amounts of 1, 3, 6, 10, 15 and 20 ml of these solutions were taken and diluted to 100.0 ml with 0.1 M potassium hydroxide for fluorometric measurement.

Fluorescence measurements were made using a Turner Model 110 Fluorometer. A Corning No. 5850 filter was used as a primary filter for isolation of the blue portion of the excitation source. A combination of Corning Yellow 2A-15 plus Wratten N.D. Filter 10 per cent 1.00 was used as the secondary filter. The calibration curves prepared from solutions starting with yellow fluorescein and with red fluorescein proved identical; these were made at three different sensitivity settings of the fluorometer. By using these calibration curves and the fluorescence measurements of the various buffer solutions saturated with fluorescein, the solubility of fluorescein was calculated.

d. Evaluation of the intrinsic solubility and the acid dissociation constants The solubility of yellow fluorescein and of red fluorescein at various values of pH is given in Table 6 and shown graphically in Figure 9. The solubility

Table 6. Solubility of yellow and red fluorescein as a function of pH

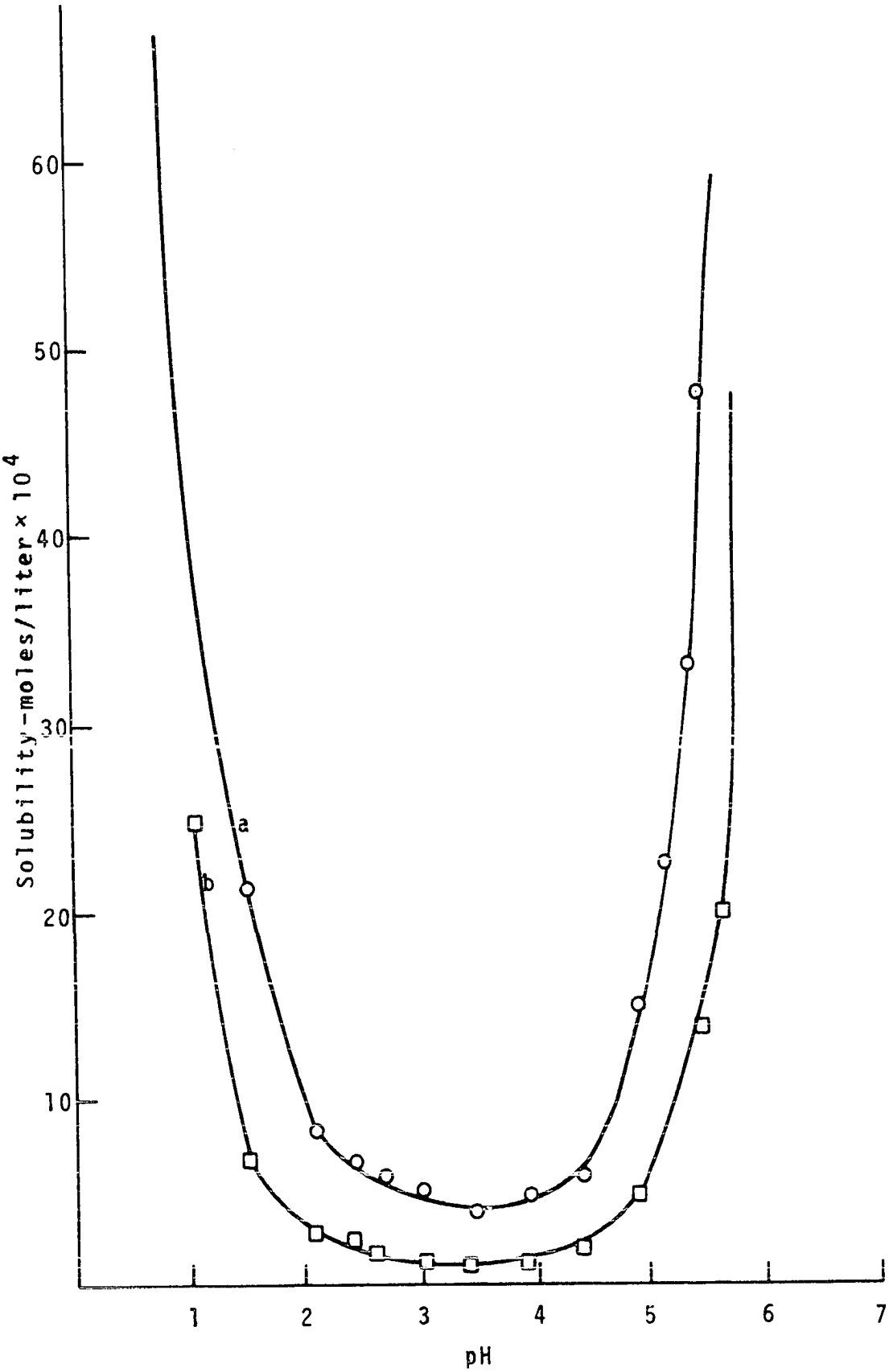
Initial pH of buffer	Yellow fluorescein		Red fluorescein	
	Final pH	moles/liter	Final pH	moles/liter
1.00	1.10	7.22×10^{-3}	1.05	2.41×10^{-3}
1.49	1.53	2.11×10^{-3}	1.51	6.92×10^{-4}
2.00	2.07	7.82×10^{-4}	2.05	2.59×10^{-4}
2.28	2.33	6.62×10^{-4}	2.35	2.50×10^{-4}
2.64	2.69	5.29×10^{-4}	2.65	1.87×10^{-4}
2.98	3.01	4.81×10^{-4}	3.01	1.69×10^{-4}
3.40	3.39	3.85×10^{-4}	3.43	1.50×10^{-4}
3.90	3.92	4.45×10^{-4}	3.92	1.62×10^{-4}
4.35	4.37	5.84×10^{-4}	4.36	2.17×10^{-4}
4.90	4.90	1.40×10^{-3}	4.90	5.06×10^{-4}
5.17	5.18	2.15×10^{-3}	--	---
5.35	5.34	3.31×10^{-3}	5.35	1.25×10^{-3}
5.55	5.53	4.72×10^{-3}	5.55	1.90×10^{-3}
6.04	6.03	1.80×10^{-2}	6.06	6.95×10^{-3}

of both the yellow form and red form of fluorescein as a function of pH exhibits a minimum at pH about 3.4. The solubility increases with decreasing pH below the minimum, that is, on the acid side of the minimum, owing to the addition of a hydrogen ion forming the univalent cation, H_3Fl^+. The solubility increases with increasing pH above the minimum, that is, on the basic side of the minimum, owing to the neutralization of one proton and the formation

Figure 9. Solubility of yellow fluorescein and of red fluorescein as a function of pH at 23°C

Curve a. Yellow fluorescein

Curve b. Red fluorescein


Solubility-moles/liter × 10^4
60
50
40
30
20
10
a
b
1
2
3
4
5
6
7
pH

of the univalent anion, HFl^-. Thus fluorescein behaves as a typical amphoteric compound and shares this characteristic with other zwitter ion substances. Following the method of Krebs and Speakman,[86] the solubility data can be used to evaluate the two acid dissociation constants involved; these constants, $K_{H_3Fl^+}$ and K_{H_2Fl}, are defined by the reactions and mathematical equations:

$$H_3Fl^+ = H^+ + H_2Fl \qquad K_{H_3Fl^+} = \frac{[H^+][H_2Fl]}{[H_3Fl^+]} \qquad (1)$$

and $$H_2Fl = H^+ + HFl^- \qquad K_{H_2Fl} = \frac{[H^+][HFl^-]}{[H_2Fl]} \qquad (2)$$

The assumption is made that the solubility of the free acid, H_2Fl, the so-called intrinsic solubility, S_i, is constant and independent of pH. At pH below the minimum, the fluorescein is present as two species, H_3Fl^+ and H_2Fl, and the solubility, S_j, is given by

$$S_j = [H_3Fl^+] + [H_2Fl] \qquad (3)$$

[The symbol S_j is adopted here deliberately for the solubility on the low-pH side of the minimum rather than the more obvious S_1 to avoid the usual confusion between the letter l and the figure 1.] At pH above the minimum, the fluorescein is present as two species, H_2Fl and HFl^-, and the solubility, S_h, is given by

$$S_h = [H_2Fl] + [HFl^-] \tag{4}$$

Another assumption is that the amount of fluorescein existing as the dianion, Fl^{2-}, is negligible compared to the amount of fluorescein existing as H_2Fl and HFl^- over the studied pH range. Because

$$S_i = [H_2Fl], \tag{5}$$

combination of equations (1) and (3) yields

$$K_{H_3Fl^+} = \frac{[H^+]\ S_i}{S_j - S_i} \tag{6}$$

and

$$\frac{S_j}{S_i} - 1 = \frac{[H^+]}{K_{H_3Fl^+}}$$

and

$$\log\ (\frac{S_j}{S_i} - 1) = pK_{H_3Fl^+} - pH. \tag{7}$$

Similarly, combination of equations (2) and (4) yields

$$K_{H_2Fl} = \frac{[H^+](S_h - S_i)}{S_i} \tag{8}$$

and

$$\frac{S_h}{S_i} - 1 = \frac{K_{H_2Fl}}{[H^+]}$$

and

$$\log\ (\frac{S_h}{S_i} - 1) = pH - pK_{H_2Fl} \tag{9}$$

Thus, a plot of log $(\frac{S_j}{S_i} - 1)$ or log $(\frac{S_h}{S_i} - 1)$ *versus* pH gives a straight line of unit slope, the intercept of which yields a value for $pK_{H_3Fl^+}$ or pK_{H_2Fl}, respectively.

The intrinsic solubility, S_i, was evaluated in two ways by using the solubility data on the low-pH side and on the high-pH side of the minimum. On the low-pH side, as the pH increases the concentration of the cation, H_3Fl^+, drops toward zero and S_j approaches S_i. Rearrangement of equation (6) gives

$$S_j = [H^+] \frac{S_i}{K_{H_3Fl^+}} + S_i \qquad (10)$$

A plot of S_j *versus* $[H^+]$ yields a straight line, the intercept at $[H^+] = 0$ being S_i.

Similarly on the high-pH side, the concentration of the anion, HFl^-, falls with decreasing pH, and S_h approaches S_i. Rearrangement of equation (8) gives

$$S_h = K_{H_2Fl} S_i \frac{1}{[H^+]} + S_i. \qquad (11)$$

A plot of S_h *versus* $1/[H^+]$ is a straight line, the intercept at $1/[H^+] = 0$ being S_i.

The two methods of evaluating the intrinsic solubility, *i.e.*, by using the solubility data on the low-pH side of the

minimum, Figure 10, and on the high-pH side of the minimum, Figure 11, led to identical results for S_i of yellow fluorescein and also for S_i of red fluorescein, Table 7.

Table 7. Values obtained for the intrinsic solubility of yellow and of red fluorescein

Data Used	Yellow fluorescein moles/liter	Red fluorescein moles/liter
Low-pH side of minimum	3.70×10^{-4}	1.45×10^{-4}
High-pH side of minimum	3.70×10^{-4}	1.45×10^{-4}

For obtaining values for the dissociation constants, it is convenient to place the two plots, log $(\frac{S_j}{S_i} - 1)$ *versus* pH and log $(\frac{S_h}{S_i} - 1)$ *versus* pH on the same graph, Figure 12 for yellow fluorescein, and Figure 13 for red fluorescein. The values obtained are given in Table 8.

Table 8. Values for the dissociation constants, $pK_{H_3Fl^+}$ and pK_{H_2Fl}, of yellow fluorescein and of red fluorescein

	Yellow fluorescein	Red fluorescein
pK for the dissociation of H_3Fl^+	2.15	2.13
pK for the dissociation of H_2Fl	4.74	4.71

Figure 10. Determination of the intrinsic solubility, Si, of yellow fluorescein and of red fluorescein using solubility data on the low-pH side of the minimum solubility

Curve a. Yellow fluorescein

Curve b. Red fluorescein

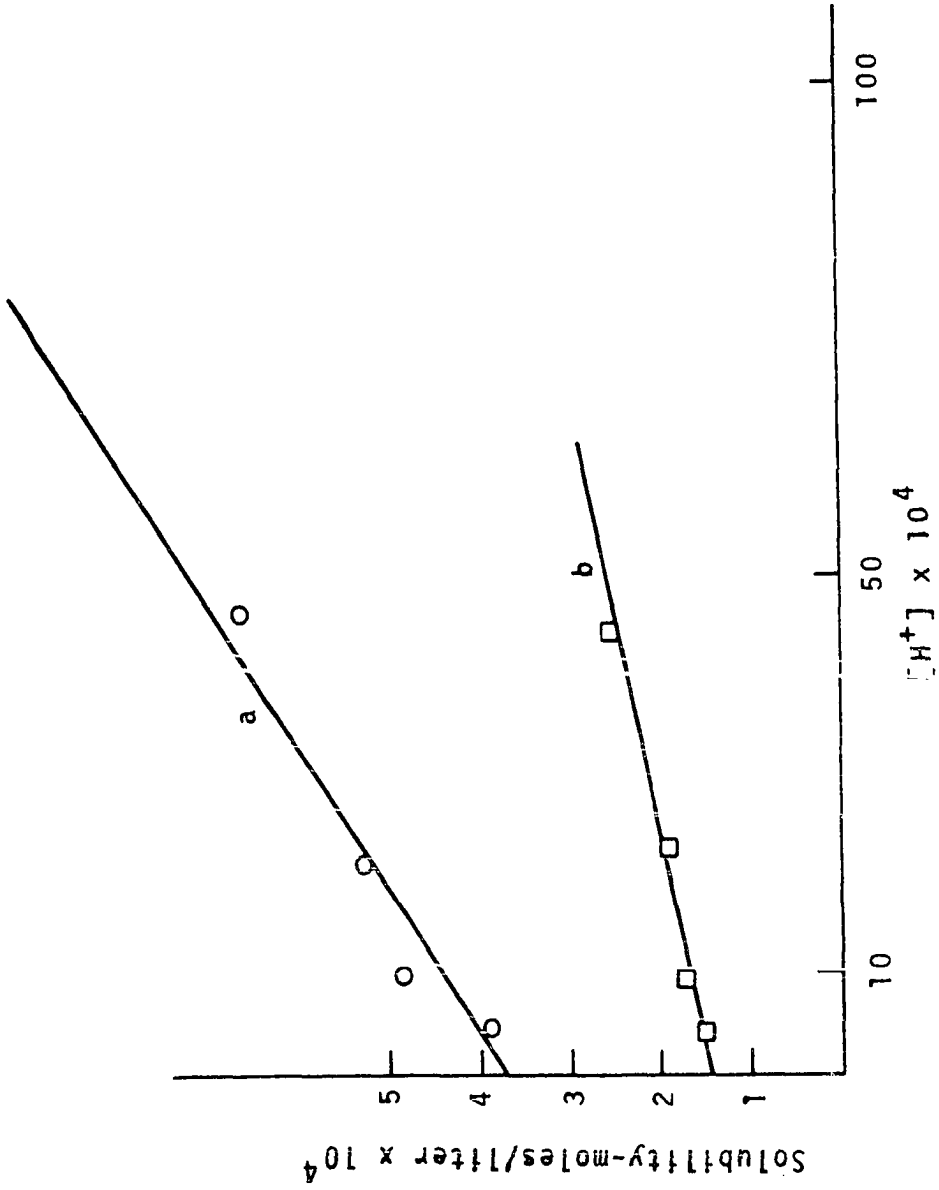

Solubility-moles/liter x 10^4
5
4
3
2
1
a
b
10
50
100
$[H^+] \times 10^4$

Figure 11. Determination of the intrinsic solubility, Si, of yellow fluorescein and of red fluorescein using solubility data on the high-pH side of the minimum solubility

Curve a. Yellow fluorescein

Curve b. Red fluorescein

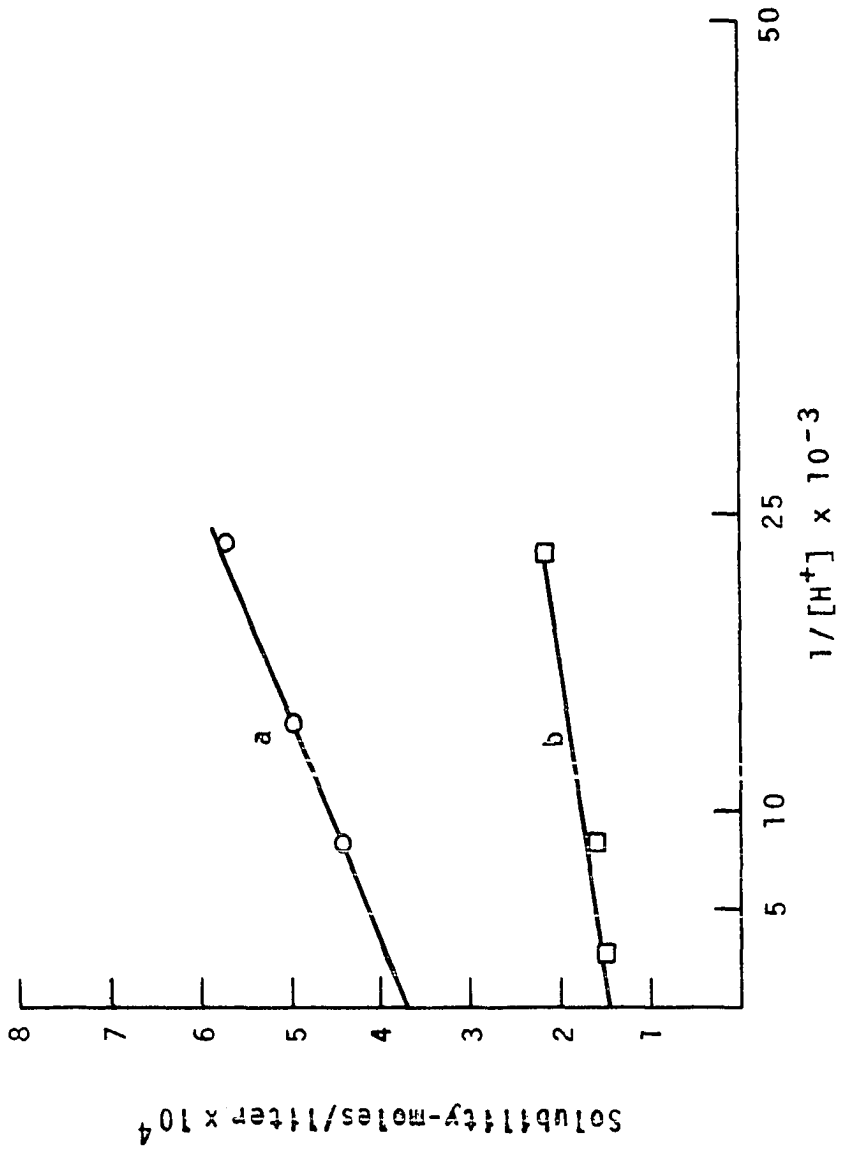

Solubility-moles/liter x 10^4
8
7
6
5
4
3
2
1
a
b
5
10
25
50
$1/[H^+] \times 10^{-3}$

Figure 12. Evaluation of the dissociation constants, $pK_{H_3Fl^+}$ and $pK_{H_2Fl^-}$, of yellow fluorescein

Curve a. $\log \left(\frac{S_j}{S_i} - 1\right)$ *vs.* pH

Curve b. $\log \left(\frac{S_h}{S_i} - 1\right)$ *vs.* pH

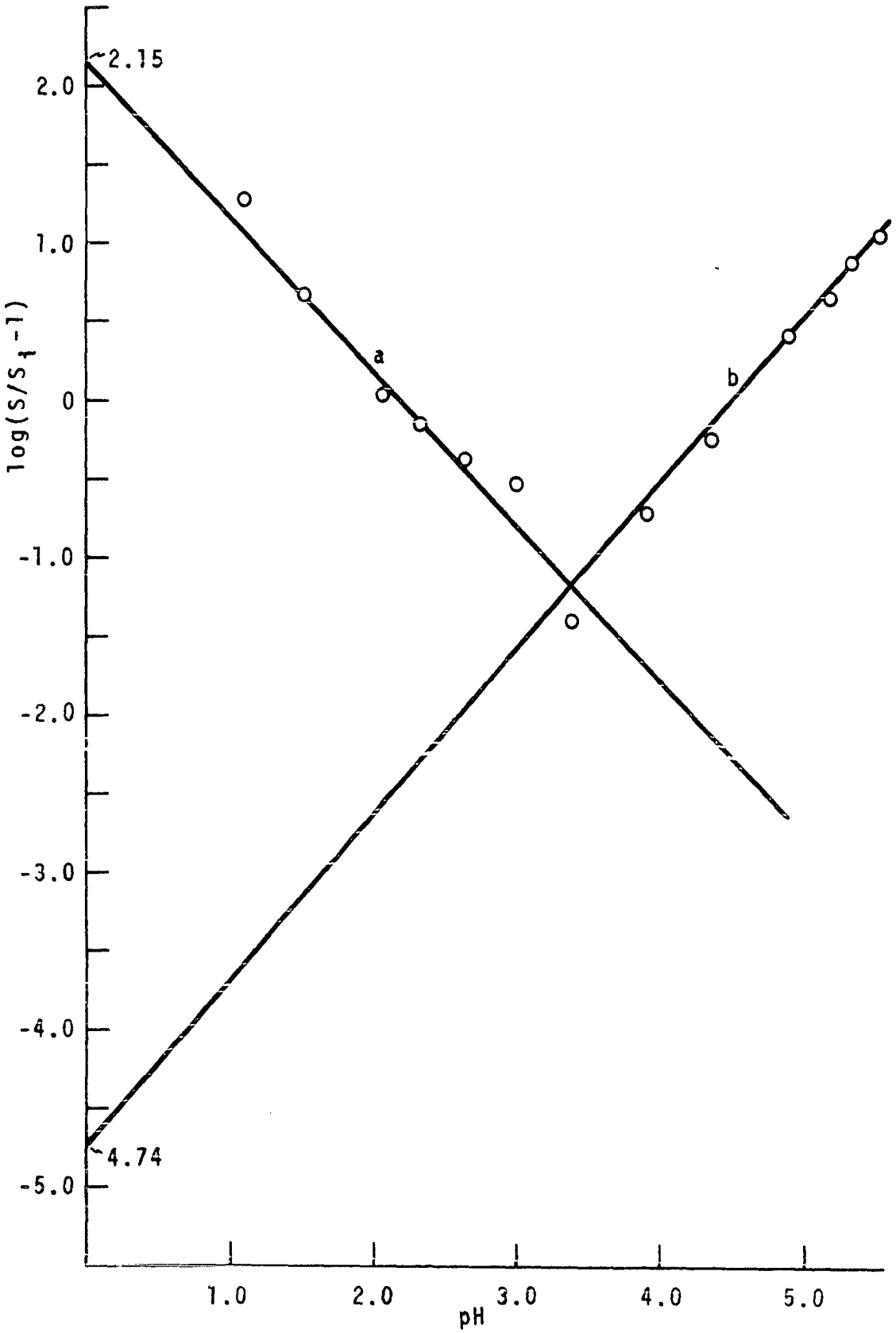
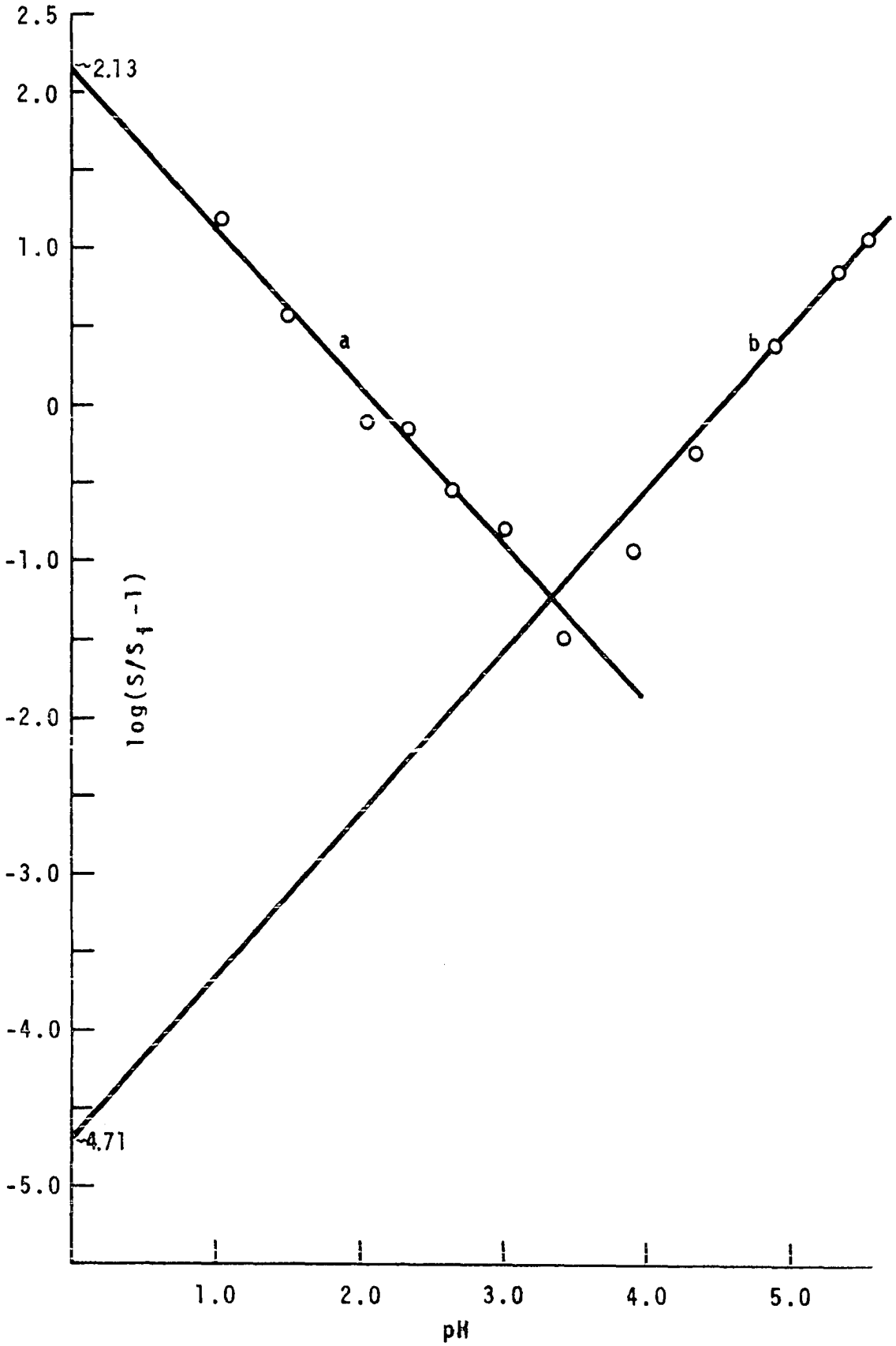

~2.15
2.0
1.0
0
-1.0
-2.0
-3.0
-4.0
-5.0
log(S/S_t-1)
a
b
~4.74
1.0
2.0
3.0
4.0
5.0
pH

Figure 13. Evaluation of the dissociation constants, $pK_{H_3Fl^+}$ and $pK_{H_2Fl^-}$, of red fluorescein

Curve a. $\log \left(\frac{S_j}{S_i} - 1\right)$ *vs.* pH

Curve b. $\log \left(\frac{S_h}{S_i} - 1\right)$ *vs.* pH


2.5
2.0
1.0
0
-1.0
-2.0
-3.0
-4.0
-5.0
~2.13
~4.71
a
b
log(S/S_f - 1)
1.0
2.0
3.0
4.0
5.0
pH

e. Discussion The solubility of both yellow and red fluorescein as a function of pH follows the typical pattern for zwitter ion compounds. The solubility curve (Figure 9) exhibits a minimum at about pH 3.4. Below this pH, that is, with increasing hydrogen ion concentration, the solubility increases owing to the formation of the monocation (Structure VIII), and above this pH, that is, with decreasing hydrogen ion concentration, the solubility increases owing to the formation of the monoanion (Structure IX). The pH at the minimum solubility is in good agreement with that of pH ∿3, obtained by Tezak and Tezak.[139] It also coincides well with the isoelectric point of pH 3.1-3.7, observed by Scharf[121] and of about pH 3, observed by Holmes.[66] A surprising result is obtained in the determination of the intrinsic solubility of yellow and red fluorescein by using two different sets of data, those on the low-pH side and those on the high-pH side of the solubility minimum (Table 7). The intrinsic solubility values are identical, irrespective of which direction is chosen in approaching the pH of the minimum solubility. The presence of a minimum in the solubility curve corresponding to the isoelectric point and the identical values obtained for the intrinsic solubility are consistent with the amphoteric nature of fluorescein.

The solubility of red fluorescein is consistently lower, by a factor of 2-2.5, than that of yellow fluorescein. Throughout the studies, the contention is that both forms of fluorescein produce, upon dissolution, the same species, namely the zwitter ion form of fluorescein, Structure VI. Since yellow fluorescein already exists in the zwitter ion form, whereas the red fluorescein must first be converted from the quinone-carboxylic acid form (Structure VII) to the zwitter ion form, it is not surprising that the solubility of red fluorescein is lower than that of yellow fluorescein.

That upon dissolution both yellow and red fluorescein produce the same species is borne out by the values for the acid dissociation constants, obtained from the solubility studies, and presented in Table 8. It is apparent that the values are identical, within experimental error, irrespective of which form of fluorescein is used.

The study of the solubility of yellow and red fluorescein as a function of pH has thus provided evidence for 1) the amphoteric nature of fluorescein in solution and 2) the conversion of both forms of fluorescein into the same, single species, that of the zwitter ion, upon dissolution.

3. Potentiometric acid-base titrations

a. Introduction Forward and back titrations of yellow and red fluorescein were carried out in water and in 50 per cent ethyl alcohol-water. It was hoped that the use

of different solvents would aid in differentiating between hydrogen atoms of different acid strength. Some titrations of phenolphthalein and dihydroxydimethylxanthene are included as aids in interpreting the results. Titrations of the colorless form of fluorescein and of 4',5'-dimethylfluorescein are also presented.

b. Apparatus and procedure A Corning Model 10 pH meter with a Beckman high-alkalinity glass electrode was used. The pH was measured against the saturated calomel electrode as reference. The pH meter was calibrated by using at least two standard buffer solutions prepared according to NBS specifications.

A weighed amount of the sample to be titrated was placed in a beaker, the appropriate solvent was added, and dissolution was effected by stirring with a magnetic stirring bar. Throughout the course of the titration, the pH was measured as soon as the needle of the pH meter attained equilibrium. In the direct titrations of fluorescein in water, solid, undissolved fluorescein was present during the early part of the titrations and stable readings of pH could not be obtained. The titration curves so obtained wavered and were not useful for evaluating the acid dissociation constants.

c. Results and discussion The alkalimetric titration curves for yellow and red fluorescein in water and for yellow and red fluorescein in 50 per cent ethyl alcohol are presented in Figures 14-17. Since both forms of fluorescein

Figure 14. Potentiometric titration of yellow fluorescein in water

Yellow fluorescein: 0.1033 g

Titrant: 0.1211 N sodium hydroxide

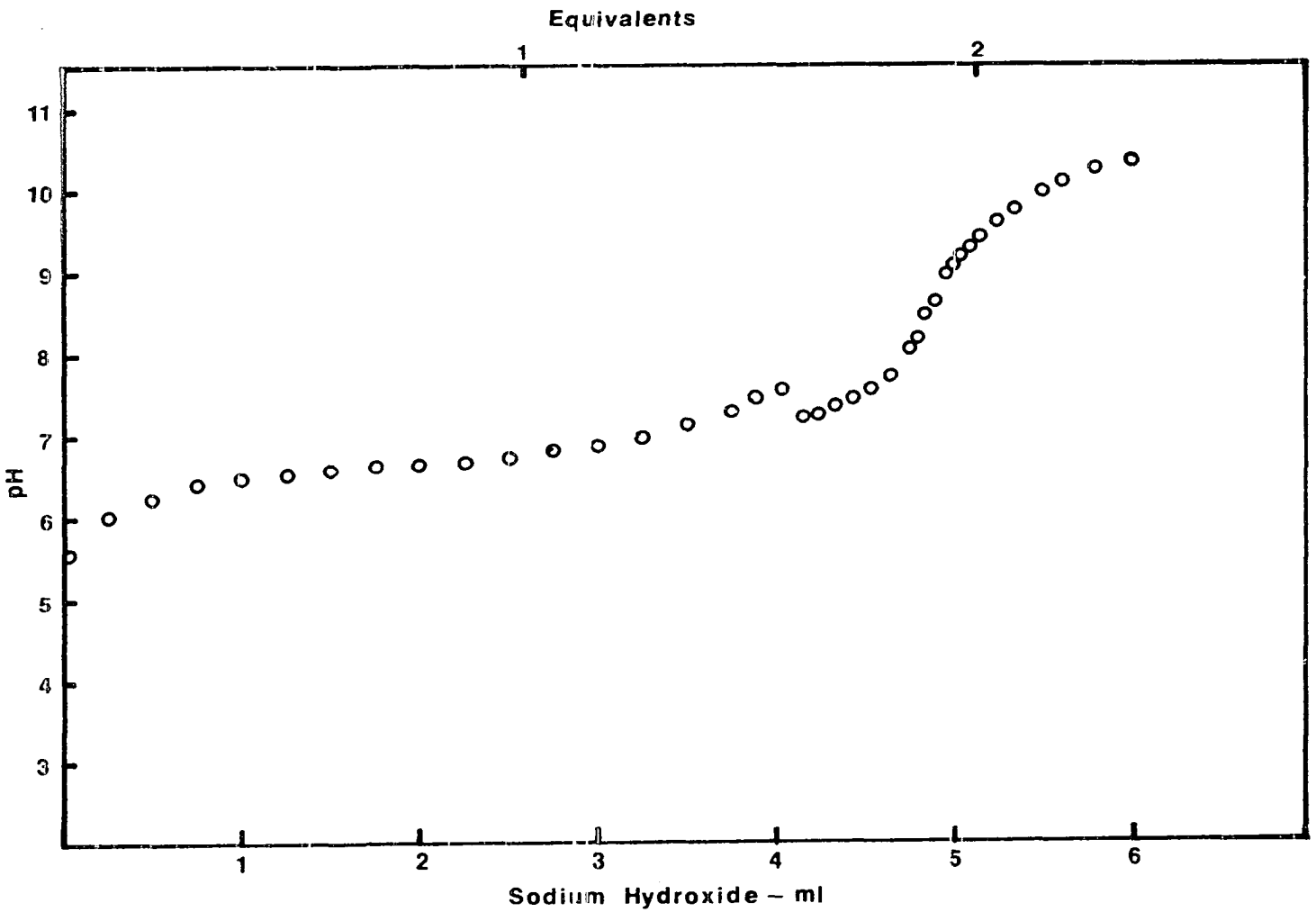

Equivalents
1
2
11
10
9
8
7
6
5
4
3
pH
1
2
3
4
5
6
Sodium Hydroxide – ml

Figure 15. Potentiometric titration of red fluorescein in water

Red fluorescein: 0.1078 g

Titrant: 0.1211 N sodium hydroxide

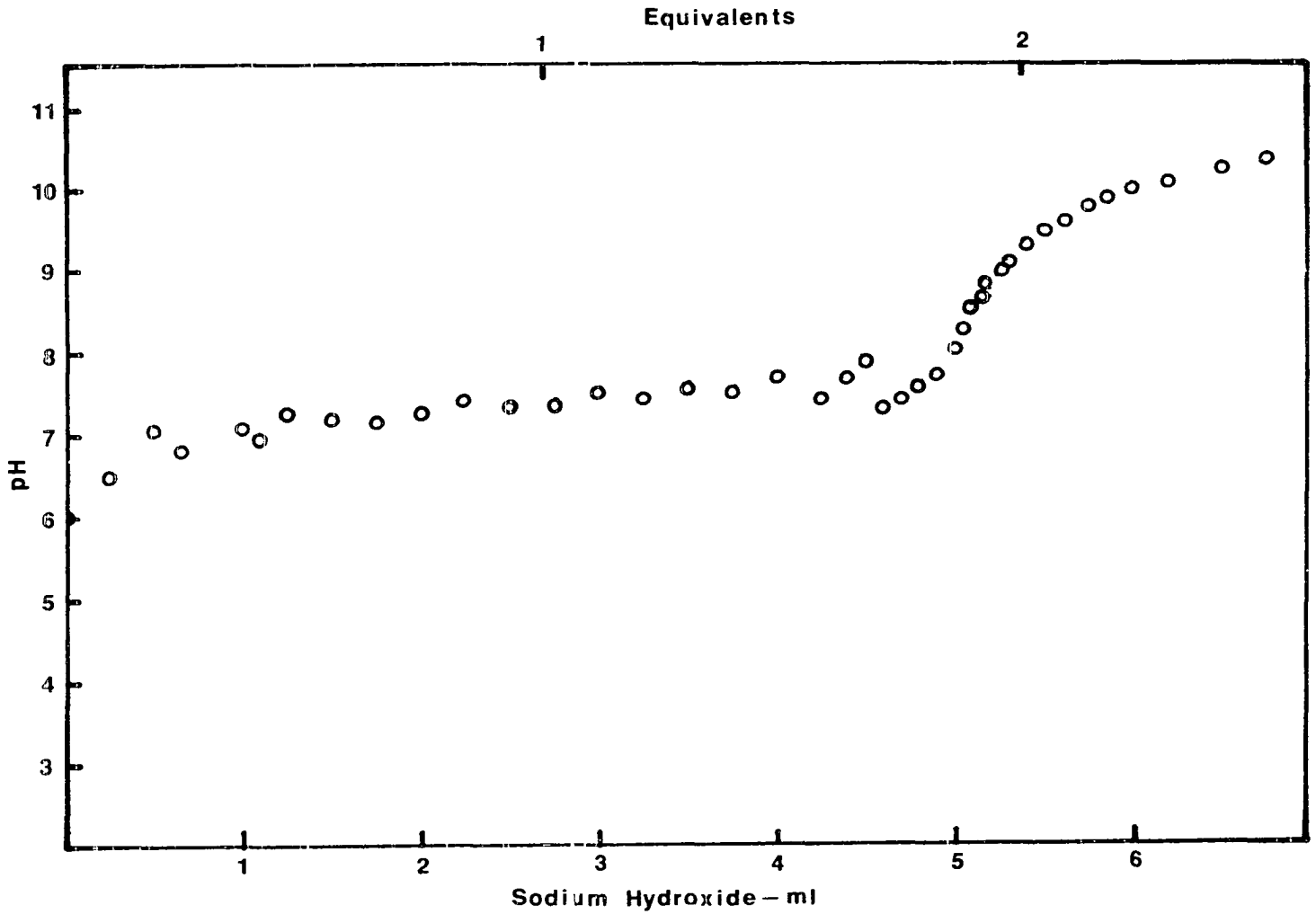

Equivalents
1
2
11
10
9
8
7
6
5
4
3
pH
1
2
3
4
5
6
Sodium Hydroxide – ml

Figure 16. Potentiometric titration of yellow fluorescein in 50 per cent ethyl alcohol

Yellow fluorescein: 0.1303 g

Titrant: 0.1211 N sodium hydroxide

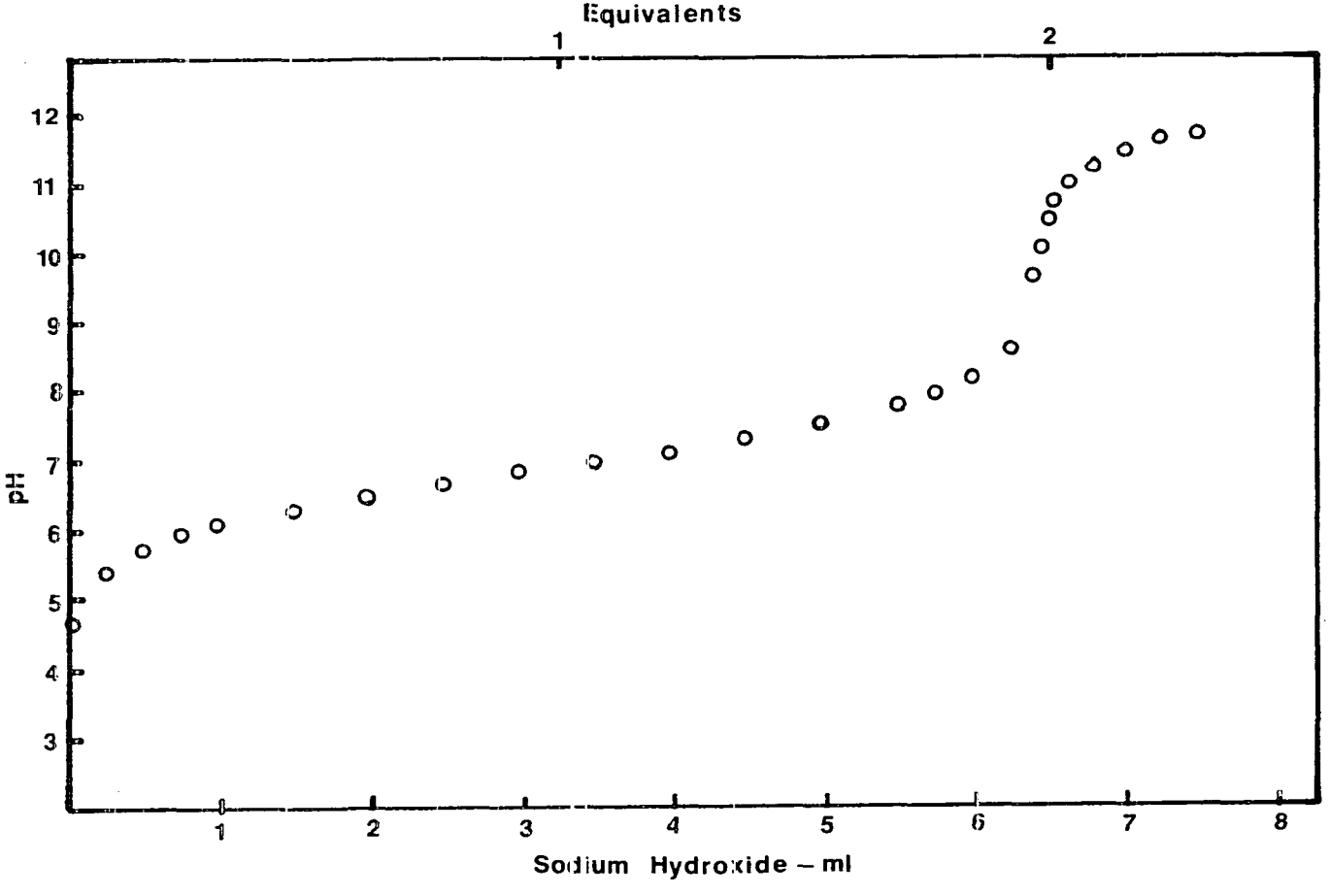
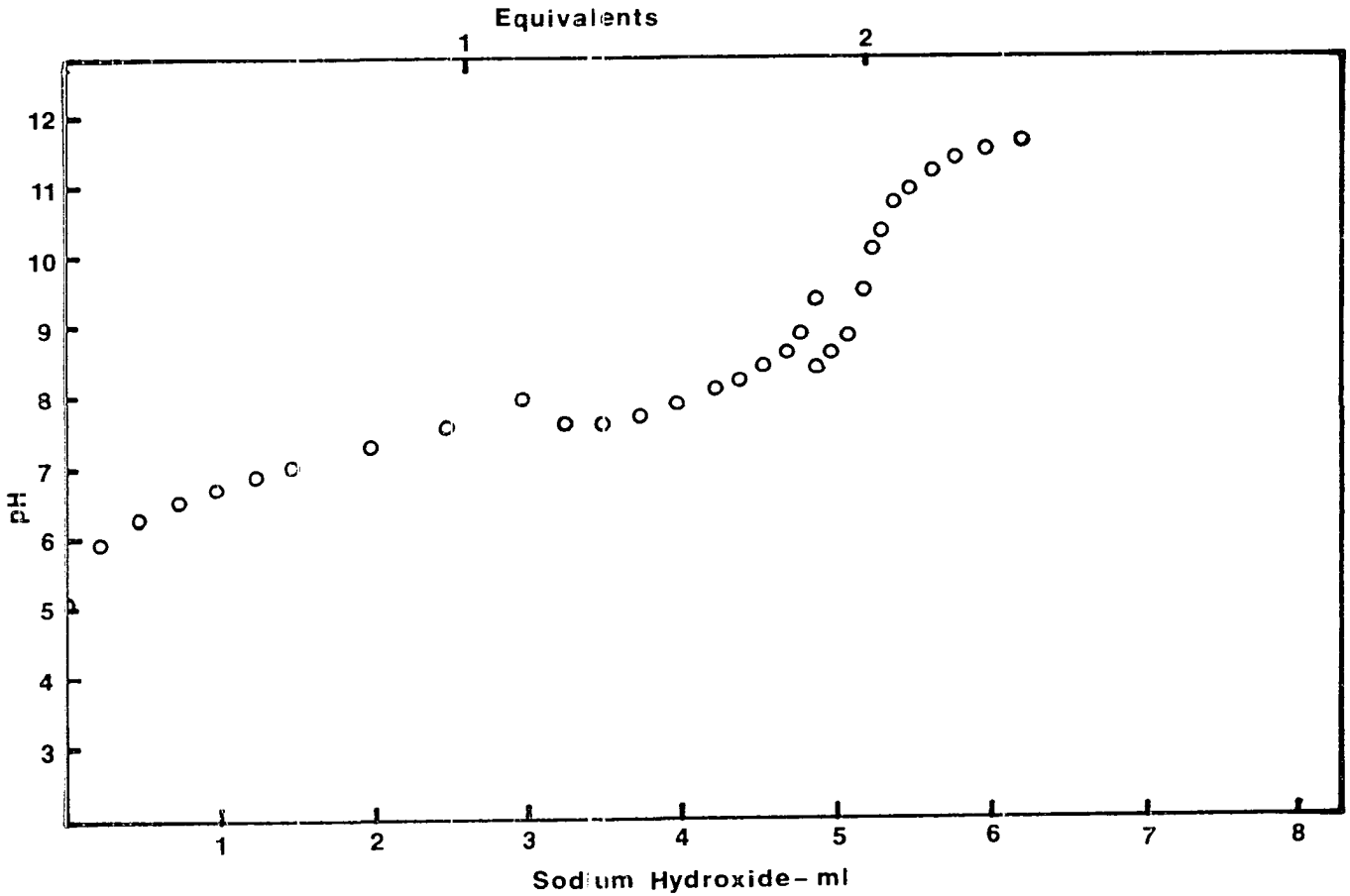

Equivalents
1
2
12
11
10
9
8
7
6
5
4
3
pH
1
2
3
4
5
6
7
8
Sodium Hydroxide – ml

Figure 17. Potentiometric titration of red fluorescein in 50 per cent ethyl alcohol

Red fluorescein: 0.1052 g

Titrant: 0.1211 N sodium hydroxide


Equivalents
1
2
12
11
10
9
8
7
6
5
4
3
pH
1
2
3
4
5
6
7
8
Sodium Hydroxide – ml

are poorly soluble in water, the curves for the direct titrations of yellow and red fluorescein in water (Figures 14 and 15, respectively) have little utility except to indicate that two equivalents of base are used at the end point and that fluorescein has two hydrogen atoms that are sufficiently acidic to be titratable in water. The better solubility of yellow and red fluorescein in 50 per cent ethyl alcohol makes the titration curves in that solvent a little smoother (Figures 16 and 17, respectively); still only one end point is found, occurring at 2 equivalents of base.

The dissolution of fluorescein in excess 0.1 N sodium hydroxide and subsequent back-titration with 0.1 N hydrochloric acid produced interesting titration curves. The back-titrations of yellow and red fluorescein in water are shown in Figures 18 and 19, respectively. The curves are identical; they are characterized by three breaks and by the appearance of a yellow precipitate just after the second break, at a pH of about 5.25. A yellow precipitate was obtained irrespective of whether the starting material was yellow or red fluorescein.

In both titration curves, the first break occurs at a pH of about 8.5 and corresponds to the titration of excess sodium hydroxide. The second break, requiring one equivalent of acid, occurs at a pH of around 5.5 and corresponds to the

Figure 18. Potentiometric back-titration of yellow fluorescein in water

Yellow fluorescein: 0.1033 g

Sodium hydroxide: 0.1211 N, 6.00 ml

Titrant: 0.1158 N hydrochloric acid

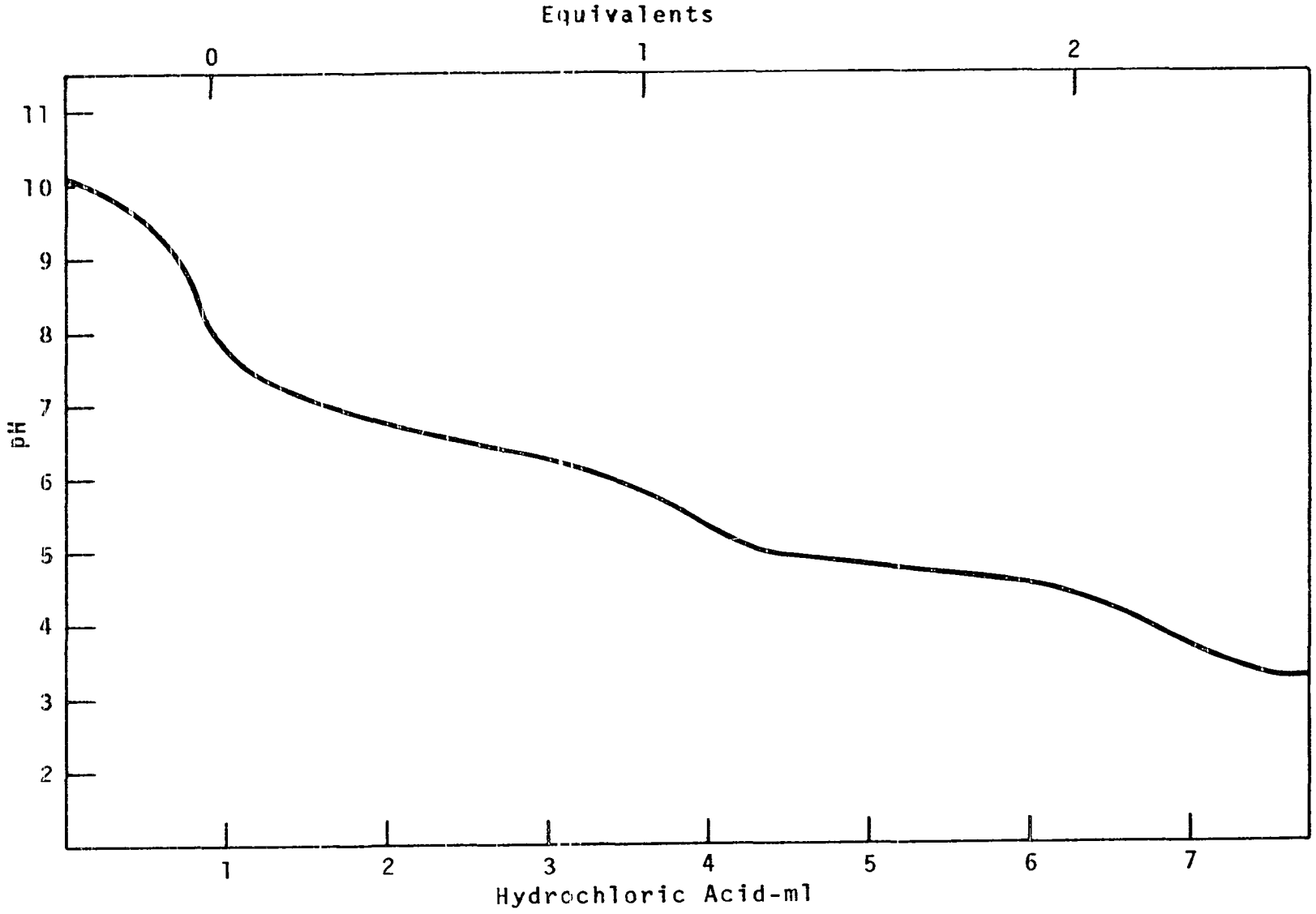
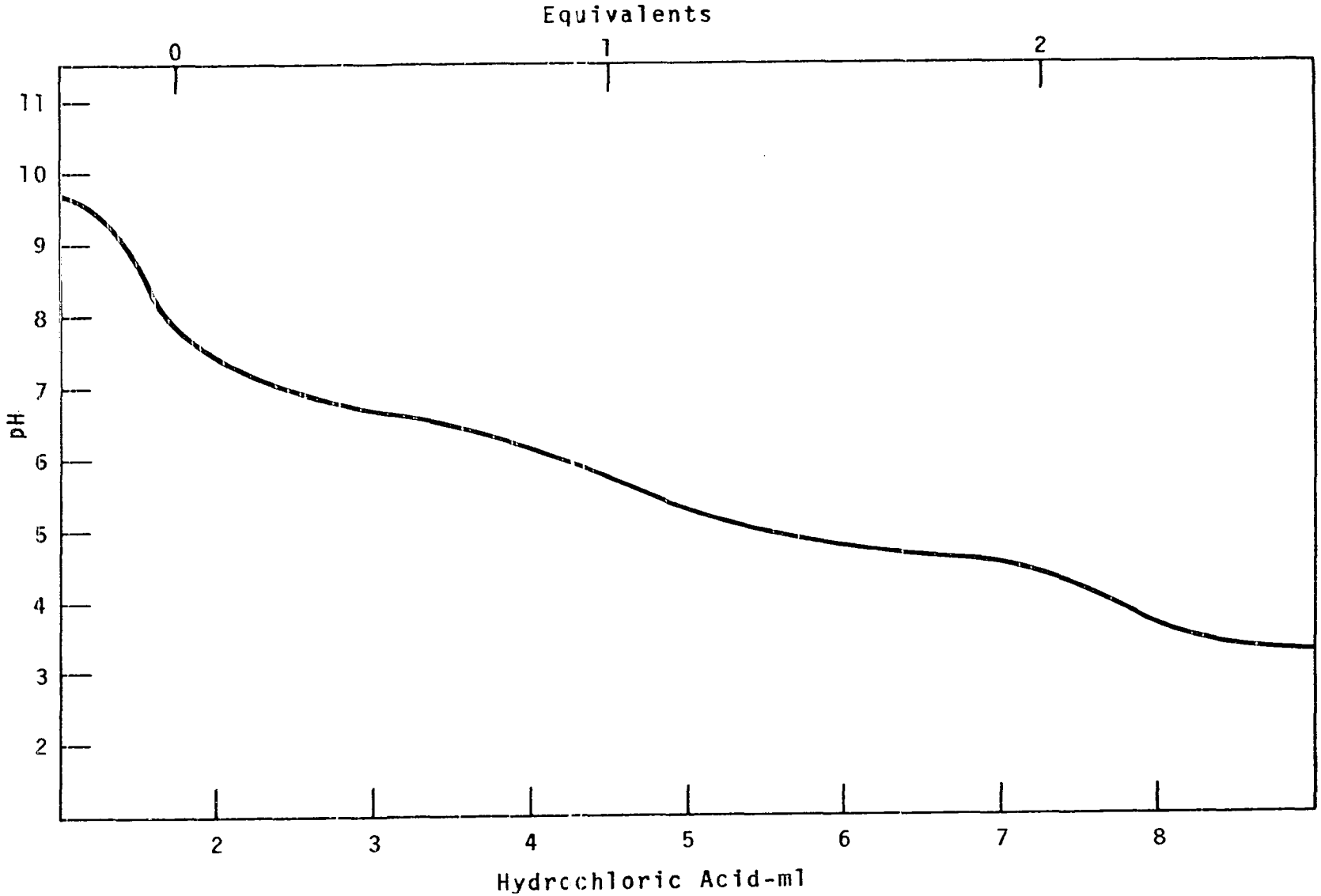

Equivalents
0
1
2
11
10
9
8
7
6
5
4
3
2
pH
1
2
3
4
5
6
7
Hydrochloric Acid-ml

Figure 19. Potentiometric back-titration of red fluorescein in water

Red fluorescein: 0.1078 g

Sodium hydroxide: 0.1211 N, 7.00 ml

Titrant: 0.1158 N hydrochloric acid


Equivalents
0
1
2
11
10
9
8
7
6
5
4
3
2
pH
2
3
4
5
6
7
8
Hydrcchloric Acid-ml

titration of the dianion Fl^{2-} to the monoanion HFl^{-}. On the basis of elementary equilibrium equations, the midpoint between the first and second break on the titration curve, occurring at pH 6.55, corresponds to the pK_{HFl^-} value, where $pK_{HFl^-} = pH + \log \frac{[Fl^{2-}]}{[HFl^-]}$. The third end-point, requiring an additional equivalent of acid, occurs at a pH of about 3.9 and corresponds to the titration of the monoanion HFl^{-} to the free acid H_2Fl. From the midpoint between the second and the third break, the pK_{H_2Fl} value is 4.75, where $pK_{H_2Fl} = pH + \log \frac{[HFl^-]}{[H_2Fl]}$. The two pK values have the same values irrespective of whether the yellow or red form of fluorescein is titrated, indicating that only a single, identical species exists in solution.

Similar back-titrations of yellow and red forms of fluorescein in 50 per cent ethyl alcohol as solvent are presented in Figures 20 and 21, respectively. The titration curves have only two breaks. The first corresponds to the titration of excess sodium hydroxide by the hydrochloric acid. The second break occurs at two equivalents of acid and corresponds to the titration of the dianion Fl^{2-} to the free acid H_2Fl. Because the solvent is 50 per cent ethyl alcohol, all species are soluble and no precipitate is observed.

In fact, the low solubility of fluorescein in water is probably the reason that the two hydrogen atoms can be

Figure 20. Potentiometric back-titration of yellow fluorescein in 50 per cent ethyl alcohol

Yellow fluorescein: 0.1303 g

Sodium hydroxide: 0.1211 N, 7.50 ml

Titrant: 0.1158 N hydrochloric acid

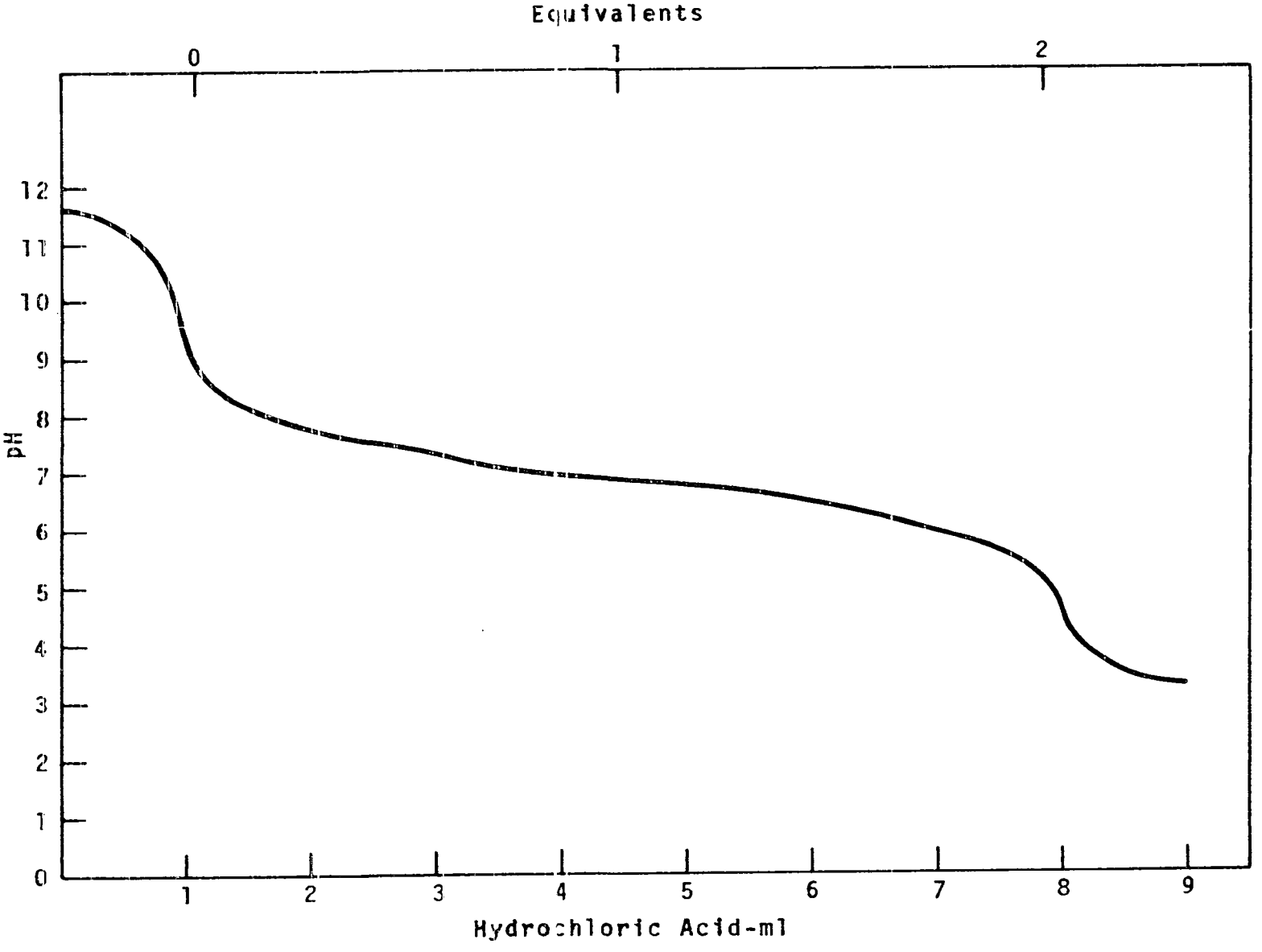
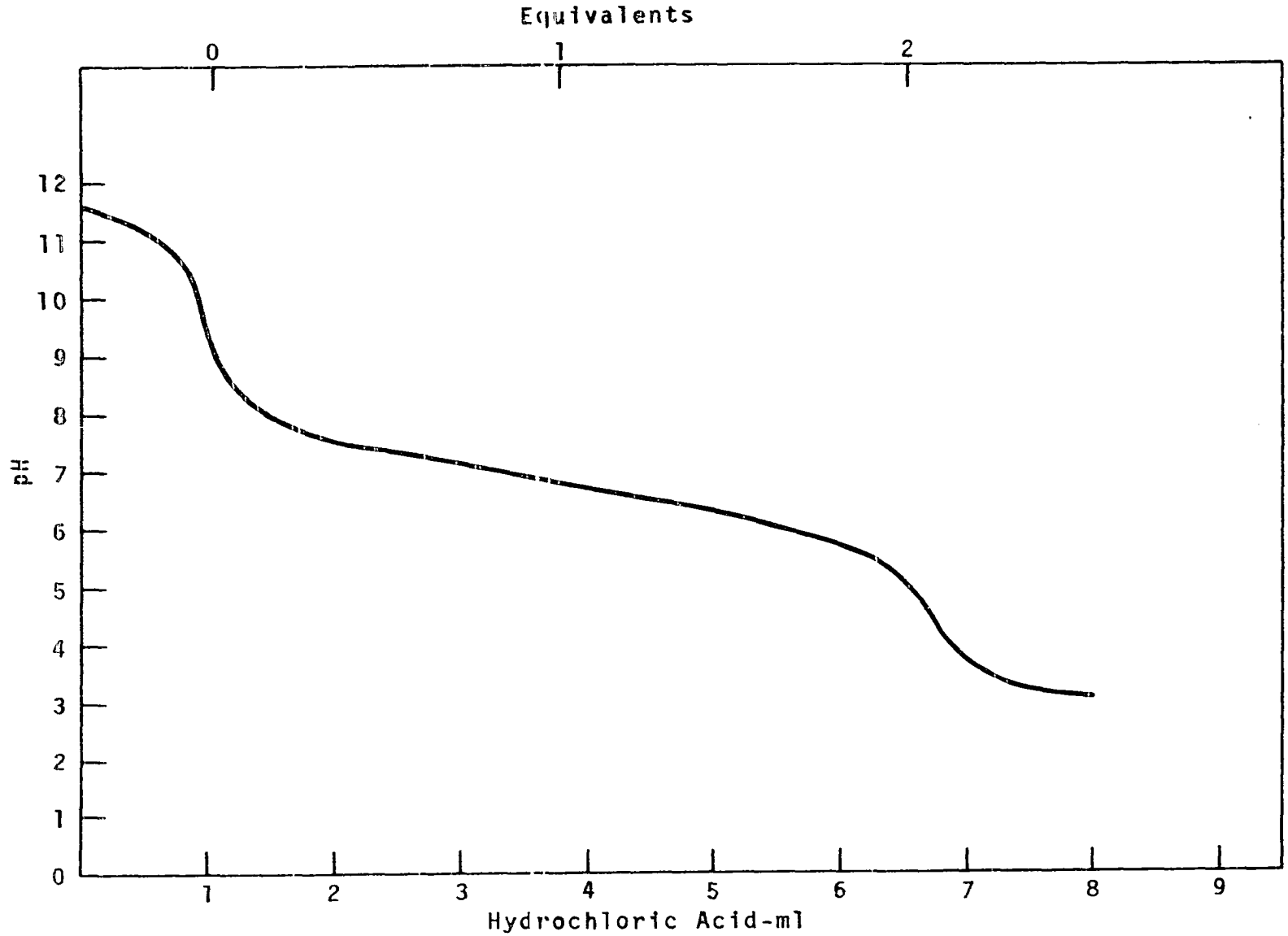

Equivalents
0
1
2
12
11
10
9
8
7
6
5
4
3
2
1
0
pH
1
2
3
4
5
6
7
8
9
Hydrochloric Acid-ml

Figure 21. Potentiometric back-titration of red fluorescein in 50 per cent ethyl alcohol

Red fluorescein: 0.1052 g

Sodium hydroxide: 0.1211 N, 6.25 ml

Titrant: 0.1158 N hydrochloric acid


Equivalents
0
1
2
pH
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
Hydrochloric Acid-ml

differentiated during titration in water but not during titration in 50 per cent ethyl alcohol. Normally, the titration of a dibasic acid will have two end-points if the two hydrogen atoms have pK values that differ by about 3 units. For fluorescein the difference in the two pK values is only 1.8. Yet because H_2Fl is insoluble, the two hydrogen atoms can be differentiated. On the other hand, in 50 per cent ethyl alcohol, the two hydrogen atoms cannot be differentiated because H_2Fl is soluble and there is not much difference in the two pK values of the first and the second hydrogen.

The titration curve of colorless fluorescein is presented in Figure 22. Because the compound is completely insoluble in water, the titration was carried out in 50 per cent ethyl alcohol. The titration curve is very similar to that for yellow (Figure 16) and red fluorescein (Figure 17) in 50 per cent ethyl alcohol. Upon dissolution, the lactone ring was opened immediately as evidenced by the yellow color of the solution and the uptake of two equivalents of base at the single end-point. The titration curve of 4',5'-dimethylfluorescein is presented in Figure 23. Again, to overcome solubility problems, the solvent was 50 per cent ethyl alcohol. The single end-point also corresponds to the uptake of two equivalents of base.

Figure 22. Potentiometric titration of colorless fluorescein in 50 per cent ethyl alcohol

Colorless fluorescein: 0.2193 g

Titrant: 0.1067 N sodium hydroxide

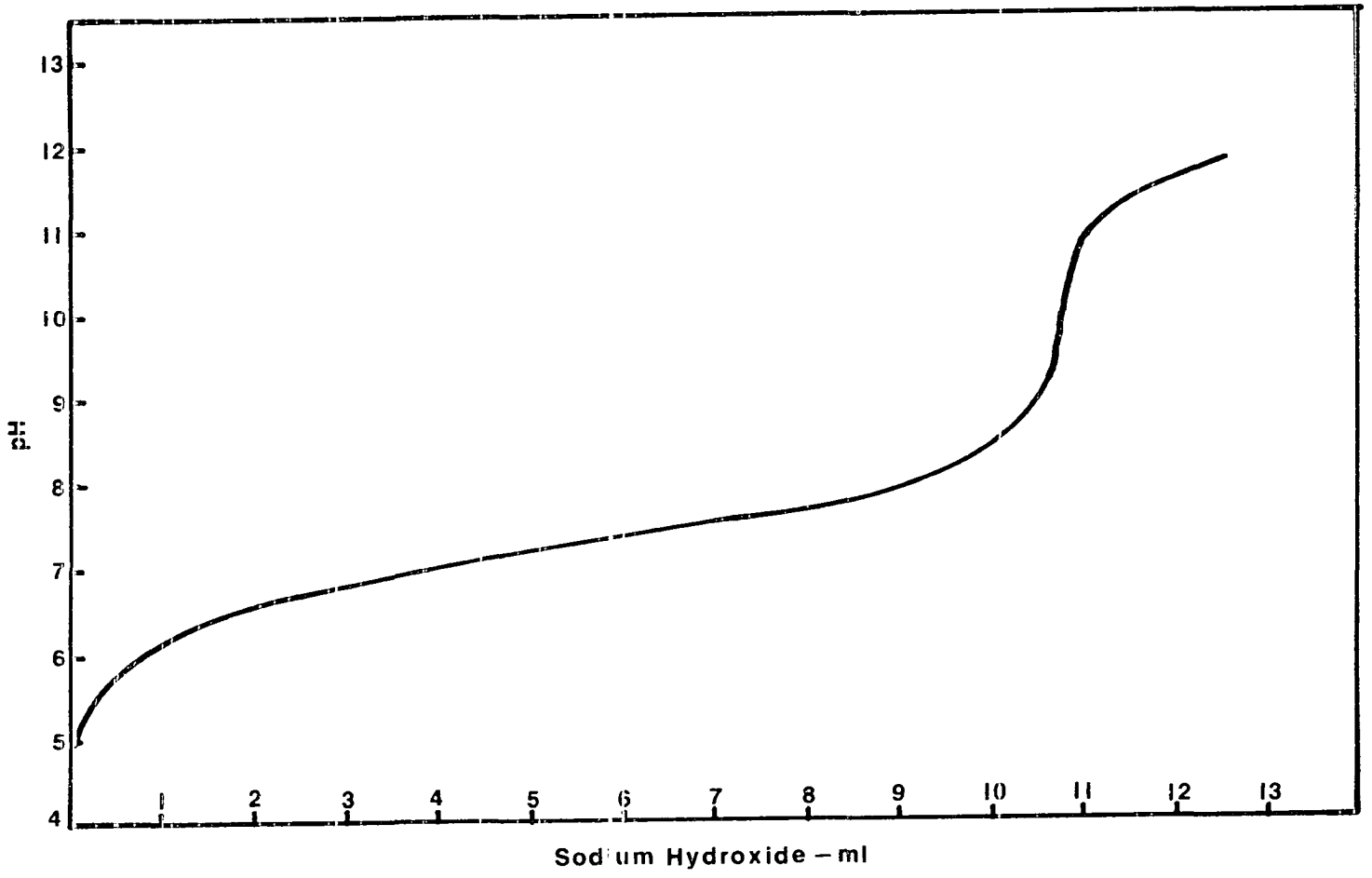
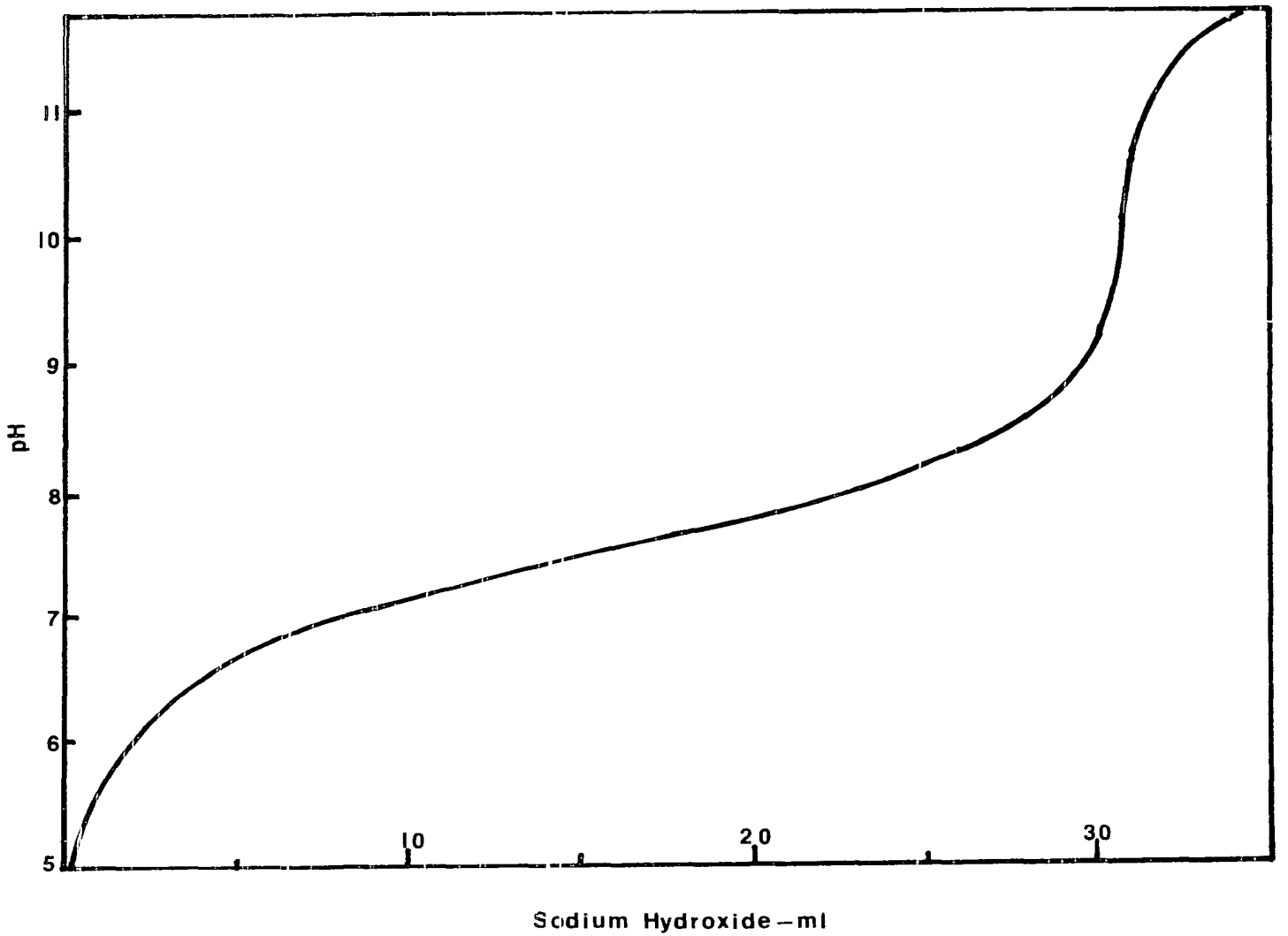

pH
13
12
11
10
9
8
7
6
5
4
1
2
3
4
5
6
7
8
9
10
11
12
13
Sodium Hydroxide – ml

Figure 23. Potentiometric titration of 4',5'-dimethylfluorescein in 50 per cent ethyl alcohol

4',5'-Dimethylfluorescein: 0.5967 g

Titrant: 0.1062 N sodium hydroxide


11
10
9
8
7
6
5
pH
10
20
30
Sodium Hydroxide—ml

On the basis of potentiometric acid-base titrations, it is evident that fluorescein exists in aqueous solution not as the colorless lactone form (Structure V) nor as the red quinone form (Structure VII) but as the yellow zwitter ion form (Structure VI), irrespective of which form of fluorescein is titrated. The solutions are always yellow, the two acid dissociation constants have the same value, and at the end of the back-titration the yellow form precipitates out.

Fluorescein in aqueous solution cannot have the closed lactone form (Structure V); such solutions would be colorless, and the phenolic hydrogen atoms of such a structure would be too weakly acidic to be titratable. For example, phenolphthalein (Structure XIV, p. 36) is a similar compound with the closed lactone structure. The two hydrogen atoms are typical phenolic hydrogen atoms with pK values of 9.64-9.96.[116] It is not quite clear if the two hydrogen atoms can be differentiated by their pK values,[80] some works give only one pK value (9.75,[90] for example) without specifying to which hydrogen atom it refers. Attempts in this work to obtain an end point by direct potentiometric titration of phenolphthalein with sodium hydroxide in both water and in ethyl alcohol as solvents were unsuccessful. Upon addition of the first increment of base, the solution became purple colored and the pH was in the alkaline region. This

observation is in contradiction to that of Dehn[26] who claimed that phenolphthalein can be titrated with up to one equivalent of base without becoming colored and that only at the start of the addition of the second equivalent of base does the solution become colored. Back-titrations of phenolphthalein are presented in Figure 24. Curve a represents the titration of excess sodium hydroxide in water with hydrochloric acid; curve b is for the same kind of back-titration, but the solvent is 50 per cent ethyl alcohol. Both curves exemplify the weakly acidic nature of the phenolic hydrogen atoms of phenolphthalein.

The titration of another model compound, 3,6-dihydroxy-9,9-dimethylxanthene (Structure XV, p. 36), is also illustrative of the weak acidity of the phenolic hydrogen atoms. Attempts of direct titration of the compound by using sodium hydroxide and tetrabutylammonium hydroxide as titrants and water, 50 per cent ethyl alcohol, 95 per cent ethyl alcohol, acetonitrile, and dimethylsulfoxide as solvents were all unsuccessful. The back-titrations of the dihydroxydimethylxanthene are presented in Figure 25; curve a is for the back-titration in water and curve b in 50 per cent ethyl alcohol. It is apparent that 3,6-dihydroxy-9,9-dimethylxanthene, which is structurally related to the closed lactone form of fluorescein, is also too weakly acidic to be titrated.

Figure 24. Potentiometric back-titrations of phenolphthalein

Curve a. Solvent: water
Phenolphthalein: 0.0899 g
Sodium hydroxide: 0.1211 N, 9.00 ml
Titrant: 0.1156 N hydrochloric acid

Curve b. Solvent: 50 per cent ethyl alcohol
Phenolphthalein: 0.1044 g
Sodium hydroxide: 0.1211 N, 9.00 ml
Titrant: 0.1156 N hydrochloric acid

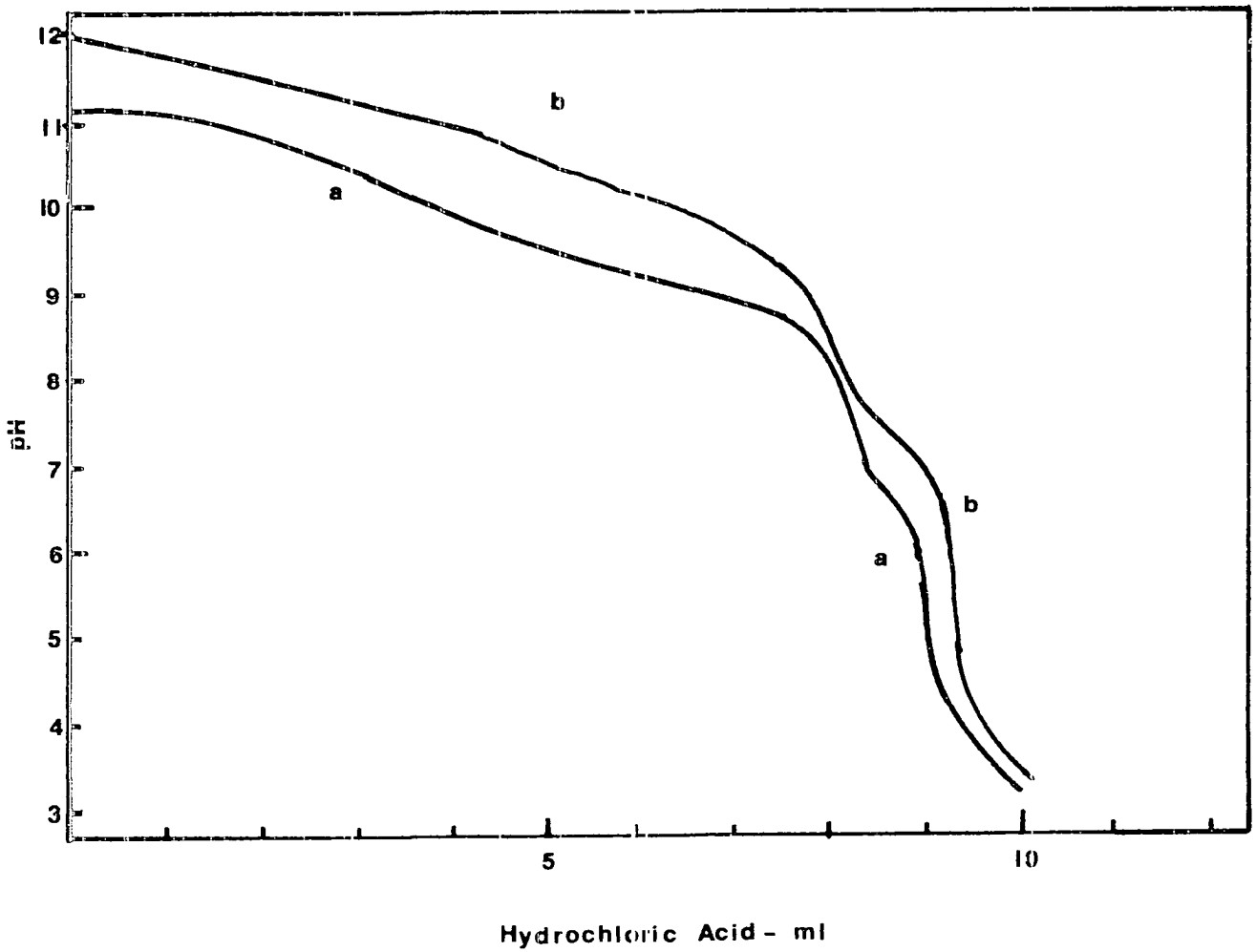

12
11
10
9
8
7
6
5
4
3
pH
b
a
b
a
5
10
Hydrochloric Acid - ml

Figure 25. Potentiometric back-titrations of 3,6-dihydroxy-9,9-dimethylxanthene

Curve a. Solvent: water

3,6-Dihydroxy-9,9-dimethylxanthene: 0.0848 g

Sodium hydroxide: 0.1211 N, 9.00 ml

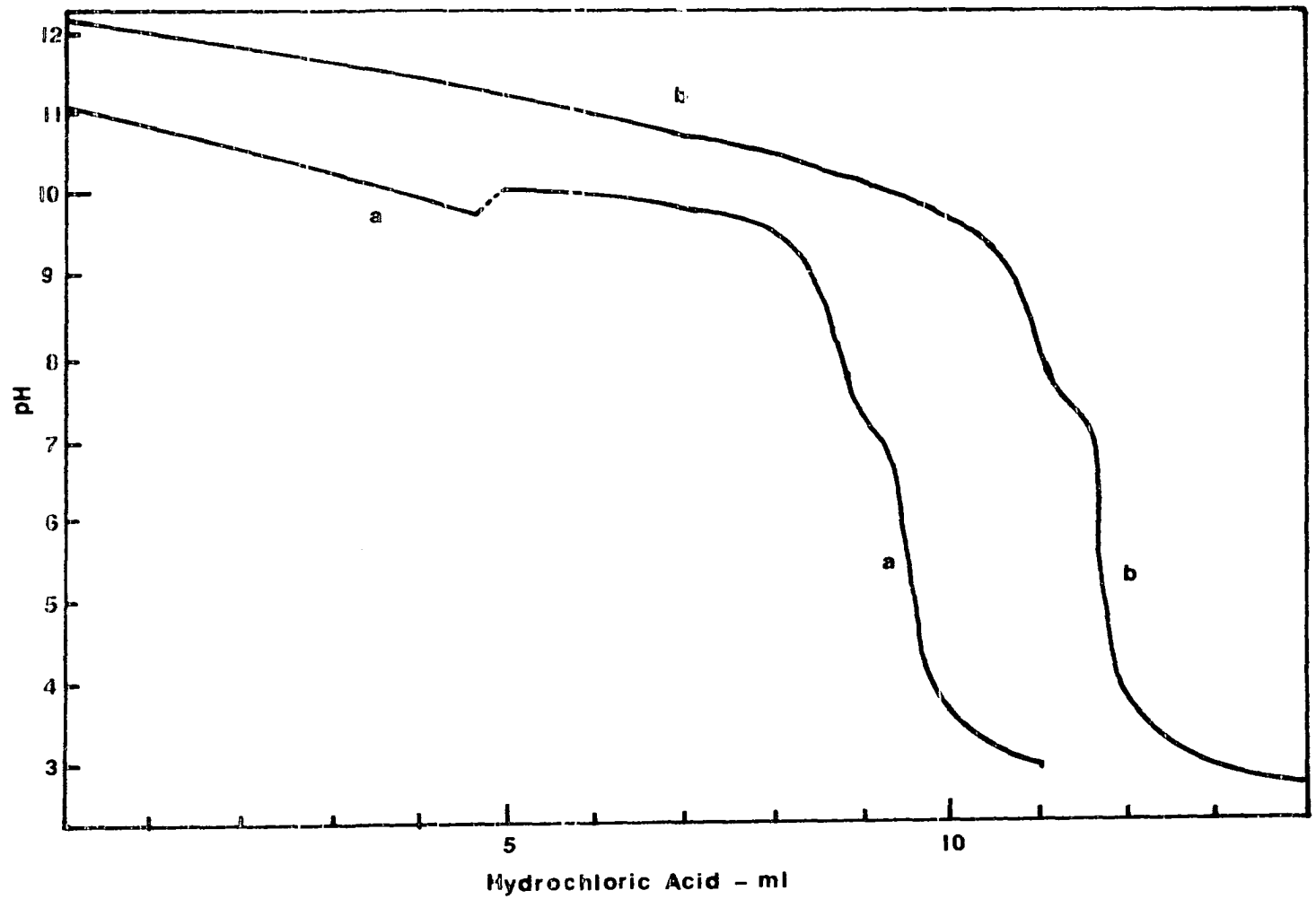
Titrant: 0.1156 N hydrochloric acid

Curve b. Solvent: 50 per cent ethyl alcohol

3,6-Dihydroxy-9,9-dimethylxanthene: 0.1005 g

Sodium hydroxide: 0.1211 N, 11.00 ml

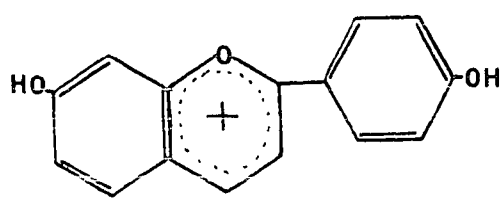
Titrant: 0.1156 N hydrochloric acid


12
11
10
9
8
7
6
5
4
3
pH
a
b
a
b
5
10
Hydrochloric Acid – ml

Yet fluorescein in aqueous solution has two titratable hydrogen atoms with pK values that are much lower than those for ordinary phenolic hydrogen atoms. Thus when fluorescein is titrated as an acid in aqueous solution, it cannot have the closed lactone form (Structure V), for which the hydrogen atoms would be too weakly acidic.

The unusually strong acidic nature of the two hydrogen atoms of fluorescein can be explained by the existence of fluorescein as the zwitter ion structure (Structure VI) in aqueous solution. The positive charge on the pyrylium-type ring of the xanthene portion of the molecule exerts a powerful influence on the phenolic hydrogen atom facilitating the dissociation of a positively charged hydrogen ion to form the monoanion (Structure IX).

An example of the effect of a positive charge of a pyrylium-type ring on the acid strength of a phenolic hydrogen atom is given by the compound 7,4'-dihydroxyanthocyanidine (Structure XXIX). Kuhn and Sperling[89] found that



XXIX. 7,4'-Dihydroxyanthocyanidine

the pK values of the two hydrogen atoms are 4.3 and 7.5. Although it is not clear which of the hydrogen atoms is removed first during neutralization, the acid strength of the two hydrogen atoms is much greater than that of normal phenolic hydrogen atoms (discussed above), owing to the positive charge of the pyrylium-type ring. In fact, the two pK values fall within the same range as the two pK values of fluorescein (4.75 and 6.55) in the zwitter ion form. Thus the zwitter ion structure of fluorescein explains the strongly acidic nature of the two hydrogen atoms of fluorescein.

It remains to be shown that the existence of the open carboxylic acid quinone form of fluorescein (Structure VII) in aqueous solution is improbable. At first glance, the existence of the carboxylic acid group would seem to fit well with the pK value of 4.75 for the first hydrogen atom, since normal carboxylic acids have pK values of that order. The simplest of the series, benzoic acid, has a pK of 4.20. However, fluorescein in the form of Structure VII must be considered as a benzoic acid substituted in the ortho position by the bulky xanthene group which is strongly electron withdrawing and capable of resonance. It is well-known that electron withdrawing groups in the ortho position increase the acidity of benzoic acid, thus lowering the pK value. Salicylic acid, for example, with a hydroxyl group

in the ortho position, has a pK of 2.97; phthalic acid, with a carboxyl group in the ortho position, has a pK of 2.89. A simple phenyl group in the ortho position lowers the pK of benzoic acid by 0.74 units. Even fluorescin (Structure XVIII, p. 69), in which the reduced xanthene portion of the molecule is not capable of resonance and thus is not as strongly electron withdrawing, has a pK value of 3-3.5 (See section II.F.3).

Hence fluorescein in the aqueous solution cannot be the quinone form with the carboxylic acid group (Structure VII), because fluorescein in this form would be a very strong acid. In fact, the very acidity of this type of structure provides some explanation to why red fluorescein which has this structure is precipitated from strongly acidic solutions. It can also be precipitated from ethanolic solutions, because in the presence of ethyl alcohol acids are weaker and the relative pK values are generally higher. Thus the red form of fluorescein can, under certain conditions, exist in ethanolic solutions without being converted to the zwitter ion form.

The resonance structures IXc,d (p. 99) are preferentially assigned over the resonance structure IXb for the monoanion, HFl^-, of fluorescein in aqueous solution. The preservation of the positive charge on the xanthene portion of the molecule, despite the additional negative charge of the

phenolate group, is a reasonable proposition for the monoanion. First of all, the very stable, aromatic nature of the pyrylium-type ring is preserved. The positively charged ring exists in the neutral, zwitter ionic molecule in the presence of a nearby negatively charged carboxylate group; it is not unreasonable to expect it to accommodate one more negative charge, the phenolate group, in the monoanion. Secondly, it helps to explain that the remaining hydrogen atom is also more acidic (pK is 6.55) than a normal phenolic hydrogen atom (usually the pK is 10-11). Of course, the leaving of the second hydrogen atom is also facilitated by the formation of the dianion (Structure Xa,c) which is strongly stabilized by resonance. Lastly, it will be demonstrated in the next section that the neutral zwitter ion and the monoanion are nonfluorescent, whereas the dianion is strongly fluorescent, indicating a fundamental and substantial difference in the structures of the fluorescent and nonfluorescent species. Assignment of Structure IXc,d to the monoanion and Structure Xa,c to the dianion conforms very well to this observation.

d. Distribution of the prototropic forms of fluorescein as a function of pH The existence of the species H_3Fl^+, H_2Fl, HFl^-, and Fl^{2-} of fluorescein in aqueous solution has been demonstrated by solubility measurements and by potentiometric titration data. To obtain an overall picture of the

distribution of each species in solution as a function of pH, the following equations were derived.

If C is the analytical concentration of the total amount of fluorescein in solution, then

$$C = [H_3Fl^+] + [H_2Fl] + [HFl^-] + [Fl^{2-}]$$

and the fraction α of each species in solution can be defined as:

$$\alpha_1 = \frac{[H_3Fl^+]}{C} \text{ and } \frac{1}{\alpha_1} = 1 + \frac{[H_2Fl]}{[H_3Fl^+]} + \frac{[HFl^-]}{[H_3Fl^+]} + \frac{[Fl^{2-}]}{[H_3Fl^+]}$$

$$\alpha_2 = \frac{[H_2Fl]}{C} \text{ and } \frac{1}{\alpha_2} = \frac{[H_3Fl^+]}{[H_2Fl]} + 1 + \frac{[HFl^-]}{[H_2Fl]} + \frac{[Fl^{2-}]}{[H_2Fl]}$$

$$\alpha_3 = \frac{[HFl^-]}{C} \text{ and } \frac{1}{\alpha_3} = \frac{[H_3Fl^+]}{[HFl^-]} + \frac{[H_2Fl]}{[HFl^-]} + 1 + \frac{[Fl^{2-}]}{[HFl^-]}$$

$$\alpha_4 = \frac{[Fl^{2-}]}{C} \text{ and } \frac{1}{\alpha_4} = \frac{[H_3Fl^+]}{[Fl^{2-}]} + \frac{[H_2Fl]}{[Fl^{2-}]} + \frac{[HFl^-]}{[Fl^{2-}]} + 1$$

The ratios can be further defined in terms of the hydrogen ion concentration $[H^+]$ and the acid dissociation constants for fluorescein. Substitution of the acid dissociation constant equations

$$K_{H_3Fl^+} = \frac{[H^+][H_2Fl]}{[H_3Fl^+]}$$

$$K_{H_2Fl} = \frac{[H^+][HFl^-]}{[H_2Fl]}$$

$$K_{HFl^-} = \frac{[H^+][Fl^{2-}]}{[HFl^-]}$$

and rearrangement yields

$$\frac{1}{\alpha_1} = 1 + \frac{K_{H_3Fl^+}}{[H^+]} + \frac{K_{H_3Fl^+}\ K_{H_2Fl}}{[H^+]^2} + \frac{K_{H_3Fl^+}\ K_{H_2Fl}\ K_{HFl^-}}{[H^+]^3}$$

$$\frac{1}{\alpha_2} = \frac{[H^+]}{K_{H_3Fl^+}} + 1 + \frac{K_{H_2Fl}}{[H^+]} + \frac{K_{H_2Fl}\ K_{HFl^-}}{[H^+]^2}$$

$$\frac{1}{\alpha_3} = \frac{[H^+]^2}{K_{H_3Fl^+}\ K_{H_2Fl}} + \frac{[H^+]}{K_{H_2Fl}} + 1 + \frac{K_{HFl^-}}{[H^+]}$$

$$\frac{1}{\alpha_4} = \frac{[H^+]^3}{K_{H_3Fl^+}\ K_{H_2Fl}\ K_{HFl^-}} + \frac{[H^+]^2}{K_{H_2Fl}\ K_{HFl^-}} + \frac{[H^+]}{K_{HFl^-}} + 1$$

By using the numerical values of

$$pK_{H_3Fl^+} = 2.14 \qquad K_{H_3Fl^+} = 7.24 \times 10^{-3}$$

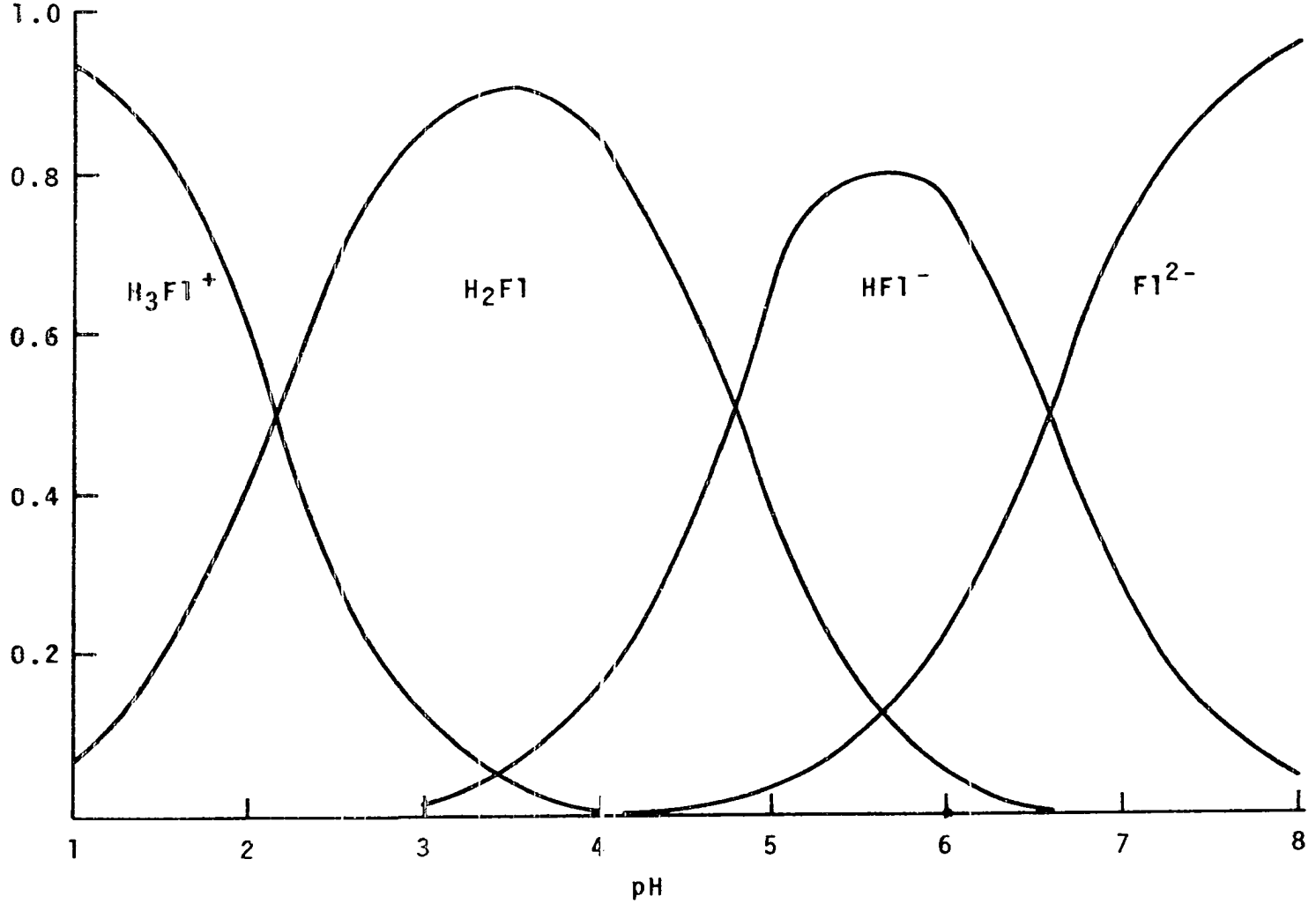
$$pK_{H_2Fl} = 4.73 \qquad K_{H_2Fl} = 1.86 \times 10^{-5}$$

$$pK_{HFl^-} = 6.55 \qquad K_{HFl^-} = 2.85 \times 10^{-7}$$

obtained by averaging the values determined by solubility measurements and potentiometric titration data, the fractions of each species can be calculated and plotted as a function of the hydrogen ion concentration. The graph is presented in Figure 26.

It can be seen from the graph in Figure 26 that the various pH values at the end points observed in Figures 18 and 19 have physical expression in terms of the graph. Thus, the maximum concentration of HFl^- occurs at a pH of about 5.6, corresponding to the end point of the titration $Fl^{2-} + H^+ = HFl^-$. Also, the maximum concentration of H_2Fl, occurring at a pH of about 3.5, corresponds to the point of minimum solubility on the curve of solubility as a function of pH (Figure 9, p. 104). The graph will also be useful in the next section for correlating the increase in fluorescence (as a function of pH) with the increase in the concentration of the dianion Fl^{2-}.

Figure 26. Relative distribution of the prototropic forms of fluorescein (H_3Fl^+, H_2Fl, HFl^-, and Fl^{2-}) as a function of pH


1.0
0.8
0.6
0.4
0.2
H_3Fl^+
H_2Fl
HFl^-
Fl^{2-}
1
2
3
4
5
6
7
8
pH

4. Fluorescence as a function of pH

a. Apparatus and procedure The excitation and emission spectra of fluorescein as a function of pH were measured by using a Bowman-Keirs Spectrophosphorimeter with an attached Moseley Autograf X-Y Recorder. The path length of the quartz cell was 1.00 cm. After inspection of the preliminary excitation and emission spectra, the emission spectrum of fluorescein was measured from pH 1.5 to pH 13. The excitation monochromator was set at 489 nm, the emission monochromator at 516 nm.

Buffers for the region pH 1.5-13 were prepared in 0.5 pH unit intervals as in the section on solubility measurements (II.D.2), by using 0.1 M solutions of hydrochloric acid, potassium acid phthalate (KHP), boric acid, and potassium hydroxide, as appropriate, and diluting with 0.1 M solutions of potassium chloride in order to keep the ionic strength constant at $\mu = 0.1$. The pH of the buffer solutions was determined, after the fluorescence measurements, by using a Corning Model 10 pH meter with a Beckman glass electrode and a saturated calomel electrode as reference.

A stock solution of fluorescein was prepared by dissolving 100.0 mg of the pure red fluorescein in 1 l. of 0.1 M potassium hydroxide. A 1.00-ml aliquot of the stock solution was placed in a 50-ml volumetric flask, 1 ml of

0.1 M hydrochloric acid was added to neutralize most of the potassium hydroxide, and the mixture was diluted to mark with the appropriate buffer for fluorometric measurement. Thus the concentration of the fluorescein solution was 6×10^{-6} moles/l.

b. Results and discussion The fluorescence curve of fluorescein as function of pH is presented in Figure 27. The fluorescence is minimal at a pH less than 4, begins to increase slowly between pH 4 and 5, rises most rapidly between pH 6 and 7, and reaches a maximum and essentially constant value at pH 8 and above. The midpoint of the fluorescence curve as a function of pH occurs at pH 6.35 and corresponds to pK_{HFl^-}, a value not too different from that obtained by potentiometric titration (6.55) in the previous section.

A more rigorous evaluation of the acid dissociation constant for the second hydrogen atom leaving the monoanion HFl^- to give the dianion Fl^{2-} is as follows: The equilibria of fluorescein H_2Fl over the pH range of interest can be defined by the reactions and mathematical equations:

$$H_2Fl = H^+ + HFl^- \qquad K_{H_2Fl} = \frac{[HFl^-][H^+]}{[H_2Fl]} \qquad (1)$$

$$HFl^- = H^+ + Fl^{2-} \qquad K_{HFl^-} = \frac{[Fl^{2-}][H^+]}{[HFl^-]} \qquad (2)$$

The assumption is made that the neutral species H_2Fl and the monoanion HFl^- do not fluoresce, and only the dianion species Fl^{2-} is responsible for the fluorescence. The assumption that HFl^- is nonfluorescent is reasonable, as seen from the inspection of Figure 27. Since the value of pK_{H_2Fl}, as determined previously, is 4.75, one would expect much more intense fluorescence at pH 4-5 than shown in the curve, if HFl^- were fluorescent.

The slight fluorescence at that pH is caused by the formation of Fl^{2-}, an expected result since the two pK values are separated only by approximately 1.6 units. In fact, calculations based on using equation for $\frac{1}{\alpha_4}$, p. 154, for a solution containing 6×10^{-6} M fluorescein showed that at pH 4 the concentration of the dianion, $[Fl^{2-}]$, is 2.5×10^{-9} M. The calculated fluorescence intensity is 0.1 relative units; the observed fluorescence at pH 4 was 2.5 relative units, which is within the experimental error of the instrument itself.

Rearrangement of equation (2) results in

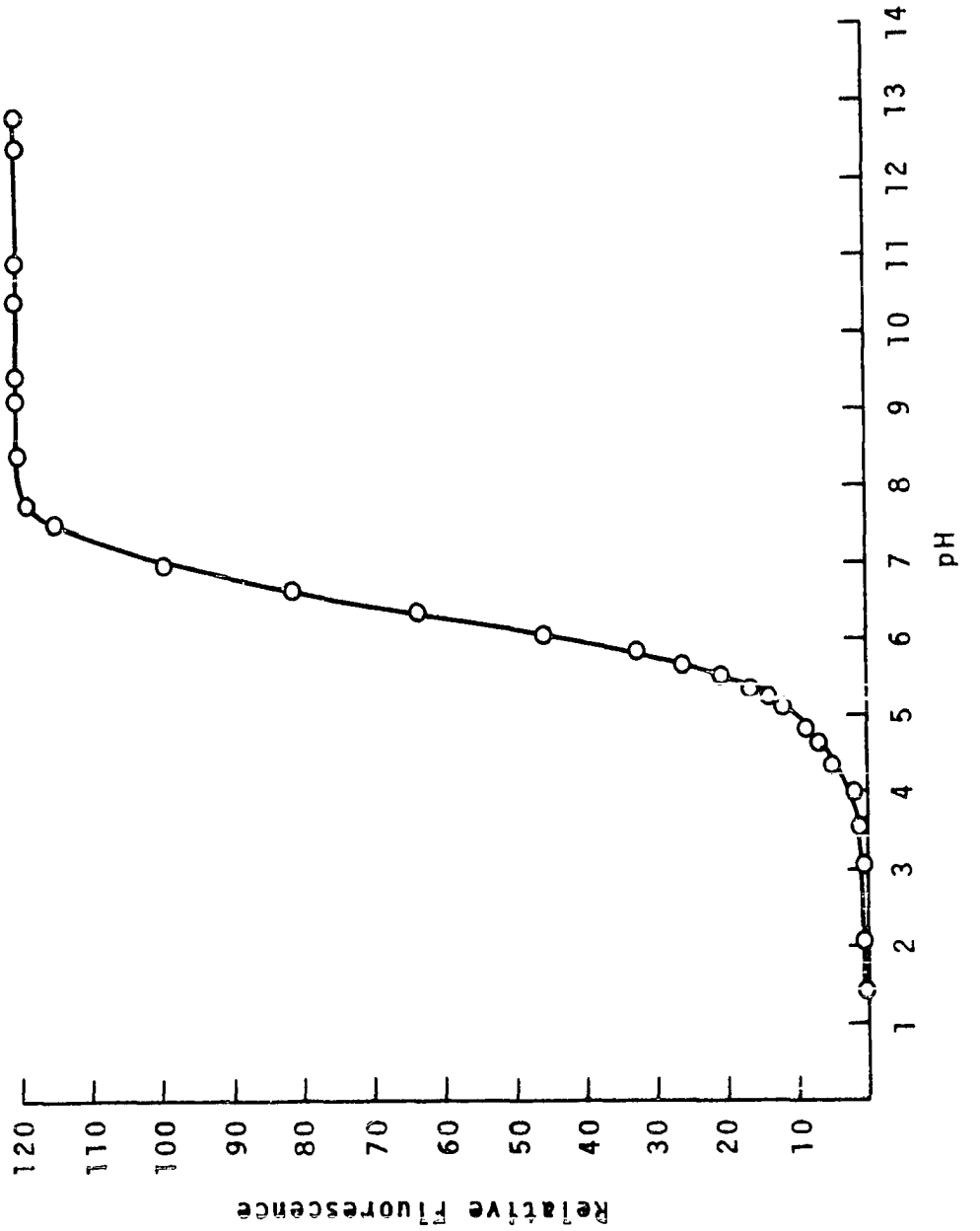
$$pH = pK_{HFl^-} + \log \frac{[Fl^{2-}]}{[HFl^-]} \qquad (3)$$

From equation (3) it is evident that when $[HFl^-] = [Fl^{2-}]$, the log term becomes zero and $pH = pK_{HFl^-}$. Thus a plot of pH vs. the log term can be used to evaluate the acid dissociation constant. With the assumptions that HFl^- is

Figure 27. Relative fluorescence of fluorescein, 6×10^{-6} M, as a function of pH

Excitation at 489 nm

Emission at 516 nm


Relative Fluorescence
120
110
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
11
12
13
14
pH

nonfluorescent, that the concentration of H_2Fl is negligible, that at pH above 8 all the fluorescein exists as Fl^{2-}, and that the analytical concentration of fluorescein at any pH can be expressed as the sum $[HFl^-] + [Fl^{2-}]$, equation (3) becomes

$$pH = pK_{HFl^-} + \log \frac{F_m}{F_d - F_m} \qquad (4)$$

where F_d is the fluorescence for the dianion Fl^{2-} alone, at pH greater than 8, and F_m is the fluorescence of the mixture of Fl^{2-} and HFl^- at any point along the curve. The experimental and calculated points for equation (4) are given in Table 9.

The plot of pH vs. the log term in equation (4) is presented in Figure 28. The slopes and intercepts of the straight-line portions were computed by the least squares method. Above pH of about 5.5 the slope of the curve is 1.07, fairly close to the expected theoretical value of 1.0. The value of pK_{HFl^-} calculated from the intercept at $\log \frac{F_m}{F_d - F_m} = 0$ is 6.28.

At pH below about 5.4, the slope of the straight line portion is 0.508, fairly close to a theoretical slope of ½. At the intersection of the two straight lines, at pH 5.35, the concentration of HFl^- is maximum and the relation holds:

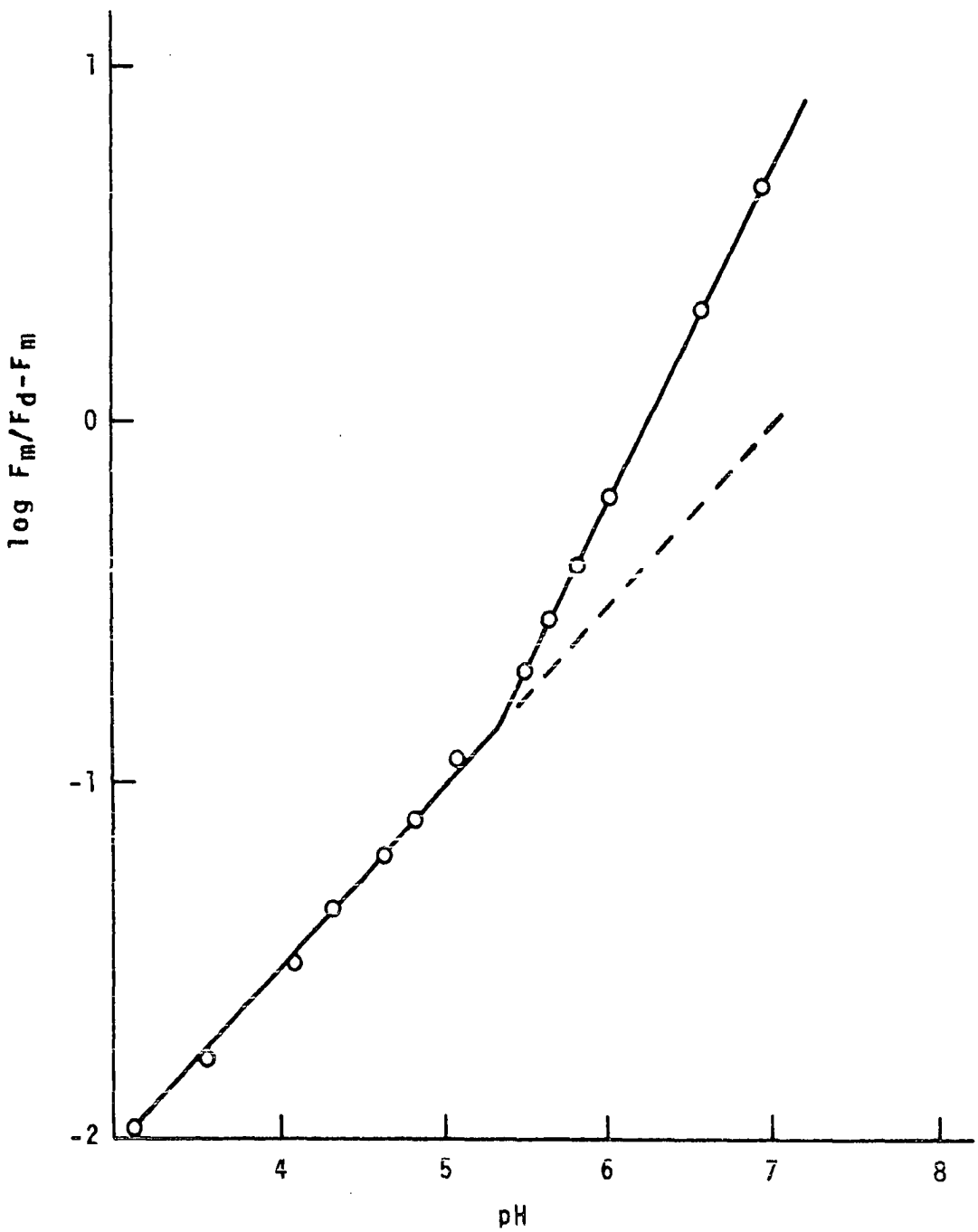
Table 9. Relative fluorescence of fluorescein as a function of pH

pH	$\log \frac{F_m}{F_d - F_m}$ experimental	$\log \frac{F_m}{F_d - F_m}$ calculated[a]	
3.11	-1.976	-1.99	
3.56	-1.772	-1.76	slope[b] = 0.508
4.05	-1.510	-1.51	intercept[b] = 7.02
4.34	-1.362	-1.36	
4.65	-1.215	-1.205	
4.84	-1.118	-1.109	
5.11	-0.955	-0.971	
5.51	-0.699	-0.722	
5.69	-0.559	-0.554	slope[b] = 1.07
5.85	-0.431	-0.404	intercept[b] = 6.28
6.05	-0.210	-0.217	
6.63	0.317	0.326	
6.99	0.673	0.663	

[a]Calculated by computer from the slope, intercept, and least squares function.

[b]Calculated by computer from the experimental points by using the least squares function.

Figure 28. Relative fluorescence of fluorescein, expressed as $\log \frac{F_m}{F_d - F_m}$, as a function of pH


1
0
-1
-2
log F_m/F_d-F_m
4
5
6
7
8
pH

$$pH = \frac{pK_{H_2Fl} + pK_{HFl^-}}{2} \qquad (5)$$

From equation (5) and the value of 6.28 for pK_{HFl^-} the value of pK_{H_2Fl} is estimated to be 4.42.

The two values of the acid dissociation constants determined by the fluorescence method (4.42 and 6.28, respectively) are lower than those determined by the potentiometric titration method (4.75 and 6.55, respectively, Section II.D.3). The differences of 0.33 and 0.27 units, respectively, can be explained by differences in the ionic strength at which the acid dissociation constants were determined. Because fluorescence measurements are sensitive to variations in ionic strength, the buffers were prepared with the ionic strength of 0.1. On the other hand, no efforts were made to control the ionic strength of the solution during potentiometric titrations. Since normally, aside from the added titrant, the solutions contained only about 100 mg of fluorescein in about 100 ml of solvent, the ionic strength was much lower than 0.1, perhaps in the order of 0.001. Since the pK values of almost all acid increase by decreasing the ionic strength, the observed differences are qualitatively explainable. From tables of pK values it is seen that upon changing ionic strength from 0.1 to 0, the pK values are

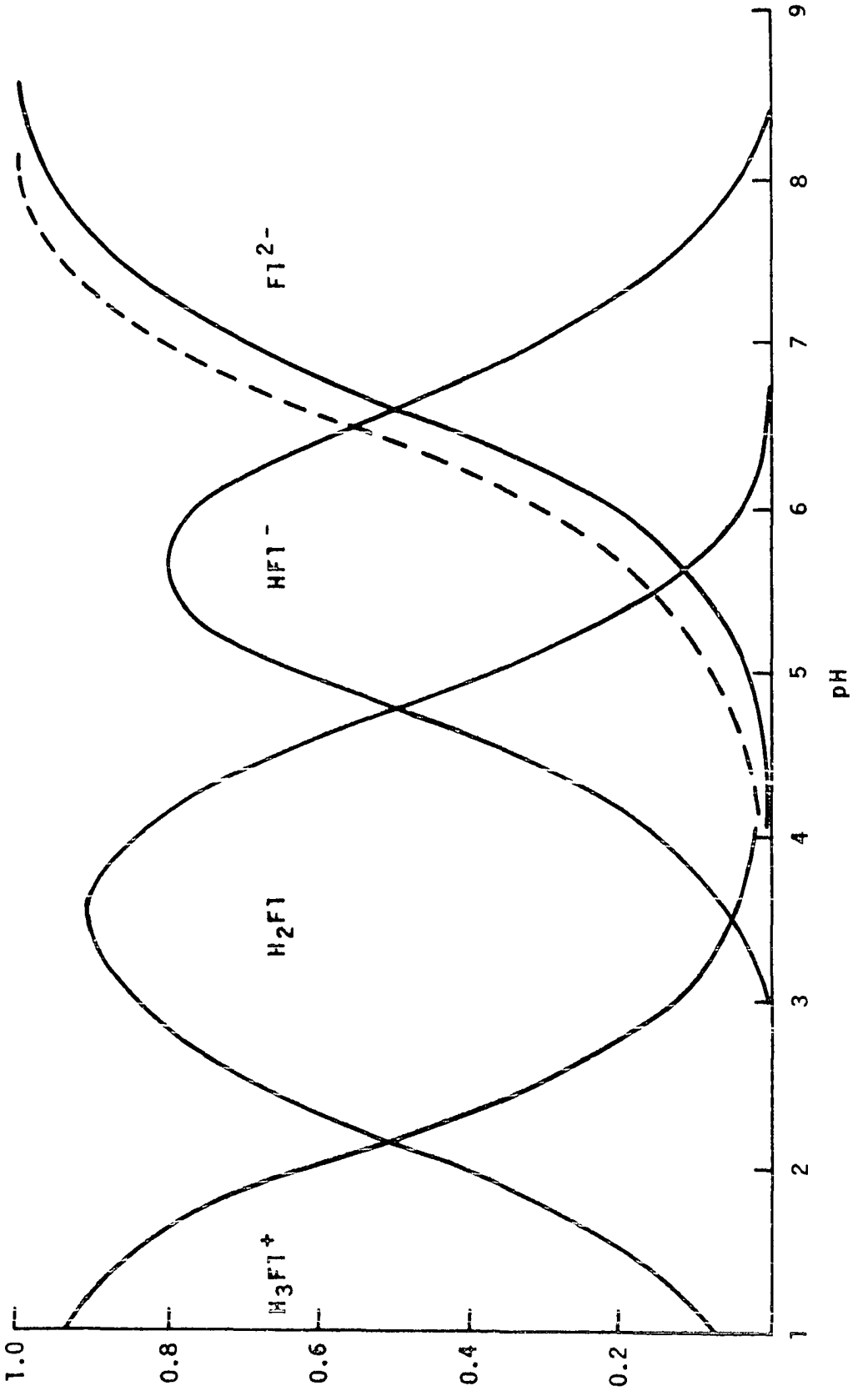
increased generally by 0.1-0.4 units. The differences in this work are of the same order.

c. Nature of the fluorescent species of fluorescein

The assumption that both H_2Fl and HFl^- are nonfluorescent and Fl^{2-} is the only fluorescent species is borne out in Figure 29 in which the plot of relative fluorescence vs. pH of Figure 27 (p. 162) was superimposed on the plot of the relative distribution of the various ionic species as a function of pH (Figure 26, p. 157). The relative increase in fluorescence as a function of pH follows very closely the relative increase of the dianion species Fl^{2-} in solution with increasing pH. The two curves are superimposable, except that the fluorescence curve is shifted to lower pH by about 0.25 pH units. The reason for the shift is in the difference in the value of pK_{HFl^-} obtained by fluorescence and by potentiometric titration, 6.28 and 6.55, respectively. Since the curves of the relative distribution of the ionic species as a function of pH were calculated by using the value of 6.55 rather than 6.28 for pK_{HFl^-}, the difference accounts for the shift.

The nonfluorescence of the monoanion HFl^- is also an additional support for the assignment of Structure IXc,d to the monoanion proposed in Section II.D.3. In the proposed structure the pyrylium-ring structure of the neutral zwitter ion form of fluorescein (Structure VI) is retained; thus it

Figure 29. Relative concentration of the dianion Fl^{2-} of fluorescein (solid line) and the relative fluorescence (dashed line) as a function of pH


1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
6
7
8
9
pH
H_3Fl^+
H_2Fl
HFl^-
Fl^{2-}

is reasonable that both the neutral species H_2Fl and the monoanion HFl^-, which share a similar structure, are non-fluorescent. It is only with the formation of the dianion Fl^{2-}, for which the structure becomes significantly different by acquiring the quinone form (Structure Xa,c) that the fluorescence of fluorescein becomes visible. The difference in the fluorescence of the dianion Fl^{2-} and the nonfluorescence of the monoanion HFl^- can be easily accounted for by the radically different structures of the two ionic forms.

5. Conclusions

In aqueous solution, fluorescein H_2Fl is an amphoteric compound that behaves as a dibasic acid with two titratable hydrogen atoms. On the basis of solubility data, potentiometric acid-base titrations, and fluorescence measurements as a function of pH, it is apparent that fluorescein exists in aqueous solution not as closed lactone ring structure nor as the open quinone form with the carboxylic acid group but rather as the yellow zwitter ion (Structure VI). The zwitter ion structure accounts for the unusually strong acidic character of the two hydrogen atoms which would normally be classified as phenolic. The zwitter ion structure, which predominates the pH region of 3-4, accounts also for the point of minimum solubility at pH 3.4, the isoelectric point at 3.1-3.6, and the amphoteric nature of fluorescein at that pH.

The monoanion HFl^- has a maximum concentration at pH of about 5.5. The monoanion is nonfluorescent, and the remaining replaceable hydrogen atom is a stronger acid than a normal phenolic hydrogen atom. The monoanion with such properties is best described by Structure IXc,d.

Above pH of about 8, fluorescein exists almost exclusively as a dianion Fl^{2-} which is assigned Structure Xa,c. The highly resonant nature of this dianion, together with its planar structure, makes it responsible for the well-known fluorescence of fluorescein. In dilute, alkaline solutions the color appears brilliant green; in concentrated solutions, the green fluorescence of the dianion disappears owing to concentration quenching, and the color becomes deep red-brown, the color of solid disodium fluoresceinate.

At pH below about 3, fluorescein exists in a cationic form H_3Fl^+, as demonstrated by the solubility of fluorescein in acids and by electrophoretic migration of the ionic species towards the cathode. On the basis of the stability of the positive charge on the pyrylium-type ring, Structure VIII is the best representation of the cationic species H_3Fl^+.

The existence of the various prototropic species, H_3Fl^+, H_2Fl, HFl^-, and Fl^{2-}, and their relative distributions in aqueous solutions over the entire pH region from 1 to 9 are

represented in Figure 26. The pH ranges in which the prototropic forms predominate show significant overlap.

That fluorescein in solution exists in a single form depending on the solvent and on the pH, but not on the starting form of fluorescein, has been confirmed by obtaining identical results for the red and yellow forms of fluorescein in solubility studies and during acid-base titrations. In the next section, II.E., NMR spectroscopy will be used to demonstrate that in dioxane solution the yellow, red, and colorless forms of fluorescein all exist also in one form, the colorless lactone form.

E. Nuclear Magnetic Resonance Spectroscopy of Fluorescein in Dioxane Solution

1. Introduction

The nuclear magnetic resonance (NMR) spectrum of fluorescein has been obtained previously, by Hefley,[58] in dimethylsulfoxide as solvent. She identified each proton of the fluorescein molecule and assigned the NMR peaks to each on the basis of chemical shift. Although the structure of fluorescein in dimethylsulfoxide as solvent is not known with certainty, it is not very likely to be the closed lactone ring form (Structure V, p. 19) assumed by Hefley. However, since it is known that fluorescein in dioxane solution exists in the lactone form, it was instructive to obtain

NMR spectra for fluorescein in that solvent and to compare them with those obtained by Hefley.

2. Experimental

a. Apparatus The Varian Model HA-100 Nuclear Magnetic Resonance spectrometer was used. The instrument has a magnet which generates a stable magnetic field of about 23,000 gauss and is provided with an external signal lock unit.

b. Materials The yellow, red, and colorless forms of fluorescein, described previously, were used. The solutions were prepared in reagent-grade dioxane; they were slightly heated to speed up dissolution of the solid.

Tetramethylsilane (TMS) was used as the reference.

3. Results and discussion

The very strong NMR signal around δ = 3-4 p.p.m. owing to the dioxane solvent swamped the spectrum in that region. However, since the peaks of the aromatic protons of interest began to appear at δ = 6 p.p.m. and higher, the solvent interference could be easily neglected.

The NMR spectra of the yellow, red, and colorless forms of fluorescein in dioxane solution were identical, indicating that in dioxane they all exist in the same form, regardless of the form of the starting material. The spectrum, depicted in Figure 30, is very similar to that reported by

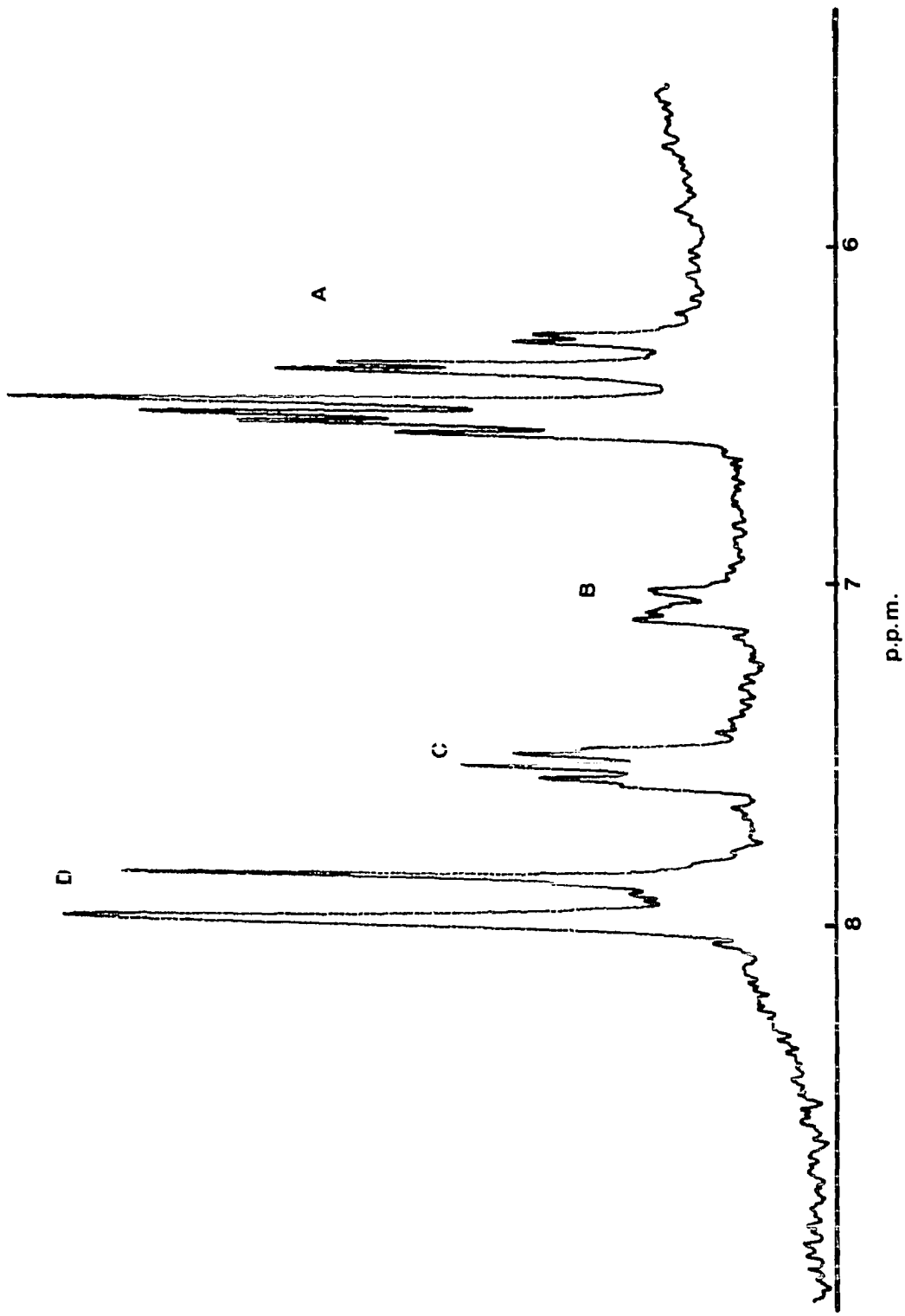
Figure 30. NMR spectrum of fluorescein in dioxane solution

A. Protons (6) at positions 1', 2', 4', 5', 7', and 8' of the xanthene rings

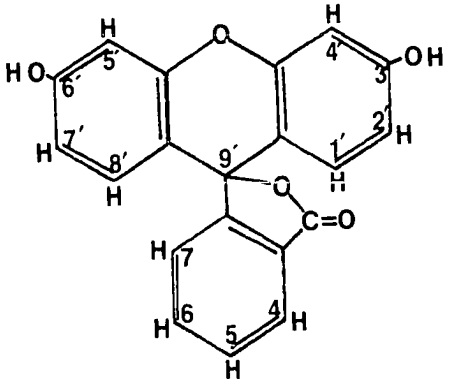
B. Proton (1) at position 7 of the phthalate ring

C. Protons (2) at positions 5 and 6 of the phthalate ring

D. Proton (1) at position 4 of the phthalate ring plus protons (2) of the phenolic groups


A
B
C
D
6
7
8
p.p.m.

Hefley; the one major difference will be discussed below. To facilitate the discussion, the structure of the colorless form of fluorescein (Structure V, p. 19) is reproduced below with numbered positions for the protons on the aromatic rings of the xanthene and the phthalate portions of the molecule.



V. Colorless fluorescein-lactone form in dioxane

The NMR spectrum of fluorescein in dioxane (Figure 30) is characterized by four groups of peaks: a multiplet (A) for 6 protons at about 6.54 p.p.m., a doublet (B) for 1 proton at 7.17 p.p.m., a triplet (C) for 2 protons at about 7.56 p.p.m., and an apparent doublet (D) at 7.86 + 8.00 p.p.m. The multiplet at about 6.54 p.p.m. integrates to 6 protons and is assigned to the protons in the positions 1', 2', 4', 5', 7', and 8' on the xanthene rings. Closer inspection reveals that the multiplet can be resolved into a quartet (for the 1', 2', 7', and 8' protons) and a singlet for the 4' and 5' protons, in agreement with Hefley's data. The doublet at 7.17 p.p.m. is assigned to the 7-proton and the triplet at

7.56 p.p.m. to the two protons in the 5 and 6 positions on the phthalate portion of the molecule, also in agreement with Hefley's observation.

The major difference in the spectra of this work and in those of Hefley's is in the next peak, an apparent doublet, occurring at 7.86 + 8.00 p.p.m. and integrating to three protons. In Hefley's work, the peak occurred at 8.05 p.p.m. and integrated to only one proton, that in the 4-position of the phthalate ring. An additional peak in Hefley's work at 9.83 p.p.m. was assigned to the acidic protons of fluorescein. In this work, no peaks were found that far downfield, indicating that the acidic protons observed by Hefley in dimethylsulfoxide have been altered by using dioxane as solvent.

The following explanation is provided for this observation. In dimethylsulfoxide, fluorescein exists probably as the zwitter ion (Structure VI, p. 19) if not as the quinone form (Structure VII). The two replaceable protons of both of these structures are much more acidic than the two replaceable protons of the lactone form (Structure V), as discussed in the preceding sections. Such acidic protons would appear in the region of 9.83 p.p.m., as observed by Hefley. In the lactone structure, however, since the two replaceable protons are less acidic, the peak in the NMR spectrum should appear closer to the region of normal

phenolic protons, namely 7 - 8 p.p.m.[35,130] Thus the apparent doublet at 7.86 + 8.00 p.p.m. integrating to 3 protons is a fortuitous composite of two peaks, one for the single proton in the 4-position of the phthalate and one for the two phenolic protons which are now much weaker in acid strength and hence have been moved upfield.

The NMR spectra of fluorescein in dioxane have demonstrated two facts, namely, 1) that solutions of fluorescein in dioxane are identical irrespective of whether the yellow, red, or colorless fluorescein is the starting material, and 2) that the fluorescein in dioxane solution exists as the colorless, lactone form.

F. Oxidation-Reduction Reactions of Fluorescein

1. Bromination of fluorescein

a. Experimental A bromination solution was prepared by dissolving 2.7842 g of primary standard potassium bromate plus 15 g of potassium bromide in exactly 1 l. of water. The normality of the solution, based on 6 equivalents per mole of potassium bromate, was 0.1000 N.

A standard solution of potassium iodate was prepared by dissolving 1.7933 g of primary standard potassium iodate in exactly 1 l. of water. The normality of the solution, based on 6 equivalents per mole of potassium iodate, was 0.1005 N.

A standard solution of sodium thiosulfate was prepared by dissolving 25.0 g of sodium thiosulfate pentahydrate plus 0.1 g of sodium carbonate in 1 l. of previously boiled water. Three drops of chloroform were added to the solution to retard spoilage caused by bacterial attack.

The solution of sodium thiosulfate was standardized by titration against the standard potassium bromate and against the standard potassium iodate solutions in the presence of 10 ml of 10 per cent potassium iodide solution, 5 ml of concentrated hydrochloric acid, and 2 ml of 2 per cent starch solution as indicator. The normality of the solution of sodium thiosulfate was 0.1109 N; the value was the same, regardless of which standard solution, potassium bromate or potassium iodate, was used for the standardization.

b. Procedure The procedure was a modified version of B. Smith[132] for the bromination of phenols. A sample of approximately one milliequivalent of yellow fluorescein was dissolved in 25 ml of glacial acetic acid. Then 25.00 ml of the standard 0.1000 N brominating solution and 5 ml concentrated hydrochloric acid were added, the flask was stoppered, and the mixture was stirred with a magnetic bar for a measured time to ensure complete bromination. After completion of the reaction, 10 ml of 10 per cent potassium iodide solution was added, and the liberated iodine was titrated immediately with standard 0.1 N sodium thiosulfate by using 2 ml of 2 per cent starch solution as the indicator.

c. Results and discussion Normal bromination of fluorescein results in the tetrabromo substituted derivative called eosin. Since each bromination site corresponds to two equivalents of bromine, owing to the reaction of Br_2 to produce the organic bromo derivative R-Br and the by-product HBr, the equivalent weight of fluorescein in this bromination reaction is theoretically the molecular weight divided by 8, or 332.3/8 = 41.54.

From the amount of liberated iodine, the amount of bromine consumed in the bromination and hence the equivalent weight of fluorescein was calculated. Incomplete bromination, as indicated by a higher equivalent weight, was mainly caused by insufficient reaction time and was eliminated by increasing the bromination time appropriately.

After bromination of 64.5 mg of fluorescein for 4 minutes, the equivalent weight was calculated as 51.80. To ensure complete bromination, the reaction time was increased to 8 minutes. Bromination of 69.7 mg of fluorescein for that time resulted in a calculated equivalent weight of 41.64, which was very close to the theoretical value of 41.54. Thus fluorescein was quantitatively brominated by a simple procedure which can be used essentially as another measure of the purity of fluorescein.

To establish the identity of the bromination product of fluorescein, the derivative was filtered, dissolved in sodium

hydroxide, precipitated by the addition of hydrochloric acid, washed with water, and air-dried. The melting point was 294-300°C; the literature[106] value for tetrabromofluorescein (eosin) is 295-296.5°C.

An additional identification was provided by preparation of the diacetyl derivative of the bromination product. The product was reacted with acetic anhydride in pyridine to yield a light-cream colored diacetyl of eosin with a melting point of 285-292°C. When recrystallized from benzene, the derivative had a melting point of 290-295°C; the literature[34] value for the diacetyl derivative of eosin is 292-295°C.

2. Direct titration of fluorescein by titanous chloride

a. Experimental The titanous chloride titrant was prepared by dilution of a commercial 20 per cent solution of titanous chloride (Matheson, Coleman & Bell) to approximately 0.1 M. The titrant was kept in a dark, well-stoppered glass bottle without any extraordinary precautions for storage. The solution was used immediately after standardization.

The titanous chloride solution was standardized against electrolytic iron. Samples of iron were dissolved in 5 ml of concentrated hydrochloric acid plus 25 ml water, iron(II) was oxidized to iron(III) by dropwise addition of potassium permanganate solution, the excess potassium permanganate was destroyed by boiling, 100 ml of 5 N sulfuric acid were added, and the solutions were deaerated by bubbling nitrogen through

them. The titrations were carried out in an enclosed vessel with three openings at the top to accommodate the burette tip, the nitrogen nozzle for sweeping the vessel with nitrogen during titrations, and an electrode, if necessary.

Two indicators were used for the titration of ferric iron by titanous chloride. When the end-point was marked by the disappearance of the red color in the presence of potassium thiocyanate, the normality of the titanous chloride solution was 0.1429 N. When the end-point was marked by the disappearance of the blue-green color of methylene blue indicator, the normality was 0.1422 N.

b. Procedure The titration of fluorescein by titanous chloride was attempted by using essentially the procedure of Knecht and Hibbert[79] for the decolorization of Eosin A. The end-point observed here, however, was the disappearance of the green fluorescence under illumination by ultraviolet light. Alternatively, the end-point was followed potentiometrically by using a platinum disk electrode against a saturated calomel reference electrode.

A stock solution of fluorescein was prepared by dissolving 833.1 mg of yellow fluorescein in 250 ml of 50 per cent ethyl alcohol. Aliquots of 25 ml were placed in the titration vessel, 10-20 ml of 20 per cent potassium sodium tartrate was added, 1 ml of 3 M sodium hydroxide was added, and the solution was titrated.

c. Results and discussion Normal reduction of fluorescein should result in the leuco form of the dye, called fluorescin, which is nonfluorescent.

During the course of the titration, the fluorescence of the solution did actually disappear, indicating a possible reduction of fluorescein to fluorescin. However, the transition was so slow, and the results were so erratic and irreproducible that no quantitative measure of the reaction was possible.

An attempt to follow the titration potentiometrically was also unsuccessful. There was no response in the potential of the solution with addition of the titrant.

3. Fluorescin - the reduced form of fluorescein

a. Preparation of fluorescin A mixture of 40 g of commercial fluorescein and 120 g of zinc dust in 500 ml of glacial acetic acid were refluxed for 1 hour with no apparent change in color. After addition of 40 g more of zinc dust, the mixture was immediately decolorized. Addition of 2.5 l. of water produced a white turbidity which began to turn brownish within one hour; however, a precipitate was not formed, even after 5 hours of standing. To prevent air oxidation of fluorescin, nitrogen was bubbled through the solution and then nitrogen was swept over the top of the solution.

The mixture was extracted three times with 200-ml portions of ether, the organic extracts were combined, evaporated partially on a hot plate, and then in a vacuum at room temperature. Addition of water to the remaining organic extract produced a pale yellowish brown precipitate which was filtered, washed, and air dried.

b. The melting point of fluorescin The behavior of the substance on heating was odd. At 110-120°C the color changed to orange, at 134-136°C the crystals foamed and subsequently solidified, and at approximately 245°C the crystals melted to a deep red liquid.

Recrystallization from alcohol-water mixtures and from hot benzene followed by drying at 110-120°C for 2 hours resulted in a yellowish brown compound with a vigorous but relatively sharp melting point at 230-235°C.

The peculiar behavior of fluorescein on heating during the determination of the melting point can be explained probably by differences in the solvents used for recrystallization. Literature values for the melting point are quite varied. Baeyer[7] reported a melting point of 125-127°C. Liebig[91] reported a melting point of 253-254°C when water of crystallization was present; in the presence of ether of solvation, he reported foaming at 130°C and then a melting point at 253-254°C. The latter observations seem most likely to agree with the behavior observed here.

c. Potentiometric acid-base titration of fluorescin

A 438.4-mg sample of fluorescin was dissolved in 25 ml ethyl alcohol plus 50 ml water and titrated potentiometrically with 0.1071 N sodium hydroxide. The titration curve is presented in Figure 31. Another sample, weighing 56.1 mg, was dissolved in 50 ml of acetonitrile, 10 ml of water was added to stabilize the reading of the pH meter, and the mixture was also titrated with sodium hydroxide. The titration curve is presented in Figure 32.

In Figure 31, the well-defined end-point at approximately 13 ml of 0.1071 N sodium hydroxide corresponds to the titration of one hydrogen atom of the carboxylic acid group. The equivalent weight of fluorescin calculated from this end-point is 315, a value not too far off from the theoretical value of 334.3. The mid-point of the titration curve, which is a measure of the strength of the acid dissociation constant of the titrated group, corresponds to approximately 4.5. At first glance this value seems to be in the normal range for hydrogen atoms of carboxylic acid groups of the benzoic acid type. However, keeping in mind that the titration was performed in about 33 per cent ethyl alcohol, and that in ethanolic solutions the apparent pH is higher than in water,[13] the value of 4.5 is too high for an approximate pK value of the first dissociation constant of fluorescin. In 50 per cent ethyl alcohol, the apparent pK

Figure 31. Potentiometric titration of fluorescin in aqueous ethyl alcohol

Fluorescin: 438.4 mg

Solvent: 25 ml ethyl alcohol plus
50 ml water

Titrant: 0.1071 N sodium hydroxide

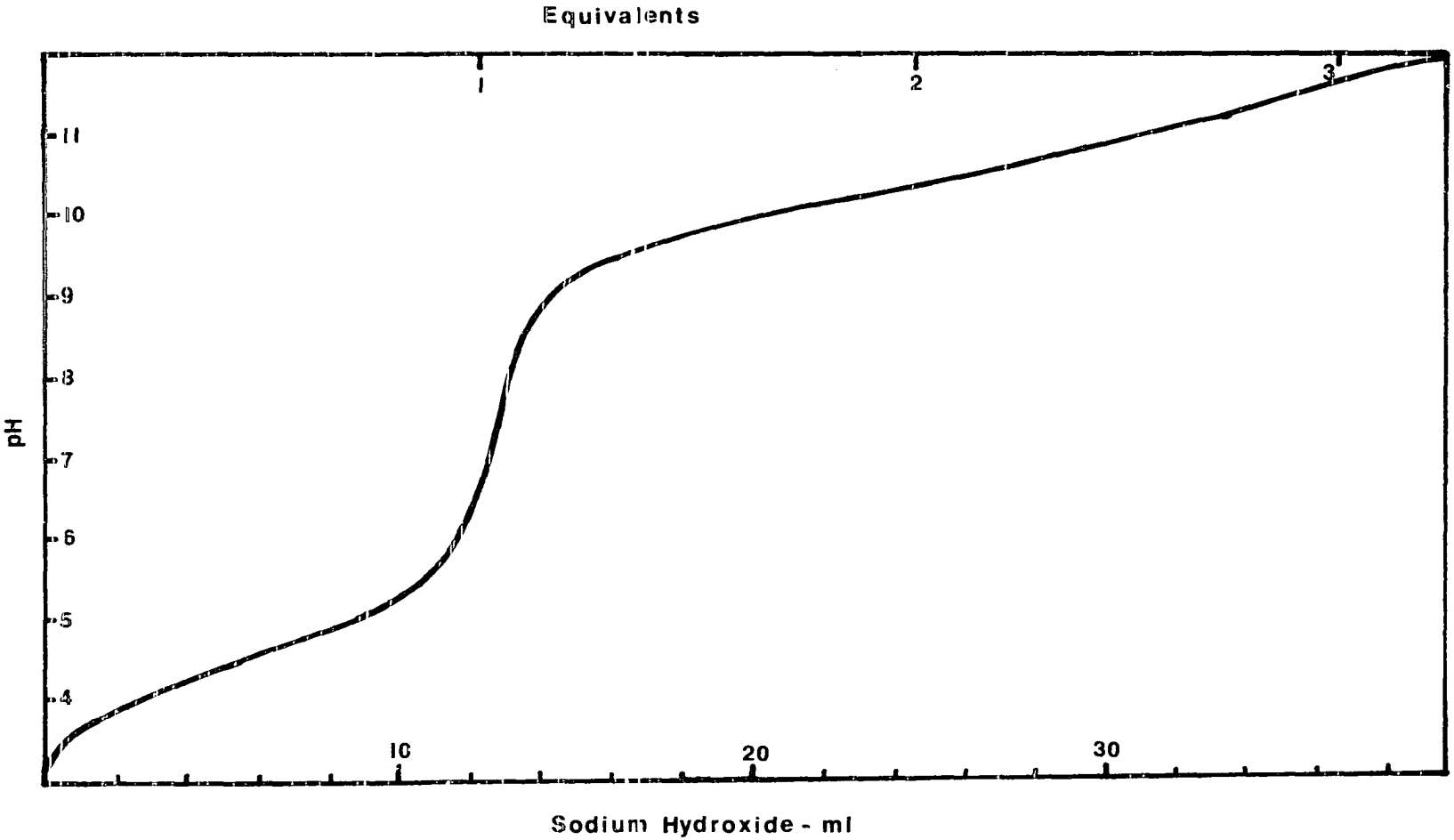
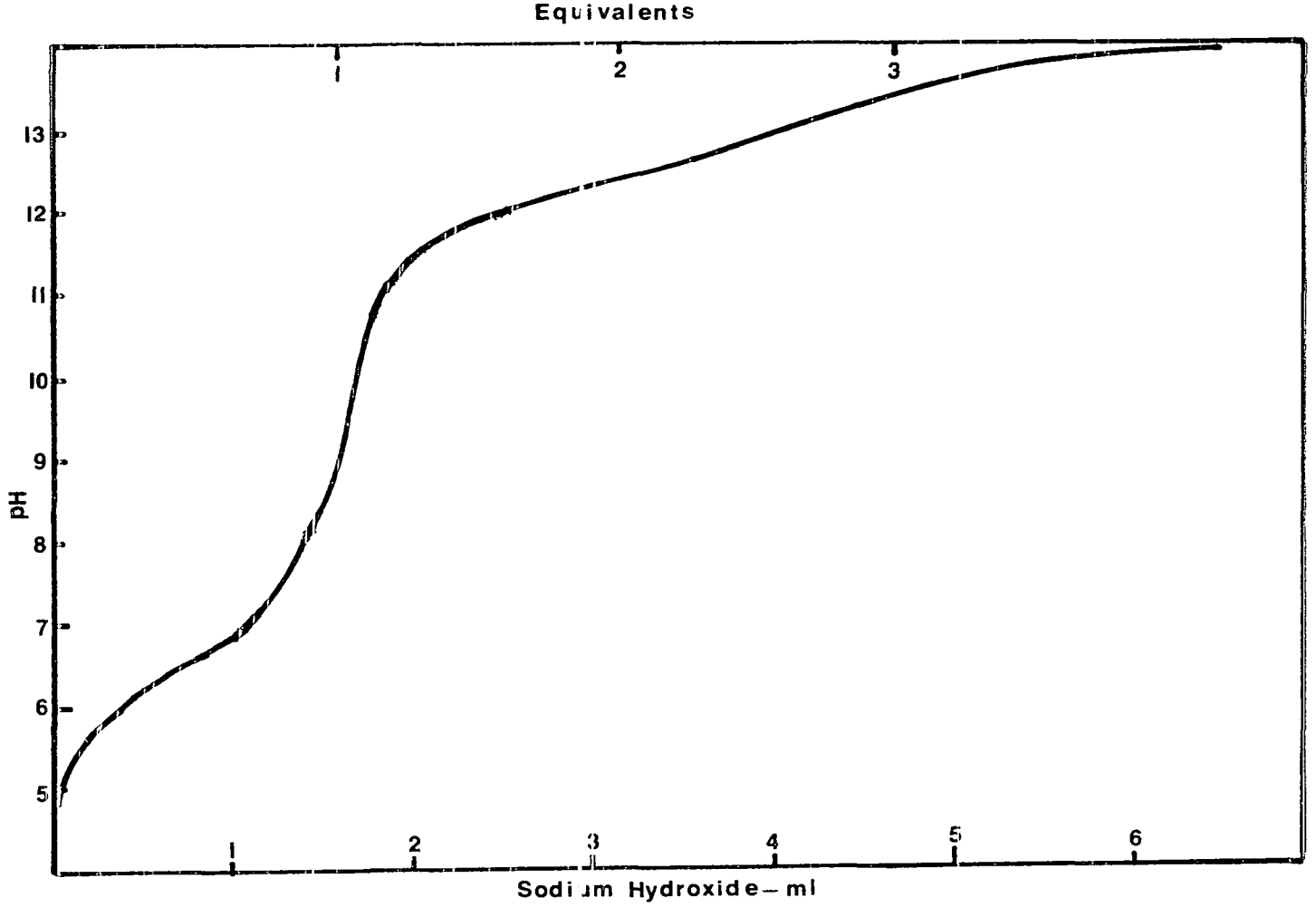

Equivalents
1
2
3
11
10
9
8
7
6
5
4
pH
10
20
30
Sodium Hydroxide - ml

Figure 32. Potentiometric titration of fluorescin in aqueous acetonitrile

Fluorescin: 56.1 mg

Solvent: 50 ml acetonitrile plus
10 ml water

Titrant: 0.1071 N sodium hydroxide


Equivalents
1
2
3
13
12
11
10
9
8
7
6
5
pH
1
2
3
4
5
6
Sodium Hydroxide – ml

values of acids are 1-2 units higher than in water.[23] For example, the pK value of benzoic acid is 5.71[69] in 50 per cent ethyl alcohol, as compared to 4.20 in water.[29] Thus the true pK value of the first dissociation step of fluorescin, corrected for the 33 per cent ethyl alcohol solvent, is probably in the range 3 - 3.5. The increased acidity of the carboxylic acid group over that of normal benzoic acid is probably caused by the effect of ortho substitution,[23] which was already discussed in Section II.D.3.

A second, very minor but still discernible, inflection point can be also seen at approximately 37 ml of base. At this point the two phenolic acid groups have been titrated. The equivalent weight of fluorescin calculated from this point, at which a total of three equivalents have been neutralized, is 110.6, a value pretty close to the theoretical 114.6 for fluorescin with three replaceable hydrogen atoms. Again, at the mid-point between the first and the second inflection point, the pH is approximately 10-10.5. After allowing for the increased apparent pH because of the ethanolic solvent, the pH value becomes approximately 9-9.5, well in the range of the acid strength of hydrogen atoms of the phenolic group.

The titration curve in Figure 32, although obtained in aqueous acetonitrile as solvent, describes the acidic properties of fluorescin in a similar manner. There are

also two end-points, one at one equivalent of replaceable hydrogen atoms from the carboxylic acid group and one at an additional two equivalents from the phenolic groups. The inflection points occur at approximately 1.60 ml and 4.65 ml, respectively. The calculated equivalent weights of fluorescin at these inflection points are 327.4 and 112.6, respectively.

The acid-base titrations of fluorescin in aqueous ethyl alcohol and in the aqueous acetonitrile are interesting in that they clearly indicate replaceable hydrogen atoms of quite different acid strength; one hydrogen atom is from a carboxylic acid group and the two phenolic hydrogen atoms are of conventional acid strength. Because in fluorescin (Structure XVIII, p. 69) the carbon atom in the 9'-position is substituted by four substituents, there are no contributions from resonance structures or conjugation systems. The two types of replaceable hydrogen atoms are quite distinct; they are also quite different from the hydrogen atoms of fluorescein itself in which the acid strength was increased because of the resonance-stabilized positive charge on the xanthene portion of the zwitter ion molecule. The rather normal acidic properties of fluorescin are in sharp contrast to those of fluorescein and help to emphasize the unusual nature of the replaceable hydrogen atoms of the original fluorescein molecule in solution, as discussed in Section II.D.3.

III. CALCEIN

A. Preparation and Characterization of Calcein

1. Preparation of Calcein

The synthesis of Calcein, 3',6'-dihydroxy-4',5'-bis[N,N'-bis(carboxymethyl)aminomethyl]fluoran, as developed by Hefley,[57] was further modified to improve the purity of the obtained product by introducing the process of freeze-drying in the final stages of isolating the compound. Because freeze-drying is carried out at a low temperature, the probability of decomposition of Calcein is decreased.

Pure Calcein was prepared by the Mannich condensation of fluorescein, iminodiacetic acid, and formaldehyde at 60-70°C using glacial acetic acid as the solvent. Both the free iminodiacetic acid (Dow Chemical Company, Midland, Michigan) and the disodium salt monohydrate (Eastman Organic Chemicals, Rochester, New York) could be used.

A sample of 30 g (0.09 moles) of yellow fluorescein, previously purified by the acetylation procedure described in the first part of this thesis, was blended with either 39.6 g of the disodium salt of the iminodiacetic acid or with 26.6 g of the free acid (0.02 moles). The mixture was dissolved in 400 ml of glacial acetic acid and poured into a 500-ml round-bottom, three-necked flask outfitted with a thermometer, a dropping flask, and a reflux condenser in each of the necks. The mixture was stirred with a magnetic

stirring bar while the temperature was maintained at 60-70°C by a heating mantle surrounding the flask. Under constant stirring, 22 ml (0.29 moles) of a 37 per cent solution of reagent-grade formaldehyde was added dropwise to the hot mixture. The mixture was stirred and kept at 60-70°C for at least 2-6 hours. The hot solution was then filtered, and the filtrate was poured into 4 l. of deionized water.

Addition of 1 M hydrochloric acid caused the precipitation of yellow-orange Calcein which was then filtered, slurried several times with deionized water, and filtered again by suction. The precipitated Calcein was purified twice by suspending the mass in 1 l. of deionized water, adding 1 M hydrochloric acid until the Calcein was dissolved, and then precipitating again by careful addition (dropwise) of 1 M sodium hydroxide to a pH of approximately 2.4. It was at this stage of the synthesis at which the losses of the final product were rather large.

The precipitate was slurried many times and washed generously with large amounts of deionized water. After filtration by suction, the compound was air dried for several hours, then freeze-dried, and finally dried at 80°C in a vacuum oven. The dried product was a deeply yellow-orange powder. It did not melt below 300°C, but appreciable charring and decomposition occurred at that temperature. The compound was stored in an evacuated desiccator over anhydrous magnesium perchlorate.

2. Characterization of Calcein

a. Elemental analysis The compound was submitted for analysis for carbon, hydrogen, and nitrogen by Huffman Microanalytical Laboratories (Wheatridge, Colorado). The results were 57.66 per cent C, 4.20 per cent H, and 4.07 per cent N. Calculated for Calcein, $C_{30}H_{26}N_2O_{13}$: 57.88 per cent C, 4.21 per cent H, and 4.50 per cent N. For Calcein with 1 water of crystallization, $C_{30}H_{26}N_2O_{13}\cdot H_2O$: 56.25 per cent C, 4.41 per cent H, and 4.37 per cent N. In view of the somewhat divergent results of elemental analysis and of the results of determination of equivalent weight by titration with alkali, the sample was resubmitted for analysis (to R. A. Chalmers, Aberdeen, Scotland). The results were 57.0 and 57.1 per cent C, 4.4 and 4.4 per cent H, and 4.7 per cent N.

b. Equivalent weight by titration with alkali Samples of Calcein (approximately 200 mg) were weighed by difference, dissolved in water or in water-alcohol mixtures, and titrated with tenth normal sodium hydroxide using a potentiometric end-point indication with a glass electrode vs s.c.e. The use of alcohol-water mixtures facilitated the dissolution of the samples, which otherwise (in plain water) did not dissolve completely until just before the end point. The results are presented in Table 10.

Table 10. Determination of the equivalent weight of Calcein by potentiometric titration with alkali

Weight of Calcein	ml of sodium hydroxide	Normality of sodium hydroxide	Equivalent weight of Calcein
0.2284 g	13.23	0.1079	160.0
0.2024 g	11.65	0.1079	161.0
0.2470 g	14.50	0.1079	157.9
0.2221 g	12.93	0.1071	160.4
0.2565 g	14.95	0.1071	160.2
		Average equivalent weight	159.9

The theoretical equivalent weight calculated for Calcein with 1 water of crystallization and 4 titratable hydrogen atoms is 160.1, in very good agreement with the average experimental value of 159.9 in Table 10.

c. Loss of weight on drying and ignition A sample of Calcein weighing 0.3220 g was ignited in a platinum crucible (previously ignited to constant weight) until completely combusted and then until constant weight was attained. The gain in weight of the crucible was 0.0002 g. The increase in weight is insignificant, indicating no residue; hence no metal impurities were present in the sample.

Attempts to remove all the water of crystallization by exhaustive drying at 80°C in vacuum were unsuccessful. A

sample of Calcein weighing 0.5208 g lost only 0.0075 g after such drying for three days. The sample (0.8131 mmoles of Calcein, calculated on the basis of 640.5 as the molecular weight for the monohydrate) lost 0.416 mmoles of water. It is thus obvious that Calcein retains water of crystallization tenaciously. During weighing procedures, it was also observed that exposure to air caused significant increase in the weight of the Calcein being weighed, apparently owing to absorption of moisture from the air.

3. Mass spectrometric study

a. Apparatus and procedure The mass spectrum for Calcein was obtained by using the Atlas CH4 Mass Spectrometer. The ionization energy was 70 eV. Because of very low volatility of the compound, the setting for maximum heating (260 units) was used in order to get a mass spectrum.

b. Results and discussion The mass spectrum of Calcein is very complex, probably owing to the high heat that was necessary to volatilize the compound. Because of the very likely decomposition of Calcein during volatilization, no parent peak is observed at 622 m/e to correspond to the molecular weight of 622.5 for $C_{30}H_{26}N_2O_{13}$. The first recordable peaks appear as a very faint sequence beginning at about 326 m/e and continuing in a typical degradation pattern of 14-m/e intervals through 312, 298, 284, 270, and 256 m/e (the last peak being very weak). The high heat

input can account for the lack of peaks above 326. The four carboxylic acid groups from the two methyleneiminodiacetic acid substituents and the carboxylate from the phthalate portion account for a mass loss of 224 mass units (4x45 + 44), from 622 down to 398. The two $CH_2N(CH_2)_2$ residues still attached to the molecule can be degraded by losses of N and CH_2, 14 mass units each. The loss of four groups of 14 mass units plus one oxygen atom or OH group (typical degradation for fluorescein) can account for a weight loss of 4x14 + 16, or 72 mass units, giving rise to the very faint group of peaks around 326 m/e (398-72). The observed sequence is then a pattern of subsequent losses of 14 mass units, corresponding to the loss of the remaining CH_2 and N groups.

The next significant degradation sequence appears at around 210, 196, and 182 m/e. The loss of the two methyleneiminodiacetic acid groups (2x146 mass units) and the phthalate portion, that is, a phenyl ring plus the carboxylate substituent (76 + 44 mass units) decreases the mass of the remaining fragment to 210 mass units, corresponding to the peak around 210 m/e. Presumably, this fragment is then degraded in a complicated pattern, and the mass spectrum below 186 m/e is too complex for analysis.

From the mass spectrum of Calcein it is evident that the molecule is extremely nonvolatile, in agreement with the characteristics of its zwitter-ion nature. The high heat

input necessary to produce enough volatilized compound for ionization results in degradation of the molecule so that the interpretation of the obtained mass spectrum has little practical utility.

4. Quantitative bromination of Calcein

a. Experimental The procedure for the quantitative bromination of Calcein is described in the section on bromination of fluorescein (p. 180). The reaction time and the amount of glacial acetic acid used as solvent were varied to obtain optimum bromination yields. Best results were obtained when 50 ml of glacial acetic acid were used as solvent for a 100-mg sample; the reaction time was not critical, 4 minutes being sufficient for quantitative bromination.

b. Results and discussion By using the procedure described previously (Section II.F.1.), a 94.0-mg sample of Calcein was brominated quantitatively within 4 minutes. The calculated equivalent weight (for consumption of 8 equivalents of bromine) was 78.20; the theoretical equivalent weight for Calcein monohydrate is 640.5/8 or 80.06.

The bromination product was isolated, washed, and identified as eosin, the tetrabromo derivative of fluorescein, by melting point (m.p. 300°C, literature value 295-296.5°C);[106] a mixed melting point with eosin obtained from fluorescein (Section II.F.1.) did not exhibit any noticeable depression.

In addition, potentiometric titration with alkali was used on the bromo derivative to obtain the equivalent weight of 329; the theoretical equivalent weight for eosin is 324.

It was surprising to discover that the two substitutent methyleneiminodiacetic acid groups are completely and so easily removed by bromination to yield eosin as the bromination product. Apparently, such facility in leaving of the substituents may be responsible for the relative instability of Calcein in alkaline solution upon standing for some time.

5. Nuclear magnetic resonance spectroscopy of Calcein in dimethylsulfoxide

a. Introduction Although the major portion of Hefley's thesis[58] was dealing with the determination of the structure of Calcein by using NMR spectroscopy, the spectra were obtained on a 60-megacycle instrument which did not have enough resolution to obtain accurate coupling constants that were expected for the 1',2' and 7',8' hydrogen atoms on the xanthene portion of the Calcein molecule. The NMR spectra for Calcein and for 4',5'-dimethylfluorescein have been repeated now on a 100-megacycle instrument.

b. Experimental The Varian Associates HA-100 NMR spectrometer was used.

The materials were the Calcein and 4',5'-dimethylfluorescein, purified as described earlier.

The solvent was deuterated dimethylsulfoxide, d_6-DMSO. The reference point was established by using tetramethylsilane (TMS).

c. Results and discussion The NMR spectra of Calcein and of 4',5'-dimethylfluorescein are reproduced in Figures 33 and 34, respectively. A detailed discussion of the assignment of NMR peaks to the different protons is given in Hefley's thesis.[58] Therefore, in this work the descriptions will be merely cursory. For the sake of comparison, the NMR spectrum of 4',5'-dimethylfluorescein (Figure 34) will be discussed first.

The spectrum of 4',5'-dimethylfluorescein is characterized by a singlet at 2.33 p.p.m., corresponding to the six methyl protons, and a beautiful A_2B_2 and $A'B_2'C'$ pattern in the aromatic hydrogen atom region, corresponding to an additional eight protons. The A_2B_2 pattern is centered at 6.54 p.p.m.; it corresponds to the four protons in the positions 1', 2', 7', and 8' on the xanthene portion of the molecule; the coupling constants are 9 Hz, typical for ortho hydrogen atoms. The peaks are in the same location as those of unsubstituted fluorescein (Figure 30, p. 176), except that the original multiplet has been reduced to a quartet and the number of protons has been decreased from six to four, indicating substitution of two protons, namely those at positions 4' and 5'.

Figure 33. NMR spectrum of Calcein in d_6-dimethylsulfoxide

A. Protons (8) of the four methylene groups of the diacetate functions

B. Protons (4) of the two linking methylene groups

C. Protons (4) at positions 1', 2', 7', and 8' of the xanthene rings

D. Proton (1) at position 7 of the phthalate ring

E. Protons (2) at positions 5 and 6 of the phthalate ring

F. Proton (1) at position 4 of the phthalate ring

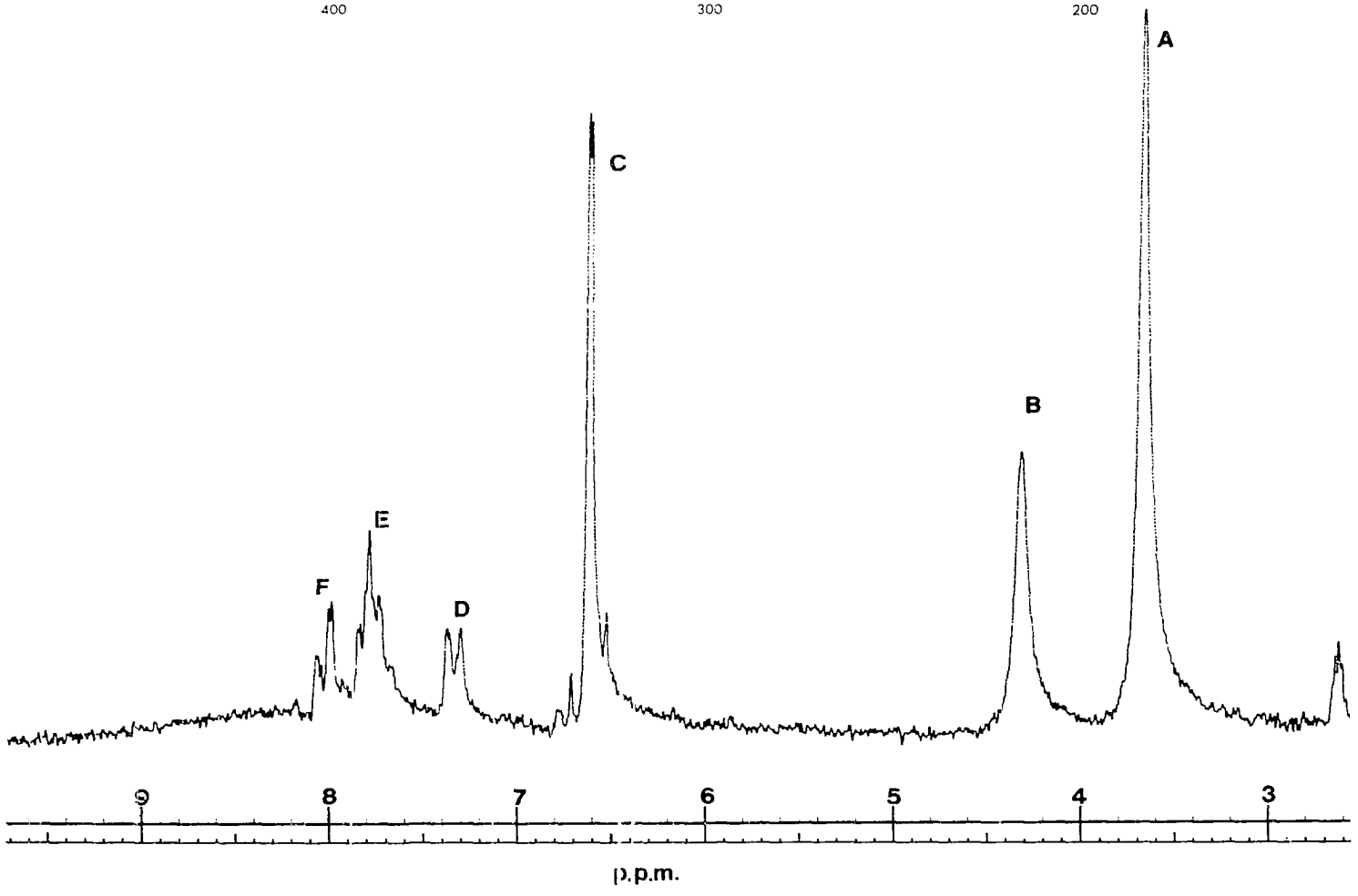

400
300
200
A
B
C
D
E
F
9
8
7
6
5
4
3
p.p.m.

Figure 34. NMR spectrum of 4',5'-dimethylfluorescein in d_6-dimethylsulfoxide

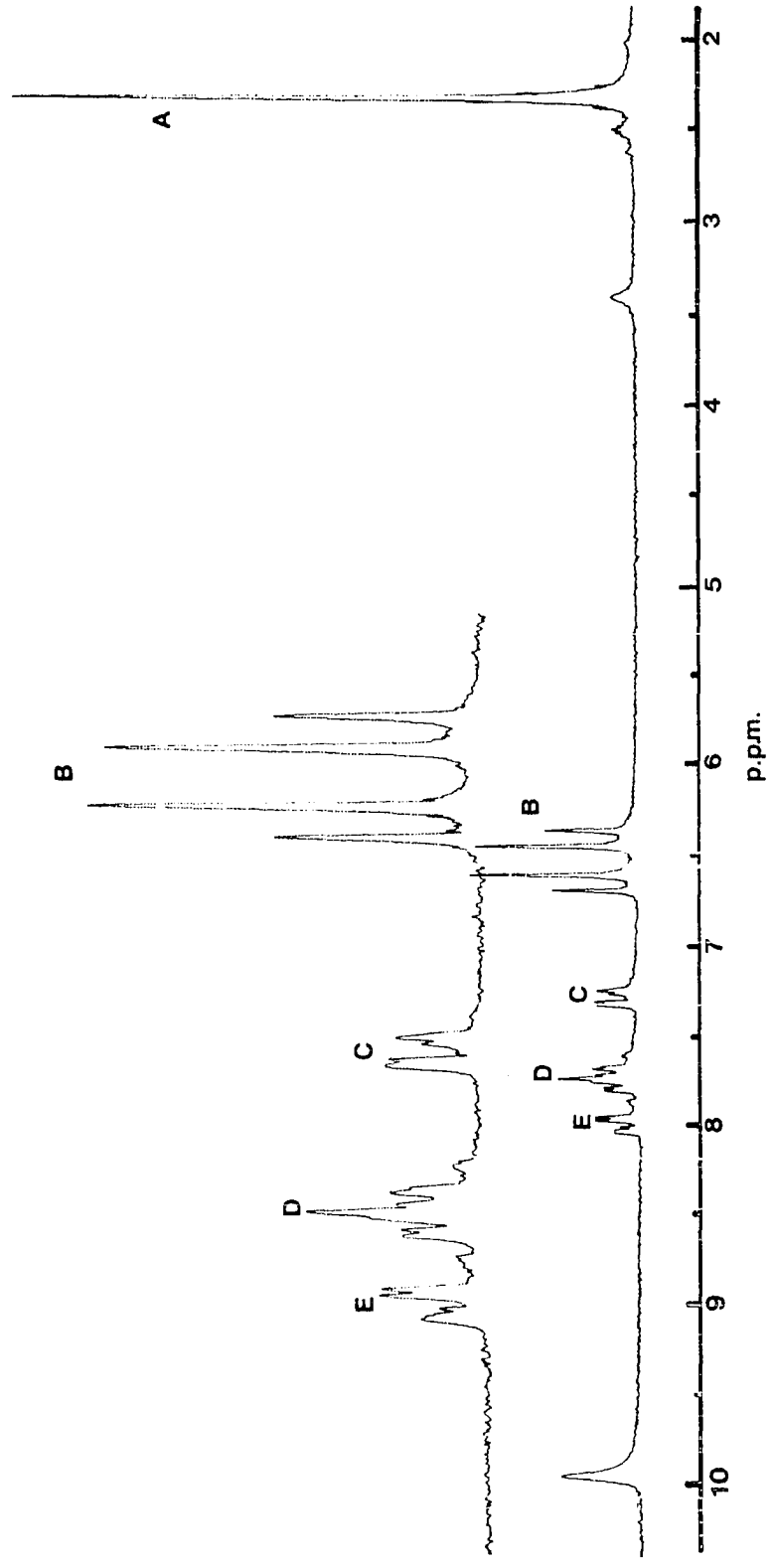
A. Protons (6) of the two methyl groups

B. Protons (4) at positions 1', 2', 7', and 8' of the xanthene rings

C. Proton (1) at position 7 of the phthalate ring

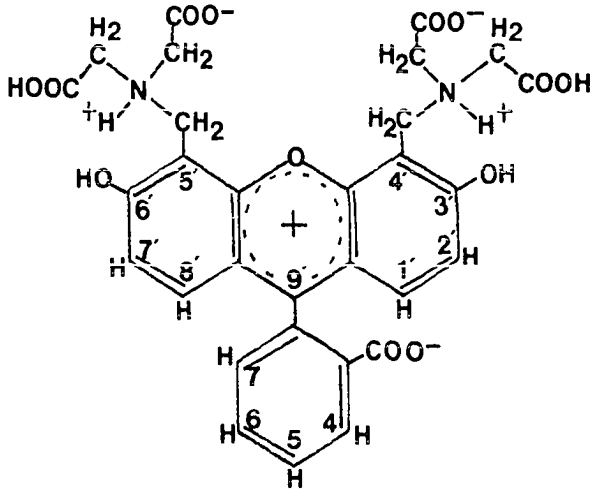
D. Protons (2) at positions 5 and 6 of the phthalate ring

E. Proton (1) at position 4 of the phthalate ring


A
B
B
C
C
D
D
E
E
2
3
4
5
6
7
8
9
10
p.p.m.

The four protons of the phthalate portion of the molecule give rise to the A'B_2'C' pattern, which is very similar to that of the unsubstituted fluorescein molecule in Figure 30. There is a doublet at 7.29 p.p.m., a triplet at 7.74 p.p.m., and a doublet at 8.00 p.p.m. The number and positions of protons (one at 7, two at 5 and 6, and one at 4, respectively) are very close to those of the unsubstituted fluorescein and, of course, to those of Calcein, discussed below. Coupling constants of 8-9 Hz for the ortho protons and of 2 Hz for the meta protons are evident.

To facilitate the discussion of the NMR spectrum of Calcein (Figure 33), the proposed structure of Calcein (Structure IV, p. 10) is repeated here, with the positions of the protons on the aromatic rings numbered. The NMR spectrum



IV. Calcein

of Calcein is very similar to that of the 4',5'-dimethylfluorescein, indicating that the substitution pattern must be very similar also. In the spectrum of Calcein, two additional peaks can be seen, a singlet at 3.57 p.p.m. corresponding to the eight protons of the methylene groups of the diacetate functions, and a singlet at 4.23 p.p.m., corresponding to the four protons of the two methylene groups linking the iminodiacetic functions to the fluorescein molecule. An A_2B_2 pattern around 6.57 p.p.m. corresponds to the four protons in the positions 1', 2', 7', and 8' of the xanthene rings, indicating that substitution has occurred in the positions 4' and 5', just like in 4',5'-dimethylfluorescein. The coupling constants are about 6 Hz, confirming the ortho location of the remaining protons. The protons on the phthalate portion of the molecule give rise to the same $A'B_2'C'$ pattern as those on the phthalate portion of the 4',5'-dimethylfluorescein molecule: a doublet at 7.28 p.p.m. corresponding to the proton at position 7, a triplet at 7.75 p.p.m. corresponding to the two protons at positions 5 and 6, and another doublet at 8.00 p.p.m. corresponding to the proton at position 4.

There is no other substitution pattern except that of the proposed structure of Calcein, with substituents in the positions 4' and 5', that can give the kind of spectrum presented in Figure 33. The multiplet of the six protons

in the unsubstituted fluorescein, seen in Figure 30, p. 176, is reduced to a symmetrical quartet for the remaining four protons. The position, symmetry, and splitting pattern are similar to those of 4',5'-dimethylfluorescein, indicating that both Calcein and 4',5'-dimethylfluorescein must be substituted in the same positions.

B. Potentiometric Acid-Base Titration of Calcein

1. Introduction

In the work of Hefley,[58] it has been elegantly demonstrated that Calcein contains six replaceable hydrogen atoms. Four of the hydrogen atoms are titratable by sodium hydroxide; the remaining two hydrogen atoms are removed as a result of chelation of two calcium ions by Calcein. During the titration of Calcein in water as solvent, insolubility of the compound until almost the attainment of the end-point and perhaps also the levelling effect of water on the relative acidity of the hydrogen atoms results in a single break in the titration curve, corresponding to four replaceable hydrogen atoms.

In this work, the low solubility of Calcein was overcome by performing the alkalimetric titrations in 50 per cent ethyl alcohol as the solvent. Besides improving the dissolution of the compound, use of this solvent permitted a more

detailed description of the titration curve, owing to the appearance of two breaks which correspond to two and to four hydrogen atoms, respectively, being titrated.

The increased details in the titration curves permit also a comparison of the acid-base behavior of Calcein with that of fluorescein and iminodiacetic acid. After all, Calcein is essentially fluorescein with two iminodiacetic acid groups attached to it by means of methylene group linkages. The acid-base behavior should therefore be a composite of the two constituents. The correspondence of the acid strengths of the various hydrogen atoms of Calcein with those of fluorescein and iminodiacetic acid is additional proof and confirmation in assigning the appropriate acid dissociation constants to the different functional groups.

This assignment of acid strengths to the various hydrogen atoms provides a basis for the description of the structure of Calcein. Specifically, the major assumption of Hefley, that the fundamental structure of Calcein is the lactone form and that the lactone ring does not open at any pH value of the solution, is refuted.

2. Experimental

a. Calcein The Calcein was the highly pure compound prepared and characterized as described previously. Solid Calcein was weighed quickly to avoid absorption of atmospheric

moisture, was dissolved in the appropriate solvent, and was titrated immediately to avoid any possible decomposition while in solution.

b. Iminodiacetic acid The acid was obtained in the free, unhydrated form from Dow Chemical Company, Midland, Michigan. Potentiometric titration of the acid, straight from the bottle, indicated a purity of 99.35 per cent. When dried for one hour at 110°C, the pure white crystals of the acid became somewhat grayish, and the purity, as determined by alkalimetric titration, dropped to 99.14 per cent.

c. Apparatus The course of the titrations was followed by using a Corning Model 10 pH meter outfitted with a high-alkalinity Beckman glass electrode and a saturated calomel electrode as the reference electrode. The pH meter was standardized by using at least two buffer solutions prepared according to NBS specifications. The titrations were performed in open beakers with free access to air.

d. Procedure The acid was weighed out into a beaker, the appropriate solvent was added, and the mixture was stirred with a magnetic stirring bar to effect dissolution. In the case of Calcein in water, dissolution was never complete until almost the attainment of the end-point. After the addition of each increment of the sodium hydroxide solution, the pH was recorded as soon as the needle of the pH meter came to rest.

3. Results and discussion

a. Titration of Calcein in water In Figure 35 the titration of Calcein in water is presented. At the end-point, occurring at 13.23 ml of sodium hydroxide, four hydrogen atoms have been replaced. The titration curve, however, is not useful for interpretation of the acidic nature of Calcein because the compound is not completely dissolved until approximately 12 ml of titrant have been added. Indeed, in the region between the addition of 4 and 9 ml of sodium hydroxide, the attainment of equilibrium was very slow. After each addition of titrant, the pH would jump up and then slowly drift down, indicating dissolution of more compound.

Although the low solubility prevents a quantitative evaluation of the titration curve, the data can be qualitatively compared with the acid dissociation constants. The equations for the stepwise dissociation of Calcein, H_6Cal, and the definitions for the pertinent acid dissociation constants are as follows:

$$H_6Cal = H_5Cal^- + H^+ \qquad K_1 = \frac{[H^+][H_5Cal^-]}{[H_6Cal]}$$

$$H_5Cal^- = H_4Cal^{2-} + H^+ \qquad K_2 = \frac{[H^+][H_4Cal^{2-}]}{[H_5Cal^-]}$$

$$H_4Cal^{2-} = H_3Cal^{3-} + H^+ \qquad K_3 = \frac{[H^+][H_3Cal^{3-}]}{[H_4Cal^{2-}]}$$

$$H_3Cal^{3-} = H_2Cal^{4-} + H^+ \qquad K_4 = \frac{[H^+][H_2Cal^{2-}]}{[H_3Cal^{3-}]}$$

$$H_2Cal^{4-} = HCal^{5-} + H^+ \qquad K_5 = \frac{[H^+][HCal^{5-}]}{[H_2Cal^{4-}]}$$

$$HCal^{5-} = Cal^{6-} + H^+ \qquad K_6 = \frac{[H^+][Cal^{6-}]}{[HCal^{5-}]}$$

The corresponding pK values, determined by Hefley,[58] are:

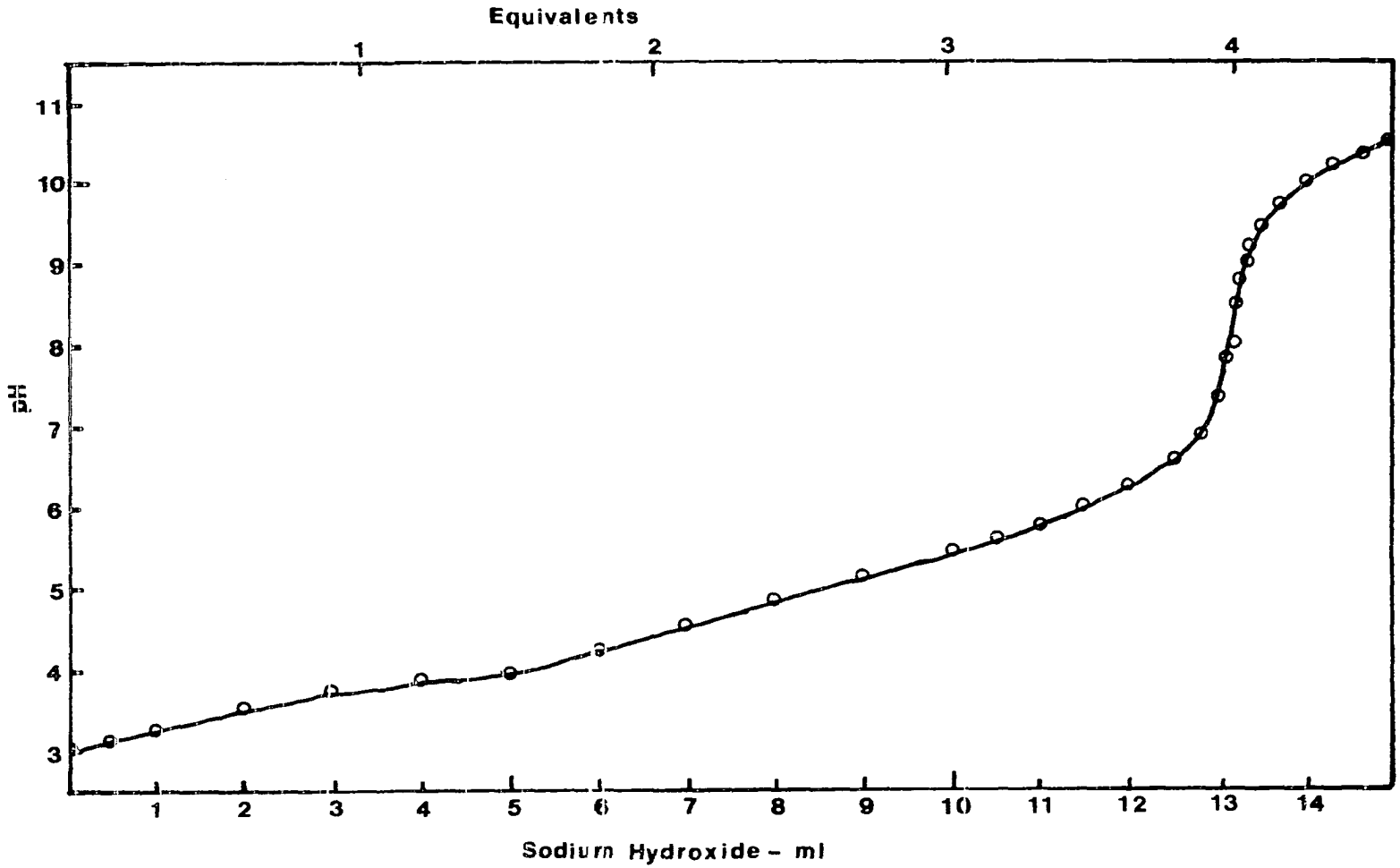
$$pK_1 = 2.74$$
$$pK_2 = 3.53$$
$$pK_3 = 4.58$$
$$pK_4 = 6.19$$
$$pK_5 = 9.88$$
$$pK_6 = 11.64$$

Elementary treatment of acid-base equilibria indicates that the mid-point of the titration curve of a tetrabasic acid with all four hydrogen atoms being titrated in a single step should occur at a pH value corresponding to the sum of the pK values divided by 4. If Calcein is considered as a tetrabasic acid with the pK values of the first four dissociation steps 2.74, 3.53, 4.58, and 6.19, then the calculated value for the mid-point of the titration curve is 4.26; in Figure 35, the value taken from the experimental

Figure 35. Potentiometric titration of Calcein in water

Calcein: 228.4 mg

Titrant: 0.1079 N sodium hydroxide


Equivalents
1
2
3
4
11
10
9
8
7
6
5
4
3
pH
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Sodium Hydroxide - ml

titration curve is 4.40. This is a remarkably good agreement considering the qualitative approach.

b. Titration of Calcein in 50 per cent ethyl alcohol

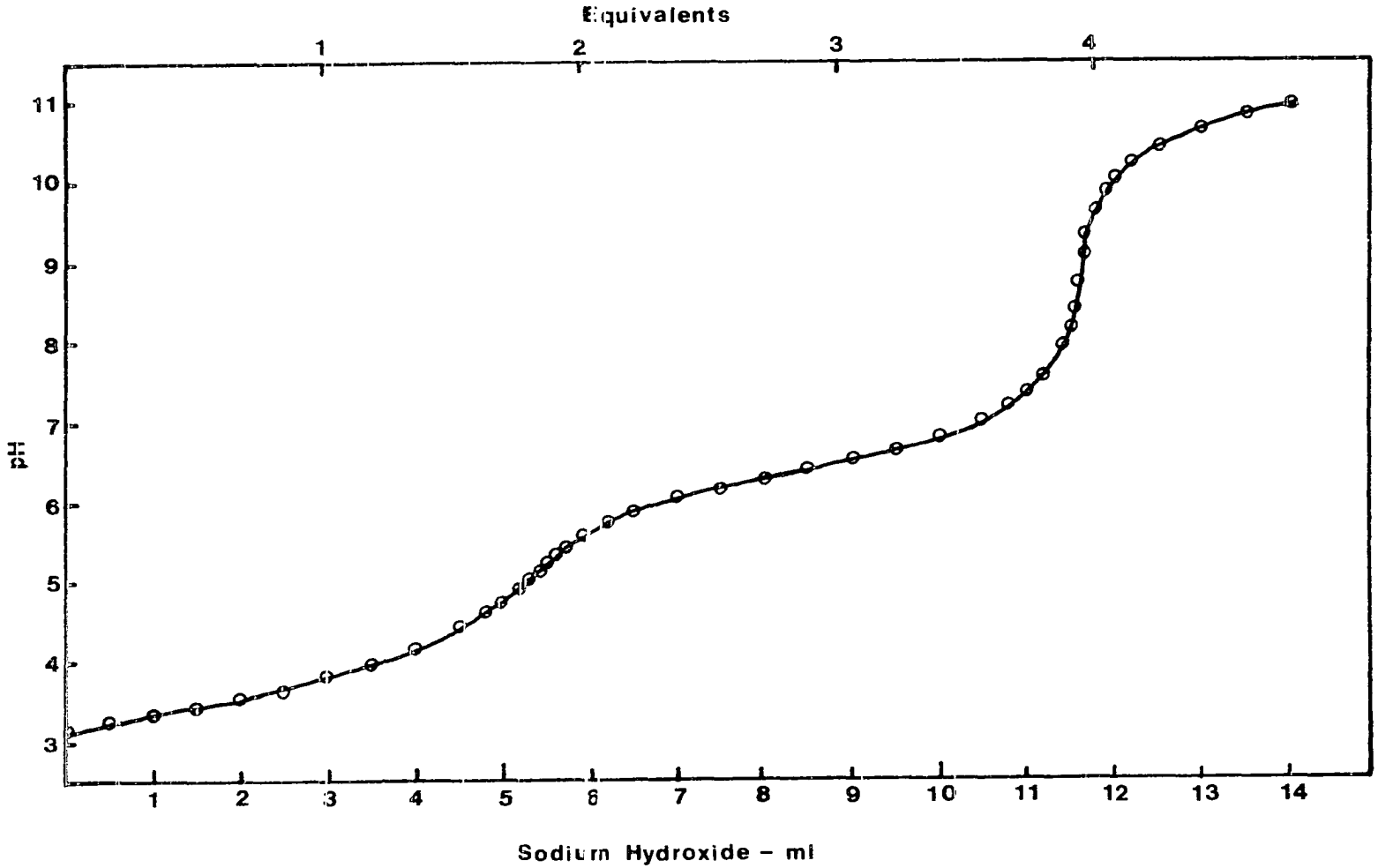
An interesting development is observed when Calcein is titrated by sodium hydroxide in 50 per cent ethyl alcohol as solvent (Figure 36). First of all, two breaks instead of one are observed in the titration curve. Furthermore, the first end-point occurs almost at one-half the value of the second end-point (5.60 and 11.65 ml of sodium hydroxide, respectively). Whereas in water all four replaceable hydrogen atoms cannot be differentiated, the use of 50 per cent ethyl alcohol as solvent allows the distinction between two groups of two hydrogen atoms with different acid strengths.

By analogous elementary treatment of Calcein as a dibasic acid up to the first end-point and as a tetrabasic acid with two hydrogen atoms already removed at the first end-point, it can be shown that the mid-point of the titration curve up to the first end-point should correspond to the sum of pK_1 and pK_2 divided by 2, while the mid-point between the first and the second end-points should correspond to the sum of pK_3 and pK_4 divided by 2. Indeed, at the first mid-point (at 2.80 ml) and at the second mid-point (at 8.60 ml of sodium hydroxide added), the pH values from the titration curve are 3.75 and 6.45, respectively; the calculated values are 3.14 and 5.39, respectively. The

Figure 36. Potentiometric titration of Calcein in 50 per cent ethyl alcohol

Calcein: 202.4 mg

Titrant: 0.1079 N sodium hydroxide


Equivalents
1
2
3
4
11
10
9
8
7
6
5
4
3
pH
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Sodium Hydroxide – ml

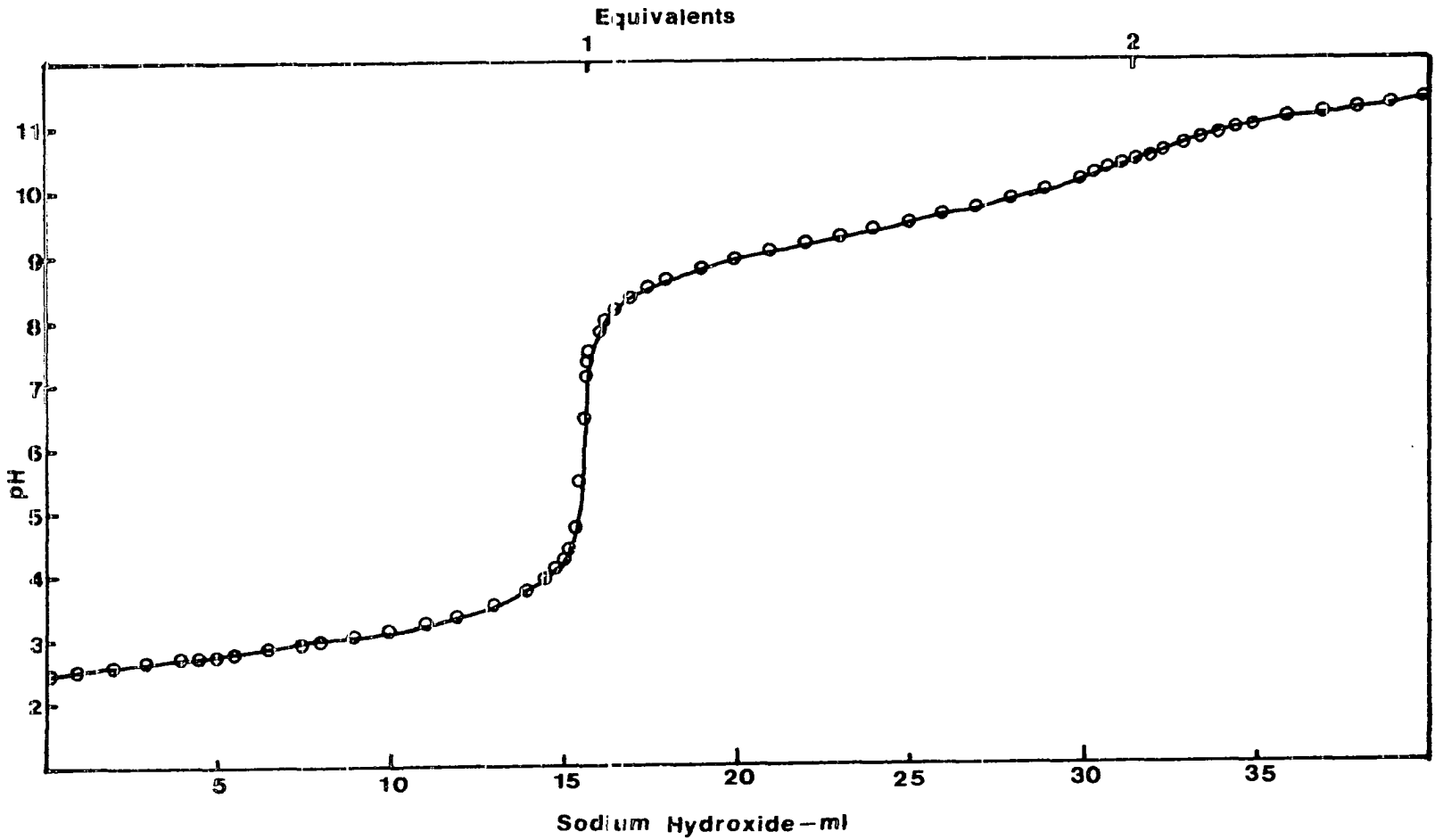
higher values of the experimental titration curve can be attributed to the fact that the titrations are carried out in 50 per cent ethyl alcohol, whereas the pK values were determined in pure water. The increase in pH while going from water to 50 per cent ethyl alcohol is reasonable, since water is more acidic than ethyl alcohol. Such behavior of acidic properties in going from water to alcohol as solvent is discussed in an excellent fashion by Bates.[13]

c. Titration of iminodiacetic acid In Figure 37, the titration of free iminodiacetic acid in water by 0.1158 N sodium hydroxide is shown. A sharp, clearly defined break at 15.64 ml of base corresponds to one titrated hydrogen atom; a second break, though ill-defined and drawn out, can be seen at approximately 31.3 ml of base, corresponding to the titration of the second hydrogen atom. The mid-point in the titration curve up to the first end-point, occurring at 7.82 ml and pH 2.98, corresponds to pK_1 of iminodiacetic acid. The mid-point between the first and the second end-points, occurring at 23.46 ml and pH 9.35, corresponds to pK_2. The two values of pK_1 and pK_2 obtained for iminodiacetic acid are in good agreement with the literature values of 2.98 and 9.89.[27] The first pK value corresponds to the neutralization of a proton from a carboxylic acid group, and the second pK value is attributed to the neutralization of an ammonium-type proton.

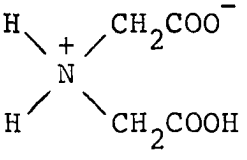
Figure 37. Potentiometric titration of iminodiacetic acid in water

Iminodiacetic acid: 0.2430 g

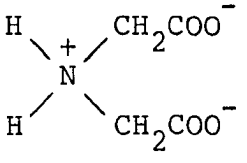
Titrant: 0.1158 N sodium hydroxide


Equivalents
1
2
pH
11
10
9
8
7
6
5
4
3
2
5
10
15
20
25
30
35
Sodium Hydroxide—ml

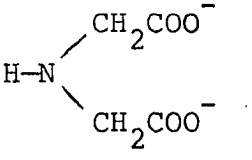
The two pK values, 2.98 and 9.35, obtained for iminodiacetic acid do not fall in the range of acid dissociation constants for carboxylic acids which usually have pK values of the order 4 to 5. The structures for the free iminodiacetic acid (H_2Imda), the monoanion ($HImda^-$), and the dianion ($Imda^{2-}$) which are in accordance with the pK values obtained are XXX, XXXI, and XXXII, respectively. The zwitter



XXX. H_2Imda



XXXI. $HImda^-$



XXXII. $Imda^{2-}$

ion nature of the free iminodiacetic acid (Structure XXX) explains the rather strong acid character of the first hydrogen atom. The presence of the positive charge on the near-by nitrogen atom accounts for the increased acid strength of the carboxylic acid group ($pK_1 = 2.98$). The removal of the proton from the ammonium-type nitrogen atom in the monoanion (Structure XXXI) to yield the doubly charged anion (Structure XXXII) accounts for the decreased acid strength of the second hydrogen atom ($pK_2 = 9.35$). Such divergent acid strengths of apparently similar carboxylic acid groups are well-known for poly(aminocarboxylic acids) which exist as zwitter ions. When protons from the carboxyl

groups are transferred to neighboring nitrogen groups, the acidity of protons remaining on the additional carboxyl groups is increased whereas the protons associated with the ammonium-type nitrogen atom become weaker acids.

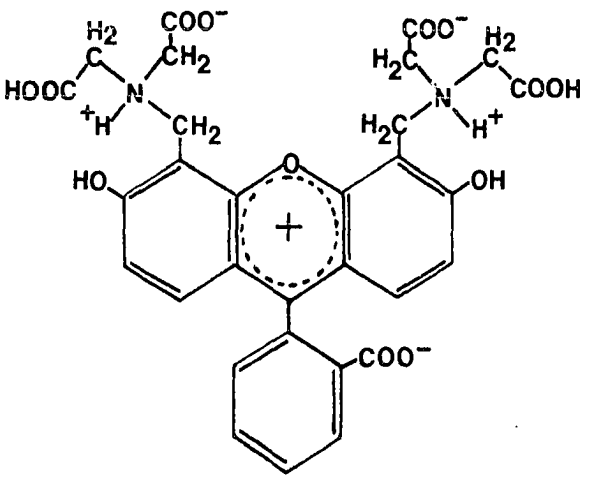
Now that the structure and acid properties of iminodiacetic acid have been described and the structure and acid characteristics of fluorescein have been elucidated (Section II.D.3), the structure of Calcein, which is composed of fluorescein and iminodiacetic acid, can be discussed. By comparing the values of the acid dissociation constants of Calcein with those of fluorescein and iminodiacetic acid, it is possible to confirm the assignment of the pK values to the different functional groups in Calcein.

d. The structure of Calcein Hefley[58] has already established the structure of Calcein, to a large degree, by providing evidence from NMR spectroscopy that Calcein is fluorescein symmetrically substituted in positions 4' and 5' by methyleneiminodiacetic acid groups. She also effectively disposed of other possible structures in which substitution occurs at different positions. Most notably, she refuted the unsymmetrical, sterically crowded, and esthetically rather unpleasing structure of Wallach et al.[151] (Structure III, p. 10). In this work, the substitution pattern determined by Hefley has been confirmed by comparing the NMR spectra of Calcein and of 4',5'-dimethylfluorescein and by

obtaining coupling constants which can be attributed only to protons in ortho position to each other. Thus, the four remaining protons on the xanthene portion of the molecule of Calcein must be in positions 1', 2', 7', and 8', and substitution must have taken place at position 4' and 5'. The evidence for the substitution pattern in the structure of Calcein, determined by Hefley and confirmed in this work, is irrefutable.

In her assignment of the structure of Calcein (Structure I, p. 10), however, Hefley made the erroneous assumption that Calcein exists in the closed lactone form and that the lactone ring does not open throughout the entire working pH range, even in alkaline solution. The error can be attributed largely to the lack of understanding of the structure and chemistry of the parent compound, fluorescein itself. Now that the nature of the fluorescein has been thoroughly studied in the first part of this thesis and the structure has been firmly established, the question of the structure of Calcein can be easily answered.

The structure of Calcein is the structure of the zwitter ion form of fluorescein bearing two methyleneimino-diacetic acid groups, themselves also in the zwitter ion form, in positions 4' and 5'. Thus Calcein is a unique triple zwitter ion (Structure IV). The structure does not only solve the problem of the lactone ring, but also provides



IV. The triple zwitter ion structure of Calcein

a ready explanation for the unusual acid strength of the third and fourth hydrogen atom of Calcein and, indeed, for the acidic characteristics of the entire molecule. In the discussion, reference will be made, by analogy, to similar compounds, the relationship to fluorescein will be emphasized, and the acidic properties of the replaceable hydrogen atoms of Calcein will be correlated with those of fluorescein and iminodiacetic acid.

The assignment of the closed lactone form to the structure of Calcein is unwarranted on the basis of both the color and the chemistry of Calcein. The color of Calcein varies from golden yellow to yellow-orange and is very similar to that of yellow fluorescein. Compounds analogous to Calcein, the so-called phthalein complexones,[1,81] are

colorless whenever the starting parent compounds and the final products bearing the methyleneiminodiacetic acid groups possess the closed lactone ring structure. The lack of color of such structures has been discussed in detail in Section II.C.4. Yet because Calcein is invariably yellow, just like the starting fluorescein, it is easy to suppose that Calcein has the same structure as yellow fluorescein. The yellow color of Calcein cannot be attributed to the substituents which exist in the zwitter ion form because a zwitter ion structure of itself is not chromophoric; amino acids, iminodiacetic acid, EDTA, and the phthalein complexones are all zwitter ions but are all white powders. The chemistry of Calcein is most interesting in the alkaline pH region in which lactone rings are not stable. This phenomenon, too, has been discussed previously. Both the color and the chemistry of Calcein point to the absence of a lactone ring.

The chemistry of Calcein encompasses acid-base reactions, reduction capability, fluorescence behavior, and chelating properties. The discussion in this section will be limited to the acid-base reactions of Calcein which are additive properties of the constituent molecules, fluorescein and iminodiacetic acid. The reactions describing the stepwise dissociation of hydrogen atoms from Calcein, H_6Cal, and the equations defining the appropriate acid dissociation constants are summarized on pages 211-212. On the basis of the ionic

structures of fluorescein and of iminodiacetic acid in various stages of ionization, structures are proposed for the ionic forms of Calcein. The structures for the ionic forms of Calcein, H_5Cal^- through Cal^{6-}, are presented in Figure 38. The assignments of the structures have been made strictly by combining the structures of fluorescein with those of iminodiacetic acid at the appropriate stage of ionization. The structures will be used as an aid in correlating the acid dissociation constants of Calcein with those of fluorescein and iminodiacetic acid.

The first two acid dissociation constants of Calcein, $pK_1 = 2.74$ and $pK_2 = 3.53$, fall in the same range as the pK_1 of 2.98 for iminodiacetic acid. They correspond to the ionization of the carboxylic acid groups (Structures XXXIII and XXXIV, respectively); the proximity of the positively charged ammonium-type nitrogen atom explains the strong acid character of the two carboxylic acid groups.

The pK_3 and pK_4 values of Calcein, 4.58 and 6.19, respectively, reflect the unusually strong acid character of the hydrogen atoms assigned to the phenolic groups. Both Hefley[58] and Wallach *et al.*[151] tried to explain the unusual acid strength on the basis of arguments put forth by Schwarzenbach *et al.*[124] for a phenol substituted by two methyleneiminodiacetic acid groups in the *ortho* positions. Such arguments are not valid in the case of the third and

Figure 38. Structures of the ionic forms of Calcein (H_6Cal)

XXXIII. H_5Cal^- (first carboxyl group ionized)

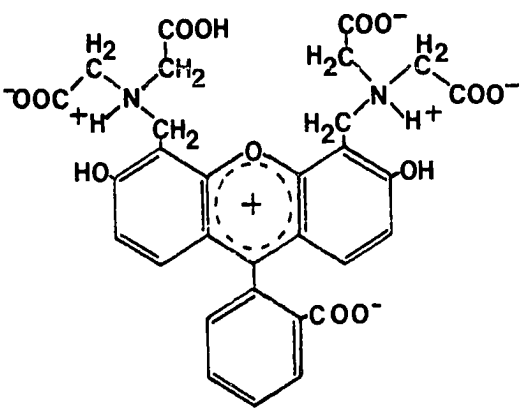
XXXIV. H_4Cal^{2-} (second carboxyl group ionized)

XXXV. H_3Cal^{3-} (first phenolic group ionized)

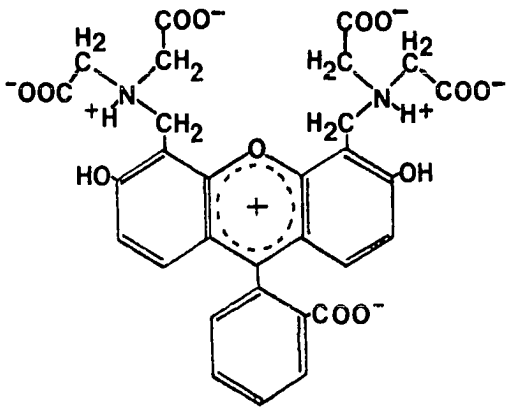
XXXVI. H_2Cal^{4-} (second phenolic group ionized)

XXXVII. $HCal^{5-}$ (first proton from ammonium-type nitrogen atom ionized)

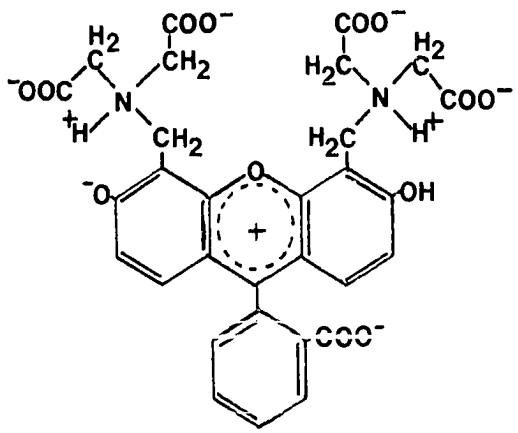
XXXVIII. Cal^{6-} (second proton from ammonium-type nitrogen atom ionized)



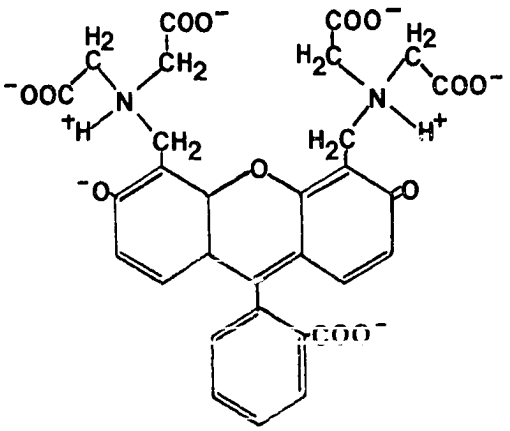
XXXIII.



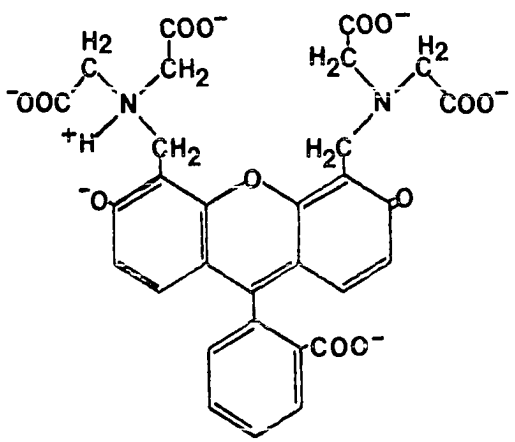
XXXIV.



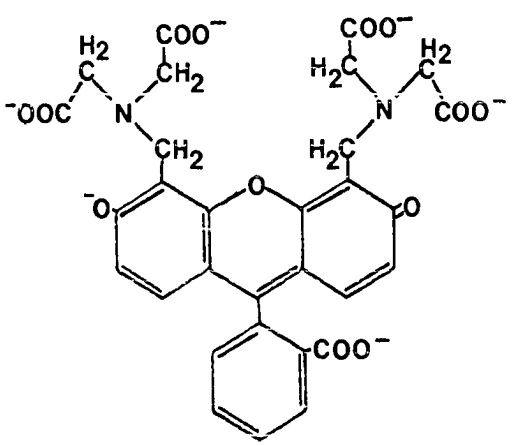
XXXV.



XXXVI.



XXXVII.



XXXVIII.

fourth hydrogen atom of Calcein. First of all, in Calcein the third and fourth hydrogen atom have each only one methylene-iminodiacetic acid group ortho to them. The presence of only one such grouping decreases the pK value of the phenolic proton from 9.92 down to 8.2;[124] this decrease is not sufficient to explain the pK_3 and pK_4 values of Calcein. Secondly, it must be remembered that the two acidic functions are not really phenolic. The nature of their acidity has been discussed in Section II.D. for fluorescein, and the effect of the positive charge on the pyrylium-type ring in the zwitter ion form of fluorescein has been identified as the cause of their increased acid character. Indeed, the acid dissociation constants of fluorescein (pK_{H_2Fl} = 4.75 and pK_{HFl^-} = 6.55) are remarkably close in value to pK_3 and pK_4 of Calcein. The slightly lower values of pK_3 and pK_4 (4.58 and 6.19, respectively) can be attributed to the proximity of the positively charged ammonium-type nitrogen groupings in the ortho positions, next to the hydroxyl groups. The closeness of the pK values for the dissociation of the same type of acidic group in Calcein as in fluorescein is remarkable, especially because different methods were used to determine them. The structures of H_3Cal^{3-} and H_2Cal^{4-} obtained after dissociation of the third and the fourth hydrogen atom described by pK_3 and pK_4, respectively, are presented in Figure 38 (Structure XXXV and XXXVI, respectively).

The pK_5 value of Calcein, 9.88, is in the same range as the pK_2 value of iminodiacetic acid, 9.35, and corresponds to the dissociation of the proton from the ammonium-type nitrogen group to give $HCal^{5-}$ (Structure XXXVII). The pK_6 value, also for a proton from an ammonium-type nitrogen group, is somewhat higher at 11.64, owing to the already high negative charge on the anion $HCal^{5-}$ which makes the last hydrogen atom a weaker acid in producing Cal^{6-} (Structure XXXVIII).

The structure of Calcein has been discussed in terms of its color, chemistry, and acid-base properties. The proposed structure (Structure IV) accounts neatly for the yellow color of Calcein, whereas analogous compounds, the so-called phthalein complexones, which possess the lactone ring structure are colorless. The open, zwitter ion structure of Calcein also disposes the problem of the lactone ring, left unanswered by Hefley. The acid-base properties of Calcein have been considered as a sum of the properties of the parent compound, fluorescein, and those of the substituents, the iminodiacetic acid groups. The six replaceable hydrogen atoms of Calcein have been related to the two replaceable hydrogen atoms of fluorescein and to the four replaceable hydrogen atoms contributed by the two substituent iminodiacedic acid groups. The unusual acid strength of the hydroxyl groups of fluorescein is also evident in Calcein; the similarity in their structures has been used to explain

this acidic character of the third and fourth hydrogen atom of Calcein. Structures for the ionic forms of Calcein have been proposed on the assumption that the ionic structures of fluorescein and of iminodiacetic acid are additive at various stages of ionization.

C. Reaction of Calcein with Aluminum

1. Introduction

Calcein has been demonstrated elegantly by A. J. Hefley[58] to be an acid with six replaceable hydrogen atoms. During the potentiometric titration of Calcein in water with a solution of sodium hydroxide, a single break is observed in the titration curve, corresponding to four equivalents of base added and, therefore, to the replacement of four hydrogen atoms that are sufficiently acidic to be titrated by sodium hydroxide in water. The two remaining hydrogen atoms are very weak acids which are too weakly acidic to be titrated potentiometrically in water; presumably they are derived from quaternary ammonium ions.

An interesting change in the acidity of the first four replaceable hydrogen atoms occurs when Calcein is titrated either in 50 per cent ethyl alcohol (Section III.B.3) and of the fifth and sixth replaceable hydrogen atoms in the presence of excess of calcium chloride (Hefley's work). In the presence of calcium ions, a compound is formed which

incorporates two moles of calcium ions per mole of Calcein. Owing to the metal-ligand coordination bonding, the two hydrogen atoms are displaced and become titratable by the base. A single, well-defined break in the titration curve is then present corresponding to six equivalents of base, four corresponding to the replacement of the original strongly acidic hydrogen atoms and two more owing to the increased acidity of the fifth and sixth hydrogen atoms. By mathematical treatment of the titration data, Hefley obtained numerical values for the six acid dissociation constants of Calcein and for the formation constants of the one-to-one and the one-to-two Calcein-calcium compounds.

It was hoped at the outset of this study to employ a similar approach in the investigation of the reaction between Calcein and aluminum chloride. At first, it would seem that an analogous series of experiments could provide useful data which could lend themselves to easy interpretation. In actuality, however, the Calcein-aluminum system was beset with difficulties owing to the complex nature of the chemistry of aluminum itself and to an unusual reaction between aluminum and the Calcein.

Unlike the relatively simple case of Calcein plus calcium, the potentiometric titration of Calcein in the presence of aluminum is complicated by the fact that trivalent aluminum itself is acidic and reacts with alkali to

form insoluble aluminum hydroxide, $Al(OH)_3$, also known as hydrous aluminum oxide, $Al_2O_3 \cdot 3H_2O$. Excess alkali dissolves such precipitated aluminum hydroxide by forming hydrated sodium aluminate, the composition of which is expressed either as $Al(OH)_4^-$ or as $[AlO_2 \cdot H_2O]^-$. The nature of the precipitation of aluminum hydroxide has been studied extensively, and many forms and structures for the insoluble species have been proposed; the nature of the hydroxide precipitated is dependent on time, temperature, pH of solution, excess of anions present, and the method of raising the pH.[22,161]

To learn the behavior of aluminum ions during titration with base under the conditions to be used in the study of the reaction of aluminum with Calcein, aluminum chloride was titrated with a solution of sodium hydroxide in both water and in 50 per cent ethyl alcohol. The titration curves obtained were useful in the interpretation of the reaction of aluminum with Calcein.

In addition to the formation of insoluble aluminum hydroxide species, an additional problem is presented by the low solubility of Calcein itself in water and most other solvents. It was previously observed, however, that appreciable quantities of Calcein can be dissolved in a solvent consisting of 50:50 water-ethyl alcohol, by volume, and the behavior of Calcein as an acid in this solvent is

known. Thus, a series of experiments was undertaken using this solvent for the titration of Calcein plus varying amounts of aluminum chloride with sodium hydroxide. This procedure proved fruitful, not only in overcoming the low solubility of Calcein and providing an understanding of the acidic character of trivalent aluminum, but also in interpreting the highly unusual Calcein-aluminum compounds formed.

Additional support for the interpretation of the behavior of the aluminum compound of Calcein was obtained from a study of the reaction of ethylenediaminetetraacetic acid (EDTA) with aluminum chloride. Although this system has been studied extensively by many workers, such as[98,120,125] for example, titrations of EDTA plus varying amounts of aluminum chloride by using sodium hydroxide as the titrant were re-examined, and the data were compared by analogy to those obtained for Calcein plus aluminum. After all, the methylene-iminodiacetic acid substituents of Calcein can be viewed as halves of an EDTA molecule. A direct comparison was used to provide evidence for the acidic properties of the aluminum compound of Calcein.

Only after the completion of these preliminary experiments could an analytically useful titration of Calcein plus aluminum be undertaken in water as the solvent. Knowing the correct combining ratio of Calcein to aluminum, the difficulty of interpretation of the aluminum compound of Calcein

was overcome. From the titration data thus obtained in water, a formation constant was calculated for the Calcein-aluminum compound.

2. Experimental part

a. Reagents The Calcein was the highly pure compound prepared and analyzed as described previously. For the titrations, solid Calcein was quickly weighed out by difference, dissolved in the appropriate solvent, and titrated immediately; alternatively, a stock solution of Calcein was prepared with a known concentration, and subsequently aliquots were withdrawn for titration. In all cases, the Calcein was used as soon as possible and on the same day on which it was dissolved.

Solid aluminum chloride hexahydrate, Analytical Reagent (Mallinckrodt), was used without further purification. A weighed amount of the compound, titrated potentiometrically with sodium hydroxide, resulted in a titration curve with a single break corresponding to three equivalents of base and a purity of 96.8 per cent. The purity of less than 100 per cent was not surprising because of the presence of six water molecules of hydration which could easily have affected the molecular weight.

A 0.1 M stock solution of aluminum chloride was prepared for all subsequent titration experiments by dissolving 24.14 g of the above compound in one liter of deionized water. The

exact molarity of this stock solution was determined by indirect titration with a solution of EDTA, as outlined in Diehl[29] (pp. 289, 393).

A 0.1 M solution of EDTA, prepared by dissolving 3.75 g of its disodium salt dihydrate in one liter of deionized water, was standardized with a standard solution of calcium chloride, prepared from primary standard $CaCO_3$; Calmagite was used as the indicator. To standardize the solution of aluminum chloride, a 3.00 ml aliquot was added to 50.00 ml of 0.01021 M EDTA, 10 ml of pH 4.5 buffer (acetic acid–sodium acetate) were added, and the mixture was heated to a gentle boil. Upon cooling, xylenol orange was added as an indicator, and the excess EDTA was titrated with 0.09985 M lead nitrate. The molarity of the lead nitrate solution was established by titration with the standard EDTA, using xylenol orange as the indicator and buffering at pH 4.5. Triplicate titrations yielded a value of 0.0995 M for the aluminum chloride solution.

For alkalimetric titrations of the free acid form of EDTA, analytical grade ethylenediaminetetraacetic acid was used without further purification or drying.

The sodium hydroxide solution, prepared by appropriate dilution of a 50 per cent concentrate, was standardized against primary standard potassium acid phthalate, using phenolphthalein as the indicator. The normality was rechecked and corrected frequently. A solution of 0.1 N

hydrochloric acid was standardized with the standard sodium hydroxide.

Deionized water, obtained by passing distilled water through a column of mixed ion exchange resin (Amberlite MB-1), was used in all the aqueous titrations. Commercial 95 per cent ethyl alcohol was diluted with the appropriate volume of water to reduce it to 50 per cent.

b. Apparatus A Corning Model 10 pH meter was used to follow the titrations. A high-alkalinity Beckman glass electrode was used to measure the pH; a saturated calomel electrode, either a sleeve type or an asbestos fiber junction type, served as the reference electrode. Before each titration, the pH meter was standardized with at least two buffers (pH 4.01, 6.86, or 9.18), prepared according to NBS specifications as outlined in Diehl,[29] p. 58. Either 250-ml or 400-ml beakers were used as the titration vessels, and the solutions were mechanically stirred by a Teflon-coated magnetic bar.

c. Procedure The acid Calcein was either weighed out and dissolved in the appropriate solvent or an aliquot of the stock solution was taken for titration. The desired molar ratio of the aluminum ion to the acid was attained by introducing a measured volume of the stock solution of known molarity by using a 10-ml buret. Often, additional solvent was added to ensure complete dissolution and adequate

submersion of the pH electrodes. After each addition of sodium hydroxide increment, the pH was read as soon as the pH meter needle came to rest. Sometimes, especially when a precipitate was being formed, it took 15 or even more minutes for the pH reading to become stabilized. When the total volume of the solution had to be known exactly, it was corrected for dilution caused by the addition of titrant and by any washings of the sides of the beaker.

3. Results and discussion

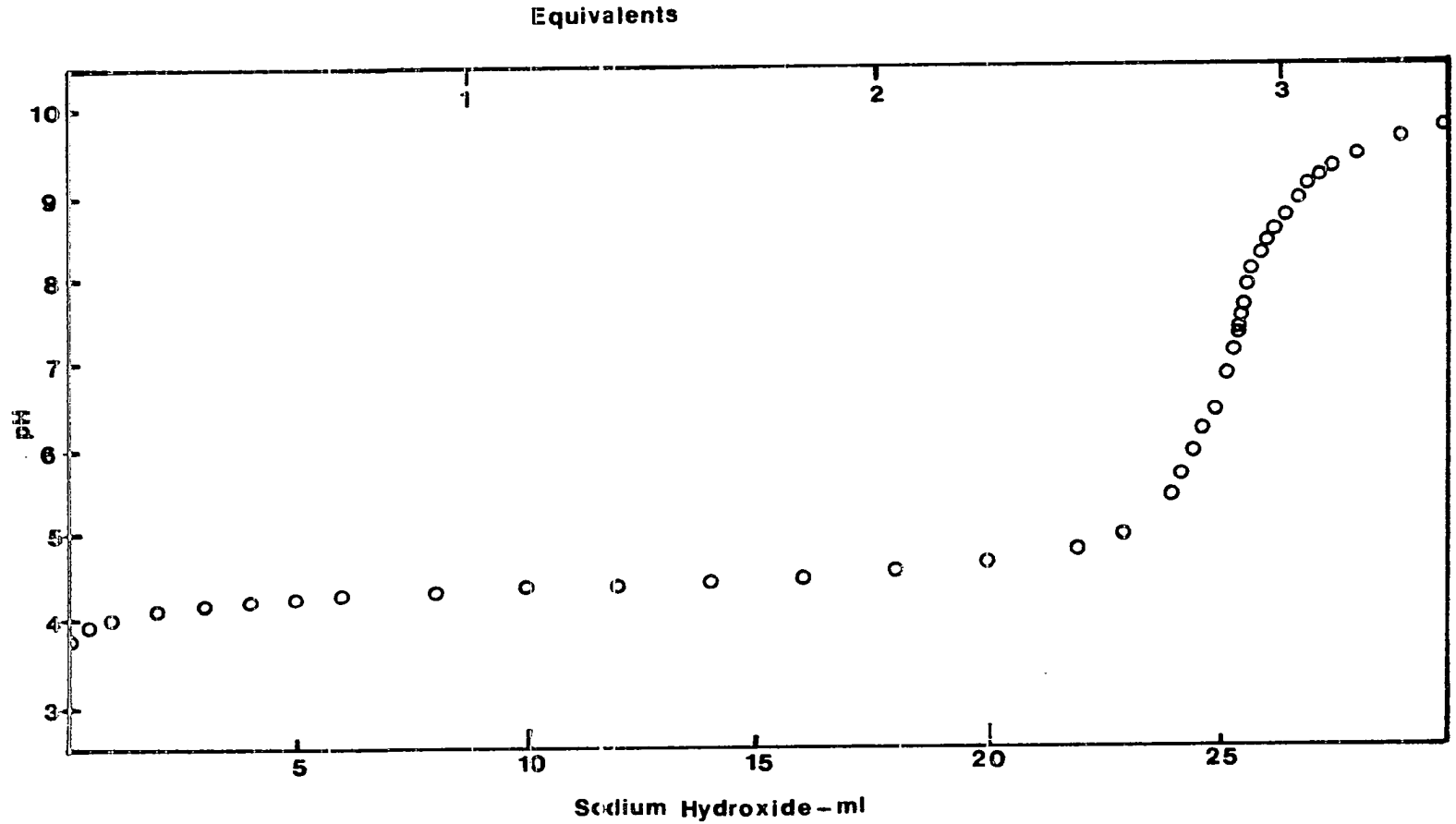
a. Titration of aluminum chloride with sodium hydroxide

Two titrations of aluminum chloride with base were carried out. One was a titration of the solid aluminum chloride hexahydrate dissolved in water; the titration curve is presented in Figure 39. It is readily seen that aluminum chloride is sufficiently acidic to lower the pH of the solution to 3.75 before the start of the titration. After the addition of approximately 5 ml of 0.1070 N sodium hydroxide, the pH rises to 4.24 and the solution begins to become cloudy. This value is close to pH 4.1, listed by Willard and Diehl,[161] p. 45, as the pH at which aluminum hydroxide begins to precipitate. Further additions of base increase the turbidity of the solution without raising the pH appreciably. In fact, the pH remains below 5 up to 23 ml of base added, indicating that a rather strong acid is being titrated. The rapid rise of pH close to the end-point,

Figure 39. Potentiometric titration of aluminum chloride in water

$AlCl_3 \cdot 6H_2O$: 0.2268 g (9.39×10^{-4} moles)

Titrant: 0.1070 N sodium hydroxide


Equivalents
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2
3
10
9
8
7
6
5
4
3
pH
5
10
15
20
25
Sodium Hydroxide – ml

which occurs at 25.50 ml and a pH of 7.30, confirms this observation. Titration beyond the end-point leads to a decrease in turbidity.

Interestingly, the end-point at 25.50 ml occurs close to the theoretical equivalence point of 26.31 ml, based on the assumption that the compound is 100 per cent pure and that the stoichiometry of the reaction is determined by the following equation:

$$AlCl_3 + 3\ NaOH = Al(OH)_3 + 3\ NaCl.$$

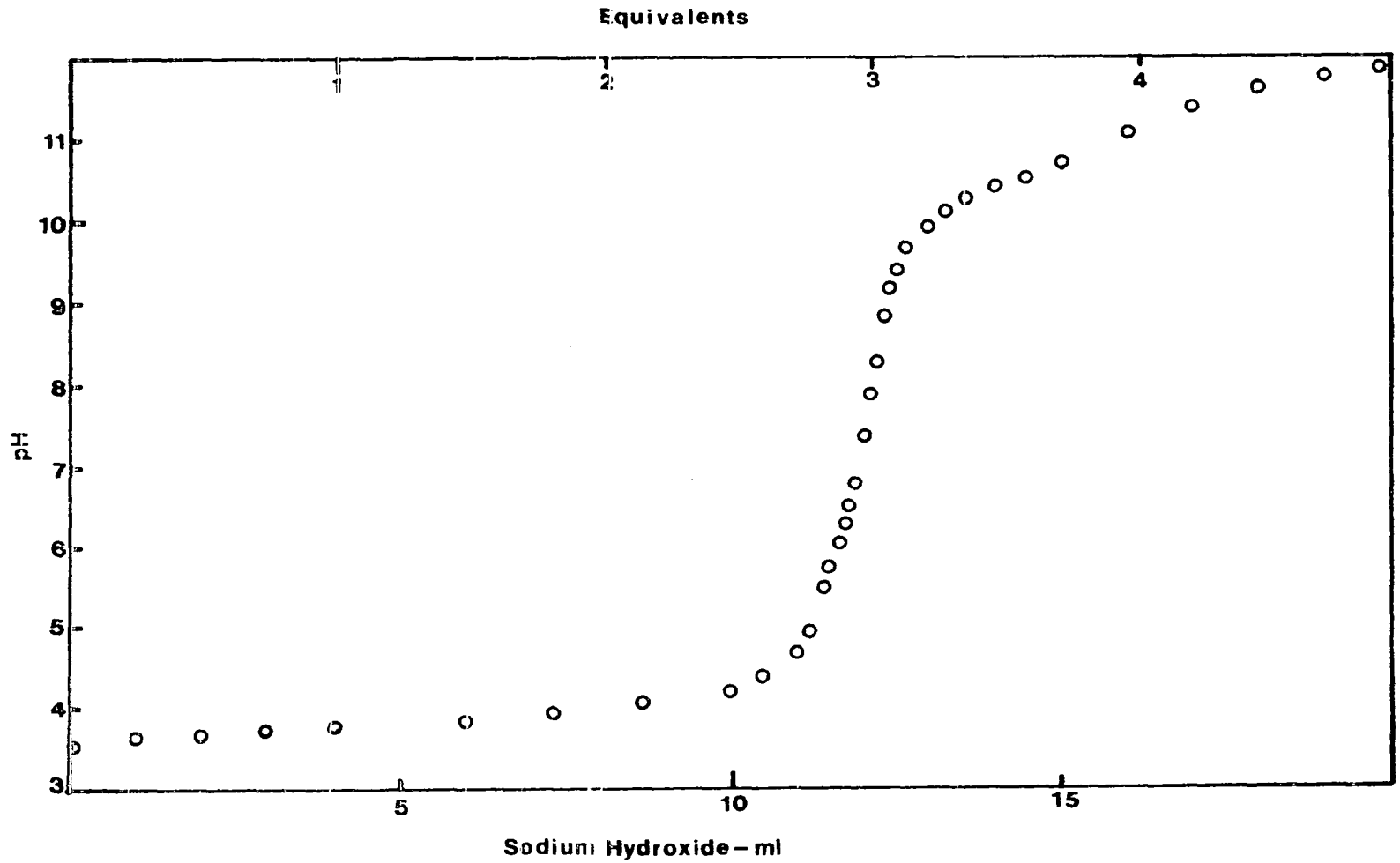
On the basis of this stoichiometry, the compound $AlCl_3 \cdot 6H_2O$, taken straight from the reagent bottle, is 96.8 per cent pure. Such a value for the purity is not unreasonable if one takes into account that six molecules of water present, which could result in a varying molecular weight, depending on the completeness of hydration.

The other titration of aluminum chloride in 50 per cent ethyl alcohol (Figure 40) illustrates this argument further. In this case, the number of moles of aluminum chloride are known precisely because of the known molarity and the volume of the aliquot taken; hence, the degree of hydration is irrelevant. The end-point occurs at 12.00 ml of 0.1227 N sodium hydroxide, very close to the theoretical value of 12.15 ml, calculated for three equivalents of base per mole of aluminum chloride. In addition, a weak though discernible

Figure 40. Potentiometric titration of aluminum chloride in 50 per cent ethyl alcohol

Aluminum chloride: 5 ml of 0.0995 M solution (4.98×10^{-4} moles)

Titrant: 0.1227 N sodium hydroxide


Equivalents
1
2
3
4
11
10
9
8
7
6
5
4
3
pH
5
10
15
Sodium Hydroxide – ml

end-point occurs at approximately 16 ml or four equivalents of sodium hydroxide. At this point, the following reaction has been completed:

$$Al(OH)_3 + OH^- = Al(OH)_4^-.$$

During the formation of the aluminate ion, one additional equivalent of base has been used up. In the process, the precipitated aluminum hydroxide has been dissolved and the solution is clear again.

In summary, then, it has been demonstrated that aluminum chloride acts as a strong acid and can be titrated by sodium hydroxide; three equivalents of base are used up per mole of aluminum. In the process, aluminum hydroxide is precipitated up to the end-point. Beyond the end-point, the precipitate is dissolved owing to the formation of the soluble aluminate ion. The processes are identical, regardless of which solvent is used, although in 50 per cent ethyl alcohol an additional break in the titration curve can be observed corresponding to four equivalents of base added.

b. Titration of Calcein in the presence of aluminum chloride Aliquots of Calcein, dissolved in 50 per cent ethyl alcohol, were titrated potentiometrically in the presence of varying mole ratios of aluminum chloride with 0.1211 N sodium hydroxide. For convenient comparison, the titration curve of Calcein alone (Curve a) and the six

titration curves with varying mole ratios of aluminum chloride (Curves b-g) are presented on the same scale in Figure 41. First, Calcein alone was titrated (Curve a), then in the presence of aluminum chloride in the folowing mole ratios of Calcein to aluminum: 1:0.75 (Curve b), 1:1 (Curve c), 1:1.5 (Curve d), 1:2 (Curve e), 1:3 (Curve f), and 1:4 (Curve g).

Inspection of Figure 41 reveals that three important end-point regions are discernible in the family of titration curves, depending on the mole ratio of Calcein to aluminum. First of all, the two breaks of Calcein alone (Curve a), at two and four equivalents of based added, become less sharply defined and shift toward more equivalents of base (Curves b,c,d) as the ratio of Calcein to aluminum is increased from 1:0 to 1:1.5. The increase in the uptake of base is not a mere titration of the added trivalent aluminum cation to the aluminum hydroxide, because no turbidity is observed up to and including Curve d. And it is demonstrated before (Figures 39 and 40) that titration of aluminum chloride with sodium hydroxide does produce a precipitate. Furthermore, the initial apparent pH of both Calcein alone (Figure 41, Curve a) and of aluminum chloride alone (Figure 40) is approximately 3.5. The fact that the addition of aluminum chloride to Calcein lowers the apparent pH to below 3 and eventually to about 2.5 indicates that a union has

Figure 41. Potentiometric titration of Calcein in the presence of varying amounts of aluminum chloride

Curve a. Calcein alone (6.29×10^{-5} moles)

Curve b. Calcein plus $AlCl_3$ in 1:0.75 mole ratio

Curve c. Calcein plus $AlCl_3$ in 1:1 mole ratio

Curve d. Calcein plus $AlCl_3$ in 1:1.5 mole ratio

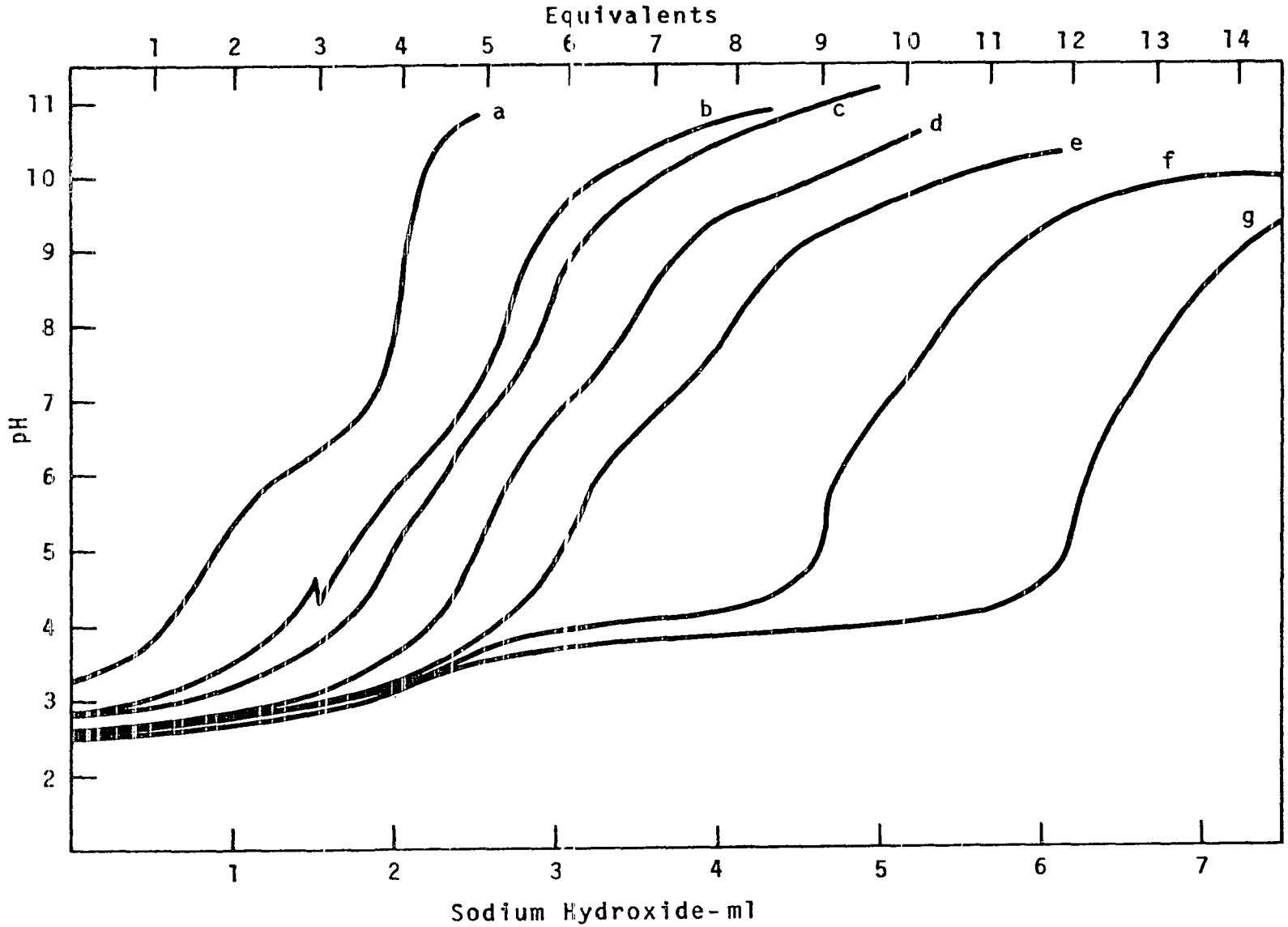
Curve e. Calcein plus $AlCl_3$ in 1:2 mole ratio

Curve f. Calcein plus $AlCl_3$ in 1:3 mole ratio

Curve g. Calcein plus $AlCl_3$ in 1:4 mole ratio

Solvent: 50 per cent ethyl alcohol

Titrant: 0.1211 N sodium hydroxide


Equivalents
1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH
11 10 9 8 7 6 5 4 3 2
a
b
c
d
e
f
g
1 2 3 4 5 6 7
Sodium Hydroxide-ml

taken place between Calcein and aluminum with the accompanying release of hydrogen ions, even in a solution that is quite acidic at the start.

Secondly, titration curves in which the mole ratio of Calcein to aluminum is 1:2 and more (Figure 41, Curves e,f,g) exhibit an additional end-point. The first, occurring at five equivalents, corresponds to the union of one mole of Calcein with 1.5 moles of aluminum, and no turbidity is observed up to this point. Beyond the first break, the solution becomes progressively more turbid, indicating that aluminum hydroxide is being precipitated. Indeed, the presence of excess aluminum, that is, aluminum which has not combined with Calcein, can be quantitatively determined from the position of the second end-point, which is shifted to more equivalents of base with increasing excess of aluminum chloride.

Finally, Curve d corresponds to the titration of a stoichiometric ratio of Calcein to aluminum, namely 1:1.5. After the first end-point, occurring at five equivalents of sodium hydroxide added, another end-point occurs at seven equivalents. These two additional equivalents of base are used up also in the subsequent titrations (Curves e,f,g) in which the reaction between Calcein and aluminum is quantitative; they correspond to the titration of the Calcein-aluminum compound itself. When excess aluminum chloride is

present, that is, in a Calcein-to-aluminum ratio greater than 1:1.5, turbidity begins to appear past the first end-point, and the end-point for the two additional equivalents occurs only after the excess aluminum has been titrated to the hydroxide. Thus, in Curve e, the two equivalents are used up in the region between 6.5 and 8.5 equivalents; in Curve f, they are titrated between 9.5 and 11.5 equivalents of base added. In Curve g, unfortunately, the region of interest between 12.5 and 14.5 equivalents is too drawn out because of the sizeable quantity of precipitated aluminum hydroxide, and no definite end-point can be detected.

The nature of the reactions with sodium hydroxide for the various ratios of Calcein to aluminum chloride will now be discussed in more detail for each case. It is evident from Figure 41 that one mole of the hexabasic acid Calcein, H_6Cal, combines with 1.5 moles of aluminum, releasing 5 moles of titratable hydrogen ions in the process; thus the combining ratio of Calcein to aluminum is 1:1.5 or, expressed better as 2:3:

$$H_6Cal + 1\ 1/2\ Al^{3+} = 1/2\ Al_3(HCal)_2^- + 5H^+ \qquad (1)$$

or

$$2\ H_6Cal + 3\ Al^{3+} = Al_3(HCal)_2^- + 10H^+ \qquad (2)$$

The same ratio (2:3) will be verified in the next Section, III.D., on the basis of fluorescence data. The first break

in the titration curves d through g of Figure 41 corresponds to the titration of these 5 hydrogen atoms.

The aluminum compound of Calcein reacts with four additional equivalents of sodium hydroxide for two reasons. First, the hydrogen atom remaining on each of the two Calcein ligands is titrated:

$$Al_3(HCal)_2^- + 2OH^- = Al_3Cal_2^{3-} + 2H_2O. \qquad (3)$$

Then, at the same time, two additional equivalents of hydroxide are used up for the following reaction:

$$Al_3Cal_2^{3-} + 2OH^- = Al_3(OH)_2Cal^{5-} \qquad (4)$$

The basis for the existence of such a hydroxy species of the Calcein–aluminum compound lies in the analogous reaction between EDTA and aluminum and will be discussed in detail below. Since the last two reactions, (3) and (4), occur simultaneously, they can be combined in the following equation:

$$Al_3(HCal)_2^- + 4OH^- = Al_3(OH)_2Cal_2^{5-} + 2H_2O \qquad (5)$$

or, for the stoichiometric ratio of 1:1.5

$$1/2\ Al_3(HCal)_2^- + 2OH^- = 1/2\ Al_3(OH)_2Cal_2^{5-} + H_2O \qquad (6)$$

Equation (6) corresponds to the reaction completed at the second end-point, as discussed above, and accounts for the

two additional equivalents of base used up. One more equation is necessary to describe the system under study, namely

$$Al^{3+} + 3OH^- = Al(OH)_3. \qquad (7)$$

This equation will be used whenever excess aluminum chloride is present.

Thus, with the aid of equations (1), (6), and (7), and the equation for the titration of Calcein alone, all the end-points occurring in the family of titration curves in Figure 41 can be interpreted. Whenever an equation is given for the release of hydrogen ions, the equation showing their neutralization with an equivalent amount of sodium hydroxide is omitted for the sake of brevity. Therefore, the total equivalents of base added will be represented either by the number of hydrogen ions released in one type of reaction or by the number of hydroxide ions used up in another type of reaction. The equations for the end-points occurring in each of the curves in Figure 41 are given below.

Calcein alone (Curve a)

First end-point, at approximately 2 equivalents:

$$H_6Cal + 2OH^- = H_4Cal^{2-} + 2H_2O$$

Second end-point, at 4 equivalents (2 plus 2):

$$H_4Cal^{2-} + 2OH^- = H_2Cal^{4-} + 2H_2O$$

Calcein plus 0.75 moles of aluminum (Curve b). There is only one-half of the stoichiometric amount of aluminum

available to combine with one-half of the Calcein. The remaining, uncombined Calcein behaves as the free acid. Thus,

First end-point, at approximately 3 1/2 equivalents:

$$1/2\ H_6Cal + 3/4\ Al^{3+} = 1/4\ Al_3(HCal)_2^- + 2\ 1/2\ H^+$$

$$1/2\ H_6Cal + OH^- = 1/2\ H_4Cal^{2-} + H_2O$$

Second end-point, at 5 1/2 equivalents (3 1/2 plus 2):

$$1/4\ Al_3(HCal)_2^- + OH^- = 1/4\ Al_3(OH)_2Cal_2^{5-} + 1/2\ H_2O$$

$$1/2\ H_4Cal^{2-} + OH^- = 1/2\ H_2Cal^{4-} + H_2O$$

Calcein plus 1 mole of aluminum (Curve c). There is still not enough aluminum for a stoichiometric reaction with the available Calcein. Hence the end-points are due to two different reactions.

First end-point, at 4 equivalents:

$$2/3\ H_6Cal + Al^{3+} = 1/3\ Al_3(HCal)_2^- + 3\ 1/3\ H^+$$

$$1/3\ H_6Cal + 2/3\ OH^- = 1/3\ H_4Cal^{2-} + 2/3\ H_2O$$

Second end-point, at 6 equivalents (4 plus 2):

$$1/3\ Al_3(HCal)_2^- + 4/3\ OH^- = 1/3\ Al_3(OH)_2Cal_2^{5-} + 2/3\ H_2O$$

$$1/3\ H_4Cal^{2-} + 2/3\ OH^- = 1/3\ H_2Cal^{4-} + 2/3\ H_2O$$

The fortuitous occurrence of the end-points at 4 and 6 equivalents of base added creates a pitfall, mentioned in the introduction of this section, in the interpretation of the reaction stoichiometry, especially when the comparison is made with the behavior of the titration of Calcein plus one mole of calcium. It is with the aid of the other titration data that a correct explanation can be provided.

Calcein plus 1.5 moles of aluminum (Curve d). Here the stoichiometry of the reaction is clean-cut and can be described as follows:

First end-point, at 5 equivalents:

$$H_6Cal + 1\ 1/2\ Al^{3+} = 1/2\ Al_3(HCal)_2^- + 5H^+$$

Second end-point, at 7 equivalents (5 plus 2):

$$1/2\ Al_3(HCal)_2^- + 2OH^- = 1/2\ Al_3(OH)_2Cal_2^{5-} + 2H_2O$$

Calcein plus 2 moles of aluminum (Curve e). Starting with this titration, there is excess aluminum present, heralded by the appearance of turbidity after approximately 5 equivalents of base have been added. In this titration curve, one can almost begin to discern the end-point at 5 equivalents; however, it is weak and smeared out over the one at 6.5 equivalents.

First end-point, at 6 1/2 equivalents:

$$H_6Cal + 1\ 1/2\ Al^{3+} = 1/2\ Al_3(HCal)_2^- + 5H^+$$

$$1/2\ Al^{3+} + 1\ 1/2\ OH^- = 1/2\ Al(OH)_3$$

Second end-point, at 8 1/2 equivalents (6 1/2 plus 2):

$$1/2\ Al_3(HCal)_2^- + 2OH^- = 1/2\ Al_3(OH)_2Cal_2^{5-} + 2H_2O$$

Again in this case, it is easy to misinterpret the reaction stoichiometry when the end-points are mistakenly taken at 6 and 8 equivalents, respectively, and the behavior is interpreted analogously to the reaction between one mole of Calcein and two moles of calcium.

Calcein plus 3 moles of aluminum (Curve f). In this case, the three breaks in the titration curve are well defined, each one being produced by a single equation.

First end-point, at 5 equivalents:

$$H_6Cal + 1\ 1/2\ Al^{3+} = 1/2\ Al_3(HCal)_2^- + 5H^+$$

Second end-point, at 9.5 equivalents (5 plus 4.5):

$$1\ 1/2\ Al^{3+} + 4\ 1/2\ OH^- = 1\ 1/2\ Al(OH)_3$$

Third end-point, at 11.5 equivalents (5 plus 4.5 plus 2):

$$1/2\ Al_3(HCal)_2^- + 2OH^- = 1/2\ Al_3(OH)_2Cal_2^{5-} + 2H_2O$$

Calcein plus 4 moles of aluminum (Curve g):

First end-point, at 5 equivalents:

$$H_6Cal + 1\ 1/2\ Al^{3+} = 1/2\ Al_3(HCal)_2^- + 5H^+$$

Second end-point, at 12.5 equivalents (5 plus 7.5):

$$2\ 1/2\ Al^{3+} + 7\ 1/2\ OH^- = 2\ 1/2\ Al(OH)_3$$

Third end-point, at 14.5 equivalents (5 plus 7.5 plus 2):

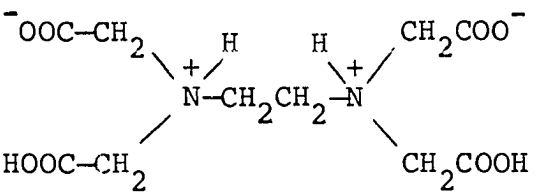
$$1/2\ Al_3(HCal)_2^- + 2OH^- = 1/2\ Al_3(OH)_2Cal_2^{5-} + 2H_2O$$

The second and third breaks in this titration curve are not easily detectable, probably because of the large amount of aluminum hydroxide that has precipitated out up to this point.

It should be noted that in titration curves for ratios of 1:2, 1:3, and 1:4 (Curves e,f,g) the end-points do not fall precisely at the theoretical number of equivalents of sodium hydroxide; they usually are a little less. This shortcoming could be partially due to the complexity of the system in which there is competition between the titration of the acidic Calcein-aluminum compound and the free excess aluminum cations. A similar discrepancy between the theoretical prediction and the actual observation is found in the titration of the acid EDTA in the presence of excess aluminum with sodium hydroxide. This reaction will be the subject of the next section.

4. Analogy to reaction of EDTA with aluminum chloride

The free acid form of ethylenediaminetetraacetic acid (EDTA) was titrated in 50 per cent ethyl alcohol with sodium hydroxide. EDTA is a tetrabasic acid and is conveniently designated by the symbol H_4Y. It exists as the double zwitter ion (Structure XXXIX), having two nonionized carboxylic acid groups and two protonated nitrogen atoms



XXXIX. EDTA

(ammonium-type groups). The common, soluble form of EDTA is its disodium dihydrogen salt, Na_2H_2Y; the free acid form H_4Y is insoluble in water. To dissolve the insoluble H_4Y, a measured amount of sodium hydroxide was added to convert it all to the soluble tetrasodium salt, Na_4Y. Addition of an equivalent amount of hydrochloric acid converted it back to the H_4Y form which did not precipitate in the 50 per cent ethyl alcohol for at least one-half hour. Thus the acid form H_4Y could be titrated immediately with sodium hydroxide.

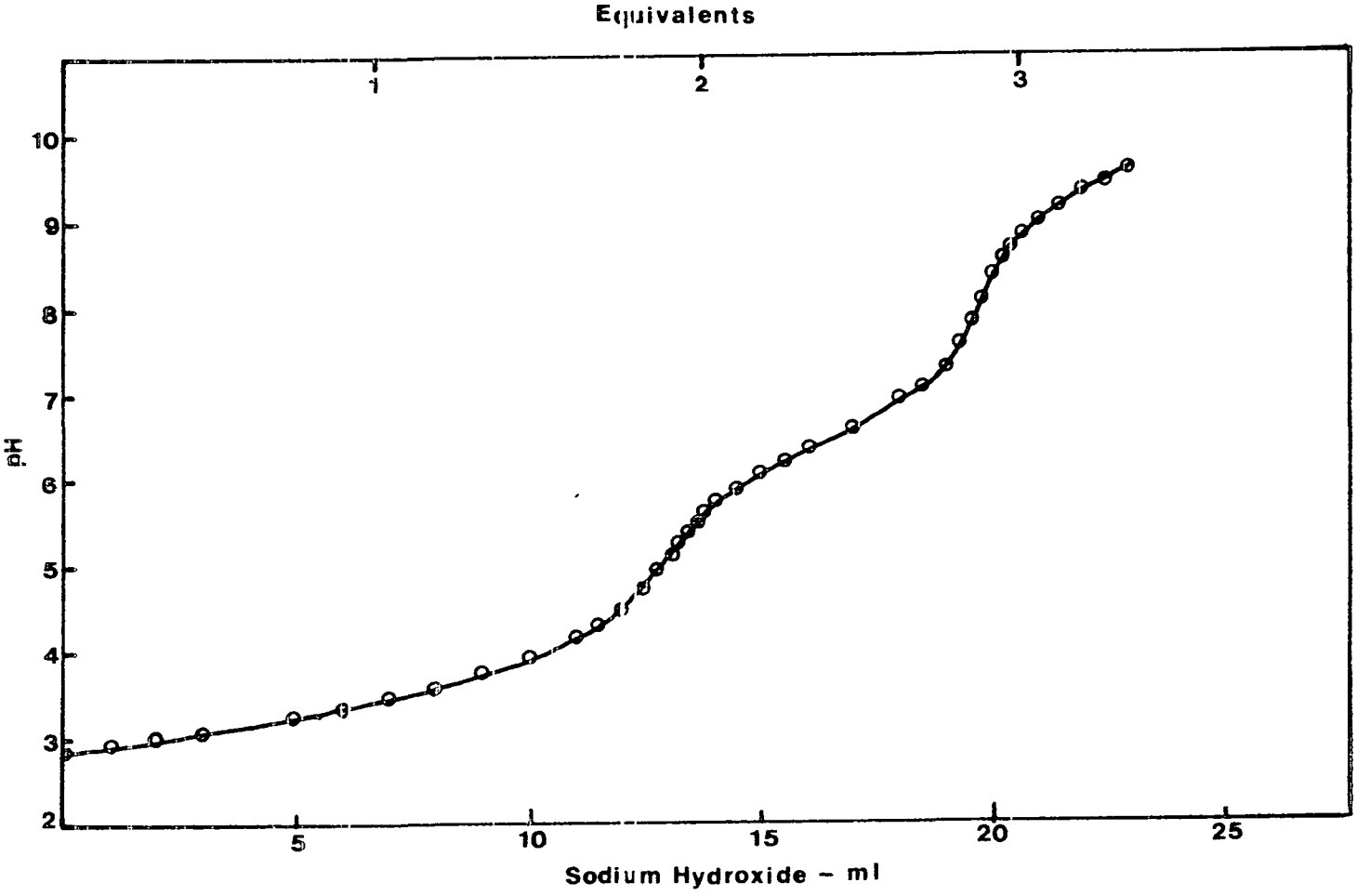
The titration curve of EDTA (H_4Y) is presented in Figure 42. It is the well-known curve with two breaks, occurring at 2 and 3 equivalents of base, respectively. At the first end-point, the two strongly acidic carboxylic groups are

Figure 42. Potentiometric titration of free ethylenediamine-tetraacetic acid (EDTA)

EDTA: 0.2452 g (0.839 mmoles)

Solvent: 50 per cent ethyl alcohol
plus 22.00 ml of 0.1227 N sodium hydroxide
plus 23.31 ml of 0.1158 N hydrochloric acid

Titrant: 0.1227 N sodium hydroxide


Equivalents
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pH
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20
25
Sodium Hydroxide – ml

neutralized. At the second end-point, only one hydrogen atom of the ammonium-type group is neutralized. The hydrogen atom remaining on the second ammonium-type group is too weak an acid to be titrated.

In the presence of one mole of aluminum chloride, the titration curve, depicted in Figure 43, is altered notably. Upon union of one molecule of EDTA with one aluminum ion, all four hydrogen atoms become strong acids and are titrated, as indicated by the first end-point at four equivalents. Although EDTA is a sexadentate ligand, that is, one with six sites of coordination for reaction with the metal ion, it is thought to be bound to the aluminum only by five coordination bonds.[98,120] The sixth position is occupied by one water molecule, while one carboxylate group is free and unattached. Thus the EDTA-aluminum compound is usually represented by $AlY(H_2O)^-$. This compound is itself moderately acidic and can be reacted with an additional equivalent of base to give the species $AlY(OH)^{2-}$. Whether the water is replaced from the coordination sphere by a more strongly bound hydroxide ion, or whether an actual ionization takes place of the type

$$AlY(H_2O)^- = AlY(OH)^{2-} + H^+ \qquad (8)$$

is not clear. In any case, this acidic behavior is well established and the pK_a corresponding to the EDTA-aluminum complex has been determined by various workers as 5.87,[17] 5.89,[125] and 6.16.[120]

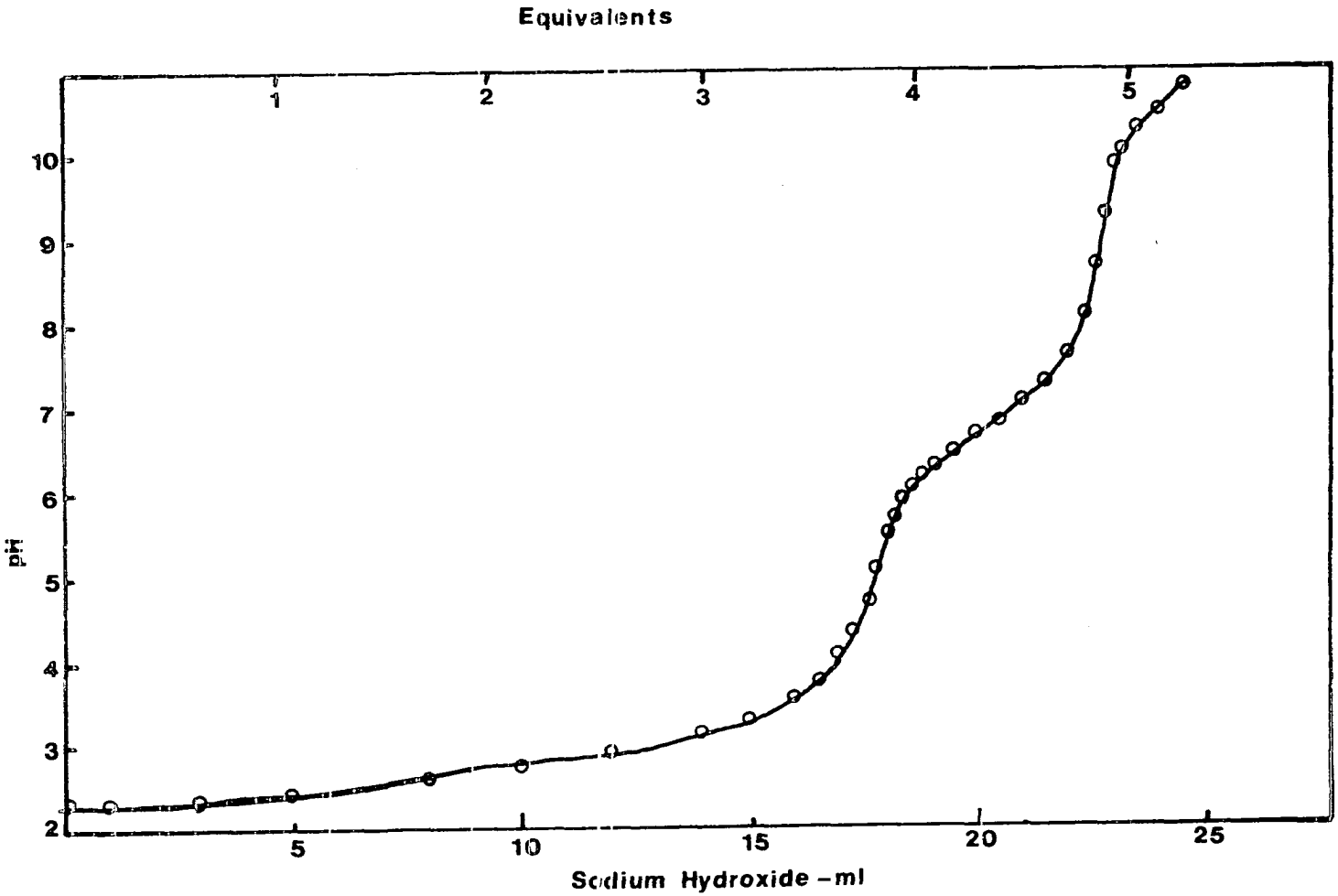
Figure 43. Potentiometric titration of EDTA in the presence of aluminum chloride (1:1 mole ratio)

EDTA: 0.1674 g (0.573 mmoles)

$AlCl_3$: 0.573 mmoles

Solvent: 50 per cent ethyl alcohol
plus 15.00 ml of 0.1227 N sodium hydroxide
plus 15.89 ml of 0.1158 N hydrochloric acid

Titrant: 0.1227 N sodium hydroxide


Equivalents
1
2
3
4
5
10
9
8
7
6
5
4
3
2
pH
5
10
15
20
25
Sodium Hydroxide – ml

This consumption of an additional equivalent of base is in fact demonstrated by the titration curve in Figure 43, in which the second end-point occurs at approximately five equivalents, one equivalent beyond the first end-point. The pH at the mid-point between the two breaks is 6.7. By elementary treatment of $AlY(H_2O)^-$ as a monobasic acid which dissociates according to equation (8), this value of 6.7 is assigned as its pK_a. This is not too far off from the literature values obtained above, considering that the solvent in this case is 50 per cent alcohol, and therefore the pK_a value should be higher than in water.

The titration curve of EDTA in the presence of two moles of aluminum chloride is depicted in Figure 44. Three distinct end-points can be observed. The first occurs at four equivalents and corresponds to the titration of the four hydrogen ions released during the formation of the EDTA-aluminum compound,

$$H_4Y + Al^{3+} = AlY^- + 4H^+. \qquad (9)$$

Beyond the first end-point, addition of more sodium hydroxide produces a turbidity that increases with further addition of base. In this region, the excess, free aluminum, i.e., that which has not combined with EDTA, is titrated to form the aluminum hydroxide which is being precipitated. The third end-point marks the formation of the $AlY(OH)^{2-}$ species from the acidic $AlY(H_2O)^-$ compound.

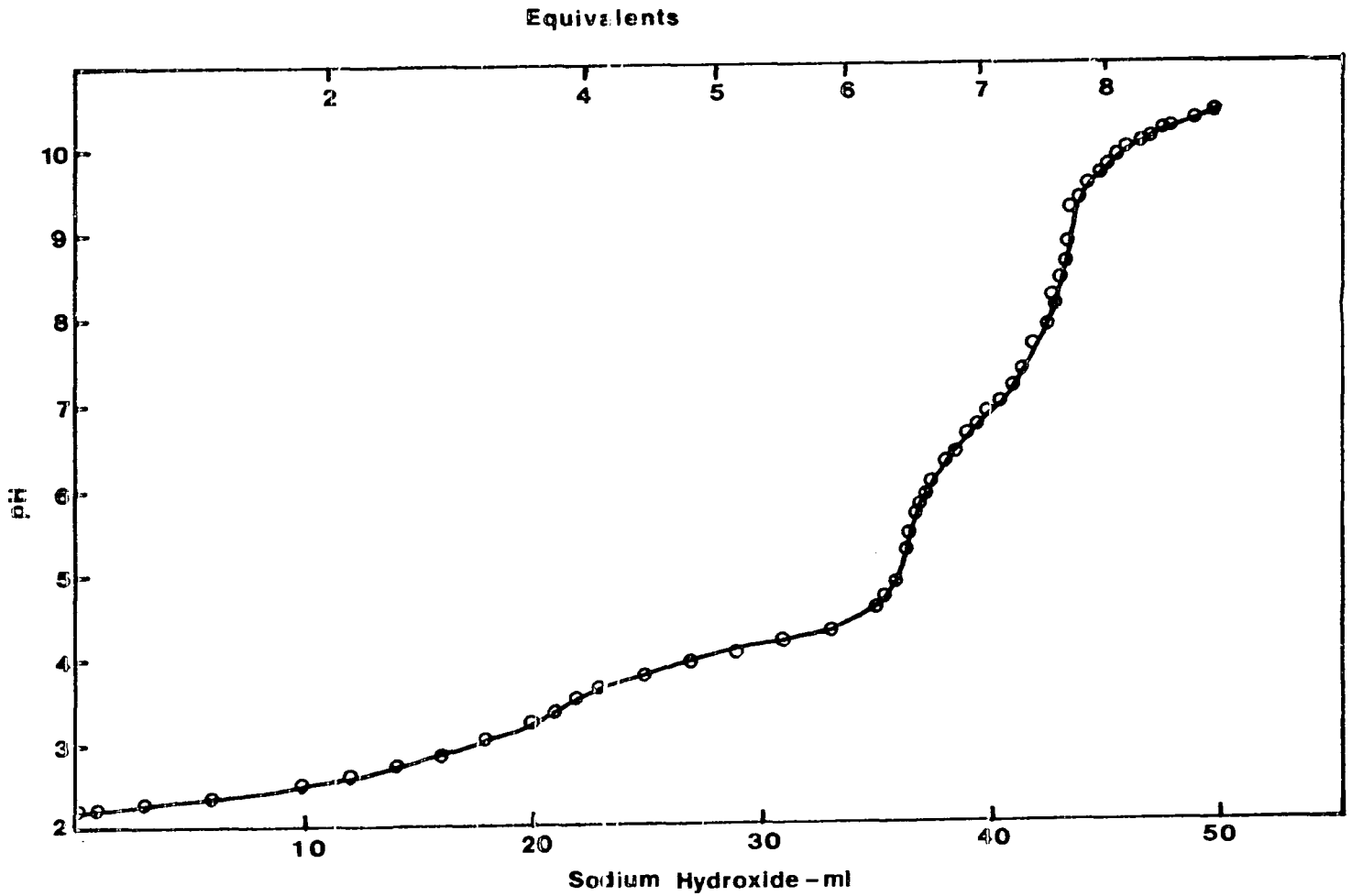
Figure 44. Potentiometric titration of EDTA in the presence of aluminum chloride (1:2 mole ratio)

EDTA: 0.2021 g (0.692 mmoles)

$AlCl_3$: 1.383 mmoles

Solvent: 50 per cent ethyl alcohol
plus 20.00 ml of 0.1227 N sodium hydroxide
plus 21.19 ml of 0.1158 N hydrochloric acid

Titrant: 0.1227 N sodium hydroxide


Equivalents
2
4
5
6
7
8
10
9
8
7
6
5
4
3
2
pH
10
20
30
40
50
Sodium Hydroxide – ml

It was noted above in the subsection dealing with the titration of Calcein in 50 per cent ethyl alcohol in the presence of excess aluminum that some of the end-points often fall slightly short of the theoretical equivalence points. This shortcoming can be also seen in these titrations of EDTA in the presence of aluminum. In Figure 44, for example, the theoretical titration end-points should occur at 4 equivalents (for the four hydrogen ions released), at 7 equivalents (for the one mole of excess aluminum being titrated to form the aluminum hydroxide), and at 8 equivalents (for the formation of $AlY(OH)^{2-}$). Close inspection of Figure 44, however, reveals that the breaks are slightly premature. Similarly in the titration of EDTA plus 1 mole of aluminum (Figure 43), the end-points, which should theoretically fall at 4 and 5 equivalents, also fall somewhat short. The purity of the EDTA used, based on the titration of EDTA in Figure 42, is calculated as 96.5 per cent and cannot account for the discrepancy. Possibly, the choice of solvent (50 per cent ethyl alcohol) could affect the kinetics of the formation of the EDTA-aluminum compound in such a way as to make the reaction appear incomplete in the titration curve. Such an explanation becomes more likely when it is recalled that, although the formation constant (pK) of AlY is relatively high at 16.13, the reaction rate is slow so that a mixture of EDTA and aluminum must be heated in order to ensure complete reaction.

This set of titration curves of EDTA plus aluminum was meant to be qualitative in nature, in order to demonstrate the acidity of the $AlY(H_2O)^-$ complex and the formation of the $AlY(OH)^{2-}$ species. Because Calcein can be considered as a derivative of fluorescein that has been substituted by two EDTA halves, this analogy can be applied to provide further evidence for the acidic properties of the Calcein-aluminum compound, $Al_3(OH)_2Cal_2^{5-}$, described before. The nature and structure of this compound will be discussed in the following section.

Now that it has been firmly established that Calcein reacts with aluminum in a 2:3 mole ratio, a potentiometric titration of Calcein in the presence of 1 1/2 mole of aluminum chloride was carried out in a totally aqueous solution. Because Calcein is rather insoluble in water, a measured excess of sodium hydroxide was added to the mixture of Calcein and aluminum chloride (in a 1:1.5 or 2:3 mole ratio), and after complete dissolution, the solution was back-titrated with standard hydrochloric acid. No precipitation occurred at any point, indicating complete conversion of the components into the 2:3 Calcein-aluminum compound. The back-titration is presented in Figure 45.

The mixture resulting from the back-titration was again titrated in the forward direction by using sodium hydroxide. The obtained curve is depicted in Figure 46. In reality, the titration curve in Figure 46 is for the same conditions as

Figure 45. Potentiometric back-titration of Calcein in the presence of aluminum chloride (1:1.5 mole ratio)

Calcein: 0.1129 g (0.1764 mmoles)

Aluminum chloride: 2.66 ml of 0.0995 M solution (0.2646 mmoles)

Solvent: 100 ml water
plus 18.00 ml of 0.1211 N sodium hydroxide

Titrant: 0.1158 N hydrochloric acid

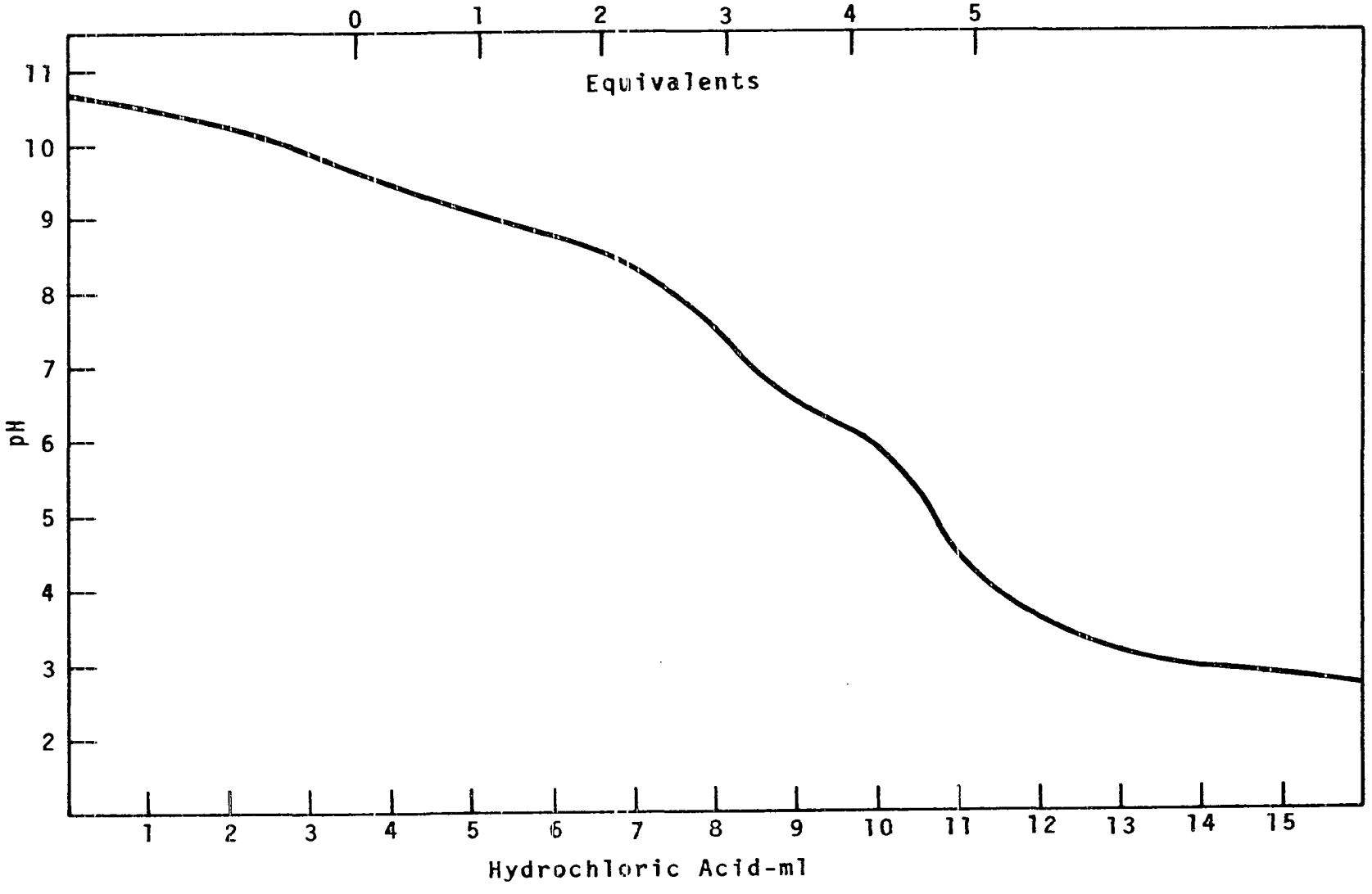

0
1
2
3
4
5
Equivalents
11
10
9
8
7
6
5
4
3
2
pH
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Hydrochloric Acid-ml

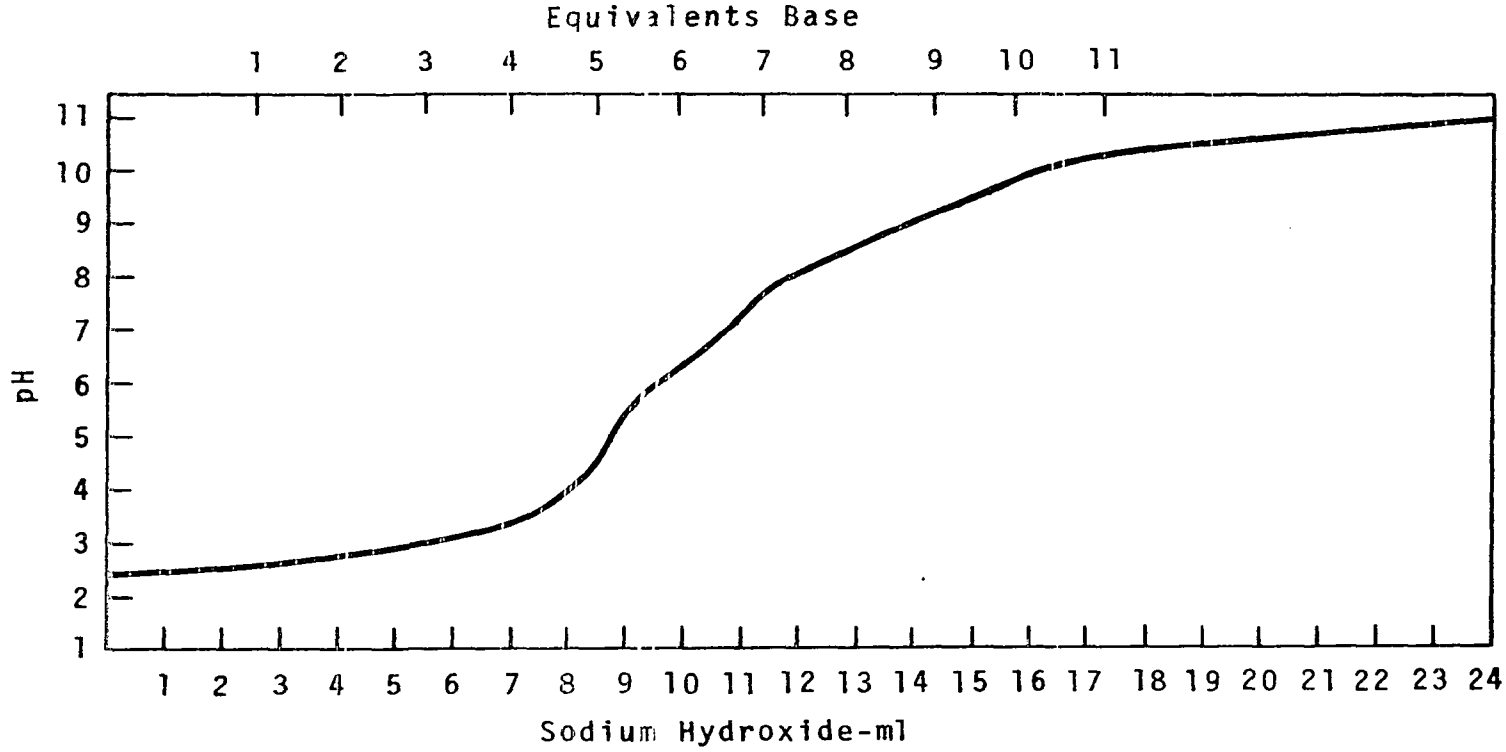
Figure 46. Potentiometric titration of Calcein in the presence of aluminum chloride (1:1.5 mole ratio)

Calcein: 0.1129 g (0.1764 mmoles)

Aluminum chloride: 2.66 ml of 0.0995 M solution (0.2646 mmoles)

Solvent: 100 ml water plus 18.00 ml of 0.1211 N sodium hydroxide plus 20.00 ml of 0.1158 N hydrochloric acid

Titrant: 0.1211 N sodium hydroxide


Equivalents Base
1 2 3 4 5 6 7 8 9 10 11
pH
11
10
9
8
7
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Sodium Hydroxide-ml

that obtained for Figure 41, Curve d, except that this time the solvent was water. In calculating concentrations, the amount of excess sodium hydroxide originally added, the amount of hydrochloric acid expended for the back-titration, and the accompanying dilution effects have been taken into account.

The pH at the start of the titration curve in Figure 46 is 2.5. Since neither Calcein by itself nor aluminum chloride alone produce such a low pH in aqueous solution, it is obvious that a reaction has taken place between Calcein and aluminum, even in an acid medium, accompanied by the release of hydrogen ions which subsequently lower the pH. The first end-point, occurring at pH 4.70, requires 8.60 ml of sodium hydroxide, fairly close to the theoretical amount of 8.42 ml for 5 equivalents. At this point Calcein has combined with aluminum in a 2:3 mole ratio, and the 5 equivalents of released hydrogen ions have been titrated.

A second end-point, occurring at pH 7.55, requires 11.20 ml of base; the theoretical amount of base required for 7 equivalents of hydrogen ions titrated at this point is 11.34 ml. A very weak and drawn out stretch of the titration curve which could possibly be interpreted as an end-point, occurs at pH 9.8 for about 16 ml of base. The theoretical amount of base required for an end-point at 10 equivalents is 15.72 ml. Further evidence that the drawn-out portion of

the titration curve at 15.72 ml could be an end-point can be deduced from the back-titration curve in Figure 45. If there is a stoichiometric reaction at 10 equivalents, then there should be an end-point at 3.56 ml of hydrochloric acid, the amount of acid equivalent to the amount of base originally added in excess of 10 equivalents, in the back-titration curve. Such indeed is the case. In addition to the end-point at about 3.56 ml by hydrochloric acid, there is an end-point at about 8.2 ml and another at about 10.9 ml, corresponding to 3 and 5 equivalents of acid added, respectively.

The reactions involved at each end-point of the forward titration curve in Figure 46 are:

First end-point, at 5 equivalents:

$$H_6Cal + 1\ 1/2\ Al^{3+} = 1/2\ Al_3(HCal)_2^- + 5H^+$$

Second end-point, at 7 equivalents:

$$1/2\ Al_3(HCal)_2^- + OH^- = 1/2\ Al_3(Cal)_2^{3-}$$

$$1/2\ Al_3(Cal)_2^{3-} + OH^- = 1/2\ Al_3(OH)_2Cal_2^{5-}$$

Third end-point, at 10 equivalents:

$$1/2\ Al_3(OH)_2Cal_2^{5-} + 3OH^- + 2H_2O = H_2Cal^{4-} + 1\ 1/2\ Al(OH)_4^-$$

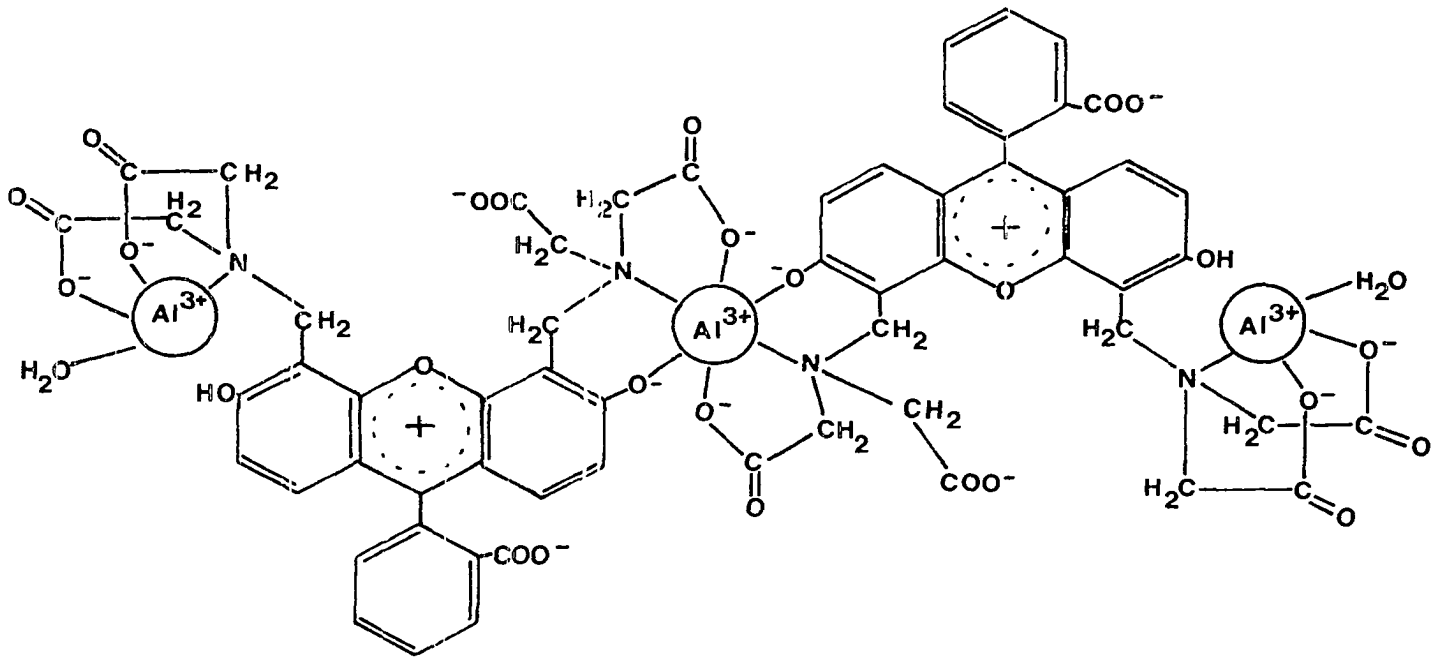
The reaction occurring at the third end-point needs further clarification. As more base is added beyond the second end-point, the Calcein-aluminum compound is decomposing. In

the process, $Al(OH)_4^-$ and H_2Cal^{4-}, the ionic species of Calcein existing at the end of a titration in water, are produced. The detailed chemistry involved in the stepwise addition of hydroxide ions to the Calcein-aluminum compound will be discussed in the section dealing principally with the structure of the Calcein-aluminum compound.

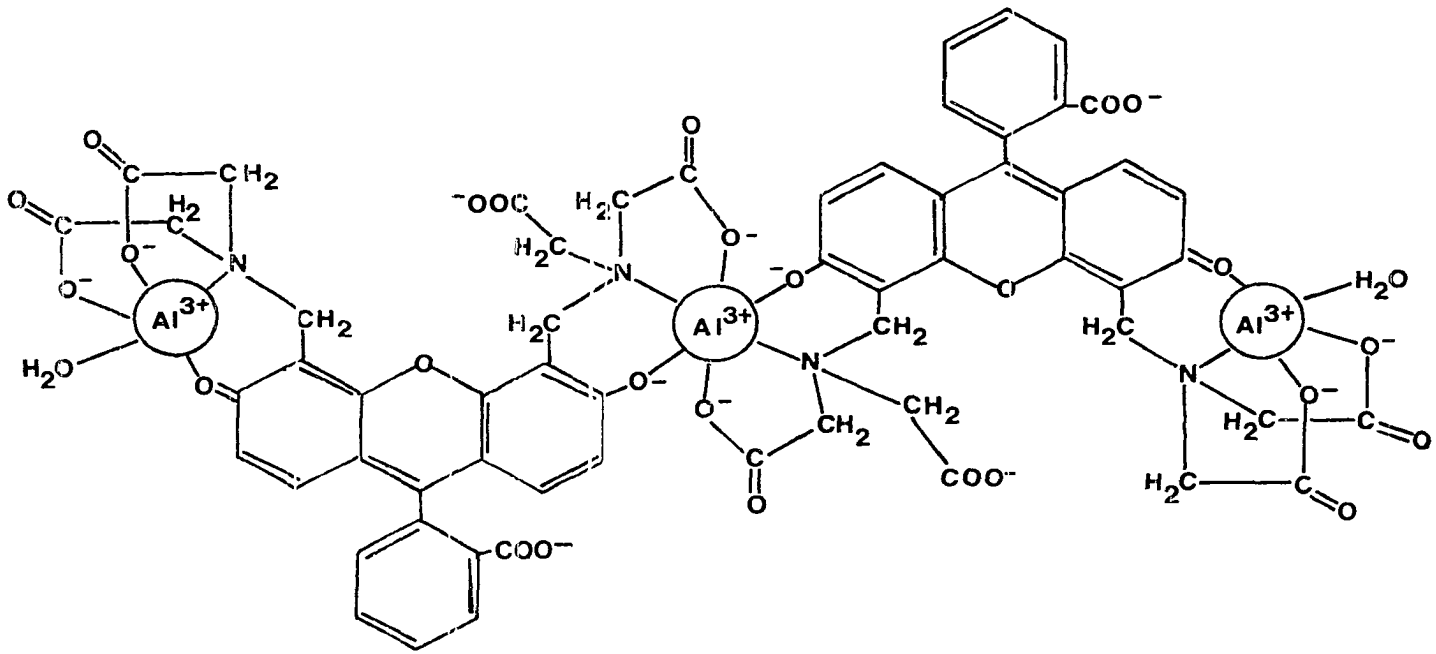
5. Structure of the Calcein-aluminum compound

The structures XL, XLI, and XLII are presented for the 2:3 Calcein-aluminum compounds existing at various values of pH. At pH 4.7, the first end-point in Figure 46, at which two molecules of Calcein have combined with three aluminum ions to produce $Al_3(HCal)_2^-$ with the accompanying release of 5 equivalents of hydrogen ions per mole of Calcein, Structure XL best describes the compound formed.

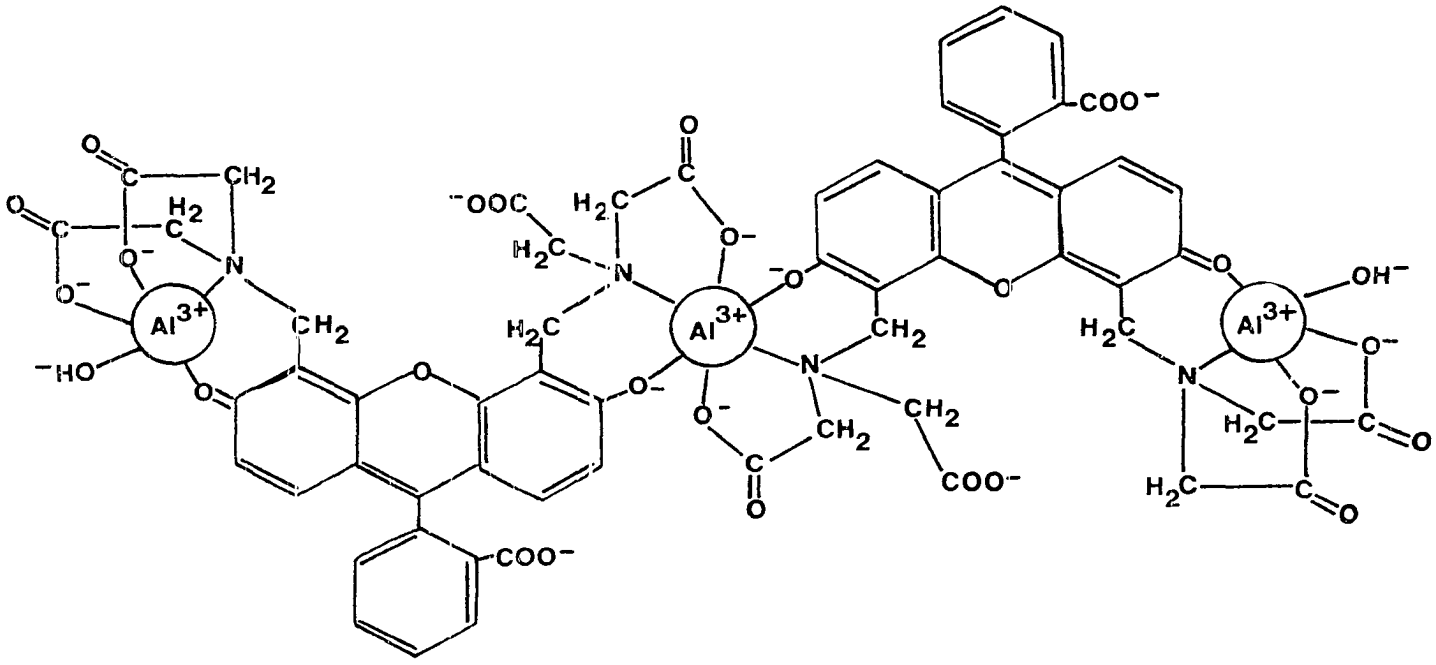
It is not unusual for aluminum to form polynuclear complexes. Therefore, Structure XL is not out of line with current understanding of the coordination chemistry of aluminum. Two types of aluminum atoms exist in the compound with Structure XL. The aluminum atom which is bridging the two Calcein molecules is hexacoordinated, to the carboxylate group, the nitrogen atom, and the phenolate group of each Calcein molecule. Each Calcein portion of the Calcein-aluminum compound has one free carboxylate group which is not bound to the aluminum atom. An uncoordinated carboxylate



XL. $Al_3(HCal)_2^-$, the 2:3 Calcein-aluminum compound at pH ∿5



XLI. $Al_3(Cal)_2^{3-}$, the 2:3 Calcein-aluminum compound at pH ~ 6.4



XLII. $Al_3(OH)_2Cal_2^{5-}$, the 2:3 Calcein-aluminum compound at pH ∿ 7.5

ligand is not unusual; it was encountered previously for the EDTA-aluminum compounds.

The other two aluminum atoms exist in a totally different environment. Each is bound to the two carboxylate functions and to the nitrogen of the remaining methyleneiminodiacetic acid groups. At this point, the second phenolic group of each Calcein portion (with a pK value of 6.19) has not been neutralized as yet, nor has it entered into coordination with the aluminum ion. Because there are only three bonds established between the aluminum ion and the iminodiacetic acid portion of Calcein, the remaining three coordination sites of aluminum are probably occupied by water molecules. Only one such water molecule is shown in Structure XL for the sake of simplicity.

Between the first and second end-points of the titration curve (Figure 46), that is between pH 4.7 and 7.5, two more equivalents of base are consumed. One equivalent of base is used up by the neutralization of the second remaining phenolic group (which has a pK value of 6.19). During this neutralization process, the Calcein molecule assumes the fluorescent para-quinone structure, with the resonance forms, and the resonating phenolate-quinone oxygen enters into coordination with the aluminum ion to produce Structure XLI. The fluorescence of the Calcein-aluminum compound at this point is maximum (see Section III.D.)

The second equivalent of base is consumed by the conversion of the $Al_3(Cal)_2^{3-}$ species into $Al_3(OH)_2Cal_2^{5-}$, which is represented by Structure XLII. This type of reaction has been discussed for the chemistry of the EDTA-aluminum compound (Section III.C.4). The pK for this type of reaction is probably not much different from that of the EDTA-aluminim compound (pK 5.89-6.16). Thus, the reaction is within the pH range between the first and the second end-points.

Since both reactions are characterized by pK values of similar magnitude, they probably occur simultaneously between pH 4.7 and 7.5. Thus it is expected that both Structure XLI and XLII exist similtaneously in that pH range. It is interesting to note that the mid-point between the first and second end-points, occurring at pH of about 6.3, corresponds to the pH of maximum fluorescence of the Calcein-aluminum compound (Figure 48), discussed in Section III.D.

6. Estimation of the overall formation constant of the 2:3 Calcein-aluminum compound

The potentiometric data of the titration curve in Figure 46 were used to calculate an approximate value for the overall formation constant of the 2:3 Calcein-aluminum compound in aqueous solution. The method is basically that of Schwarzenbach and coworkers,[124] as modified by Hefley.[58] Because of the highly complex nature of the reaction of aluminum with Calcein, it was necessary to make several assumptions which

can result in a formation constant that must be looked upon only as an estimation. The assumptions are that 1) the 2:3 Calcein-aluminum compound is formed directly and not stepwise, 2) aluminum exists in solution as the free metal ion without formation of any hydroxy species, 3) only the H_6Cal and $HCal^{5-}$ species of Calcein are important in the reaction, and 4) the $HCal^{5-}$ species is described by the Calcein anion with the remaining hydrogen atom being that of the second phenolic function, that is the hydrogen atom corresponding to K_4.

The pertinent acid dissociation constants of Hefley[58] and the accompanying equations defining the ionization steps of Calcein, presented previously on pp. 211-212, are again summarized to facilitate the discussion:

$$(1)\quad H_6Cal = H_5Cal^- + H^+ \qquad K_1 = \frac{[H^+][H_5Cal^-]}{[H_6Cal]} = 1.84 \times 10^{-3}$$

$$(2)\quad H_5Cal^- = H_4Cal^{2-} + H^+ \qquad K_2 = \frac{[H^+][H_4Cal^{2-}]}{[H_5Cal^-]} = 3.4 \times 10^{-4}$$

$$(3)\quad H_4Cal^{2-} = H_3Cal^{3-} + H^+ \qquad K_3 = \frac{[H^+][H_3Cal^{3-}]}{[H_4Cal^{2-}]} = 2.6 \times 10^{-5}$$

$$(4)\quad H_3Cal^{3-} = H_2Cal^{4-} + H^+ \qquad K_4 = \frac{[H^+][H_2Cal^{4-}]}{[H_3Cal^{3-}]} = 6.4 \times 10^{-7}$$

$$(5)\quad H_2Cal^{4-} = HCal^{5-} + H^+ \qquad K_5 = \frac{[H^+][HCal^{5-}]}{[H_2Cal^{4-}]} = 1.3 \times 10^{-10}$$

$$(6)\quad HCal^{5-} = Cal^{6-} + H^{+} \qquad K_6 = \frac{[H^+][Cal^{6-}]}{[HCal^{5-}]} = 2.3 \times 10^{-12}$$

The overall formation of the compound can be described by

$$3\ Al^{3+} + 2\ H_6Cal = Al_3(HCal)_2^- + 10\ H^+ \qquad (7)$$

or

$$3\ Al^{3+} + 2\ HCal^{5-} = Al_3(HCal)_2^- \qquad (8)$$

and the overall formation constant K_f is

$$K_f = \frac{[Al_3(HCal)_2^-]}{[Al^{3+}]^3[HCal^{5-}]^2} \qquad (9)$$

Now the equilibrium constant for the simultaneous dissociation

$$H_6Cal = HCal^{5-} + 5\ H^+ ,$$

where $HCal^{5-}$ is the form discussed above under assumption 4), is

$$K_1K_2K_3K_5K_6 = \frac{[HCal^{5-}][H^+]^5}{[H_6Cal]} = 10^{-32.37} \qquad (10)$$

Rearranging yields

$$[HCal^{5-}] = \frac{10^{-32.37}[H_6Cal]}{[H^+]^5} \qquad (11)$$

which is substituted into equation (9) to yield, after rearrangement, the equation for the formation constant

$$K_f = \frac{[Al_3(HCal)_2^-][H^+]^{10}}{10^{-64.74}[Al^{3+}]^3[H_6Cal]^2} \qquad (12)$$

In the titration curve (Figure 46) it is assumed that at the mid-point the amount of H_6Cal left is one-half of the amount originally present, that the reaction between aluminum and Calcein is complete, and thus there is no $HCal^{5-}$ left in solution.

At the mid-point, the pH is 2.88 and the other values were calculated from the titration data by taking into consideration any dilution effects:

$$[Al_3(HCal)_2^-] = 2.41 \times 10^{-4}$$

$$[Al^{3+}] = 7.23 \times 10^{-4}$$

$$[H_6Cal] = 4.82 \times 10^{-4}$$

Thus

$$K_f = \frac{(2.41 \times 10^{-4})(10^{-2.88})10}{(10^{-64.74})(7.23 \times 10^{-4})^3(4.8 \times 10^{-4})^2}$$

$$K_f = 2.4 \times 10^{48}$$

Although the value may seem high at first glance, it is reasonable when compared to the formation constant of the aluminum compound with EDTA, for which $K^{Al}_{AlY^-}$ is $10^{16.13\ 125}$ or $10^{16.68\ 98}$.

7. Conclusions

The nature of the chelating reaction between Calcein and aluminum has been studied by potentiometric titrations of

Calcein by sodium hydroxide in the presence of varying mole ratios of aluminum. The insolubility of Calcein was overcome by using 50 per cent ethyl alcohol as solvent for the titrations. The acidic nature of the aluminum cation has been reexamined by potentiometric titration of aluminum with alkali in aqueous and ethanolic media. Despite the formation of insoluble aluminum hydroxide, a surprisingly sharp and accurate end-point, occurring at three equivalents of alkali, has been obtained for the titration of aluminum chloride. A second end-point, though weak but discernible, corresponding to one additional equivalent of alkali consumed, has been observed during the titration of aluminum in 50 per cent ethyl alcohol. Beyond the end-point corresponding to the three equivalents of alkali, the gradual disappearance of turbidity has been attributed to the dissolution of aluminum hydroxide and simultaneous formation of the aluminate ion, $Al(OH)_4^-$.

The family of curves obtained by titrating Calcein in the presence of various mole ratios of aluminum has been used to elucidate the combining ratio of Calcein to aluminum. In addition, the reaction of EDTA and aluminum has been reexamined, also in 50 per cent ethyl alcohol, to help interpret the obtained results. Calcein reacts with aluminum in a 2:3 mole ratio. The stoichiometry was adduced on the basis of the number of end-points, their relative positions, the

appearance and disappearance of turbidity, and the general shape of the titration curves obtained. Equations relating the neutralization equivalents to the acid-releasing and acid-consuming reactions have been presented for each titration curve.

The nature and structure of the 2:3 Calcein-aluminum compound has been discussed. The Calcein-aluminum chelate has of itself acidic properties, as evidenced by the uptake of additional alkali. The acidic nature of the Calcein-aluminum chelate has been ascribed to two sources. In one, a water molecule in the coordination sphere of the bound aluminum atom is converted by alkali to a hydroxide; such a reaction is common to the analogous EDTA-aluminum compound. The second source of an acidic hydrogen atom is ascribed to the simultaneous neutralization of the phenolic group remaining on the Calcein portion of the compound. The combined neutralization reactions from these two sources explain the existence of a second end-point in the alkalimetric titration of Calcein in the presence of a stoichiometric amount (i.e., a mole ratio of 1:1.5) of aluminum chloride.

An accurate titration of the stoichiometric amounts of Calcein and aluminum has been obtained in water. The vexation of insolubility was overcome by first dissolving the measured amounts of Calcein plus aluminum chloride in a

known amount of excess alkali, then back-titrating the mixture to a predetermined point, and finally titrating the completely dissolved substances in the forward direction by alkali.

From the data obtained by this titration in water, the overall formation constant for the 2:3 Calcein-aluminum compound has been estimated. The assumptions included: 1) that the 2:3 Calcein-aluminum compound was formed simultaneously and not stepwise, 2) the neglect of any possible hydroxy species of aluminum, 3) the belief that only the H_6Cal and $HCal^{5-}$ species of Calcein were important in the compound formation, and 4) that the acidic function remaining in the $HCal^{5-}$ species belonged to the second phenolic group and its acidity was defined by K_4. Because of the many major assumptions, the value of the formation constant for the reaction $3\ Al^{3+} + 2\ HCal^{5-} = Al_3(HCal)_2^-$, estimated as 2.4×10^{48}, is only an approximation.

The application of potentiometric titrations to obtain a family of curves for various mole ratios of Calcein to aluminum has proved a fruitful method in characterizing an otherwise complicated reaction system involving acidic properties of Calcein, acidic properties of aluminum itself, insolubility of Calcein, and precipitation of aluminum hydroxide. The additional line of evidence drawn from analogous reactions of EDTA with aluminum was also helpful in elucidating some of the reaction steps.

D. Fluorescence of Calcein plus Aluminum

1. Experimental

a. Fluorescence as a function of pH A series of 39 buffers in the pH region 1.5 to 13.0 was prepared using 0.1 M solutions of hydrochloric acid, potassium chloride, potassium acid phthalate, boric acid, and potassium hydroxide. The buffer combination for each pH range was as follows:

pH range	buffer combination of 0.1 M
1.5 - 2.5	HCl, KCl
3.0 - 3.7	HCl, KHP
4.0 - 6.2	KOH, KHP
6.5 - 7.0	KHP, KOH, H_3BO_3
7.3 -13.0	H_3BO_3, KOH

Use of potassium salts in the preparation of buffers minimized residual fluorescence of Calcein, while use of 0.1 M solutions assured a constant ionic strength. The pH of the buffer solutions was measured before and after dilution, using a Corning Model 10 pH meter, a Beckman glass electrode, and a saturated calomel electrode as reference. The pH meter was standardized with standard buffer solutions.

A fresh stock solution of Calcein was prepared from the pure compound, analyzed previously, by dissolving 6.4 mg in 100 ml of deionized water. The resulting solution, 10^{-4} M in Calcein, was used within a few hours after preparation.

A segment of aluminum wire (Alcoa, 99.99 per cent pure) was sanded with emery paper to remove any oxide. A portion of it, weighing 0.3193 g, was dissolved in 10 ml of concentrated hydrochloric acid with mild heating, and then diluted to 1 liter. A 10-ml aliquot of this concentrate, diluted to 1 liter, produced a stock solution that was 1.183×10^{-4} M in aluminum.

A solution of 0.01 M EDTA was prepared by dissolving 7.48 g of disodium (ethylenedinitrilo)tetraacetic dihydrate in 2 l. of water. A 3.0-ml aliquot of this solution, diluted to 1 l., produced a stock solution that was 3×10^{-5} M in EDTA.

Using 50-ml volumetric flasks, two sets of buffered series were prepared. Into the first set, 1 ml of the stock solution of Calcein, 1 ml of the EDTA solution, and 2 ml of the stock solution of aluminum were added and the appropriate buffer was used to dilute to mark. The second set was prepared in the same manner, only without any aluminum added. After dilution, the volumetric flasks contained solutions that were 2×10^{-6} M in Calcein, 6×10^{-7} M in EDTA, and 4.7×10^{-6} M in aluminum. Studies of fluorescence were made using a Bowman-Keirs Spectrophosphorimeter with an attached Moseley Autograf X-Y Recorder; the quartz cells used were 1.00 cm in path length. Excitation spectra were obtained with the emission monochromator set at 510 nm, while emission spectra were obtained with the excitation

monochromator set at 490 nm. Relative fluorescence measurements were made using several sensitivities of the instrument, excitation set at 490 nm and emission at 510 nm. Measurements in the alkaline region had to be made immediately after mixing, because the residual fluorescence insreases noticeably with time. The residual fluorescence was seen even in the presence of small amounts of EDTA and probably resulted from the leaching of alkaline earth ions from the glassware. Larger amounts of EDTA suppressed this fluorescence which was greatest in the alkaline region of pH.

b. Fluorescence as a function of aluminum concentration A series of solutions was prepared in 25-ml volumetric flasks using varying amounts of aluminum and a constant amount of Calcein, dissolved in a buffer of pH 2.5. The solutions were prepared as follows: 1 ml of the solution 10^{-4} M in Calcein was added to each flask, then the solution 1.183×10^{-4} M in aluminum in consecutive increments of 0.1 ml; the flask was diluted to volume with the buffer of pH 2.5, consisting of 0.1 M hydrochloric acid and 0.1 M potassium chloride. Fluorescence measurements were made as before, with the excitation monochromator set at 490 nm and the emission measured at 510 nm.

c. Fluorescence of Calcein plus aluminum as a function of time The relative fluorescence measurements on solutions containing 4×10^{-6} M Calcein and varying amounts of 10^{-6} M aluminum were made immediately after mixing, 5 hours

after mixing, and 50 hours after mixing. The monochromator settings of the spectrophosphorimeter remained unchanged at 490 nm for excitation and 510 nm for emission. There was a visible difference in the solutions after standing; most of the greenish color has disappeared.

2. Results and discussion

The relative fluorescence of Calcein alone (Curve a) and of Calcein in the presence of excess aluminum (Curve b) as a function of pH is presented in Figure 47. The fluorescence of Calcein as a function of pH follows the pattern established previously by Hefley;[58] the fluorescence begins to rise at about pH 4, attains a maximum between pH 7 and 9, and then decreases until it is zero at pH 13. The fluorescence of Calcein in the presence of excess aluminum rises more steeply in the acid pH region. It is maximum at pH 6.0-6.5. After pH 7, the fluorescence curve assumes the shape of the curve for free Calcein, and by pH 9 the two curves are superimposable.

An interesting result is obtained (Figure 48) when a potentiometric titration curve (Figure 46) is superimposed on the fluorescence curve of the Calcein-aluminum compound. The maximum fluorescence of the Calcein-aluminum compound, found at pH 6.0-6.5, corresponds to the mid-point between the first and second end-points of the titration curve, approximately pH 6.3. At that point, the concentration of

Figure 47. Fluorescence of Calcein and of Calcein plus aluminum as a function of pH

Curve a. Calcein (2×10^{-6} M)

Curve b. Calcein (2×10^{-6} M) plus aluminum (4.7×10^{-6} M)

Excitation at 490 nm

Emission at 510 nm

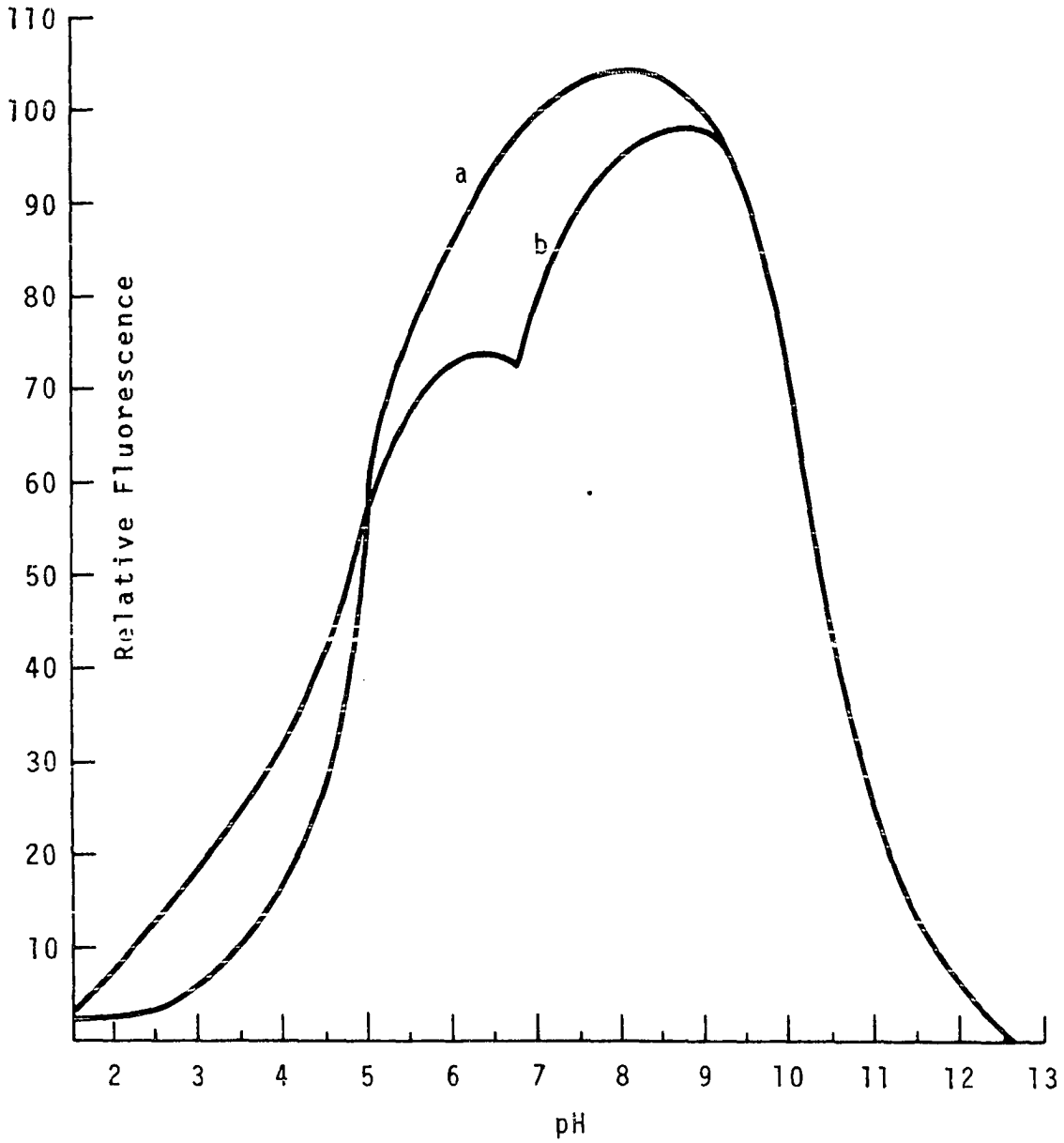
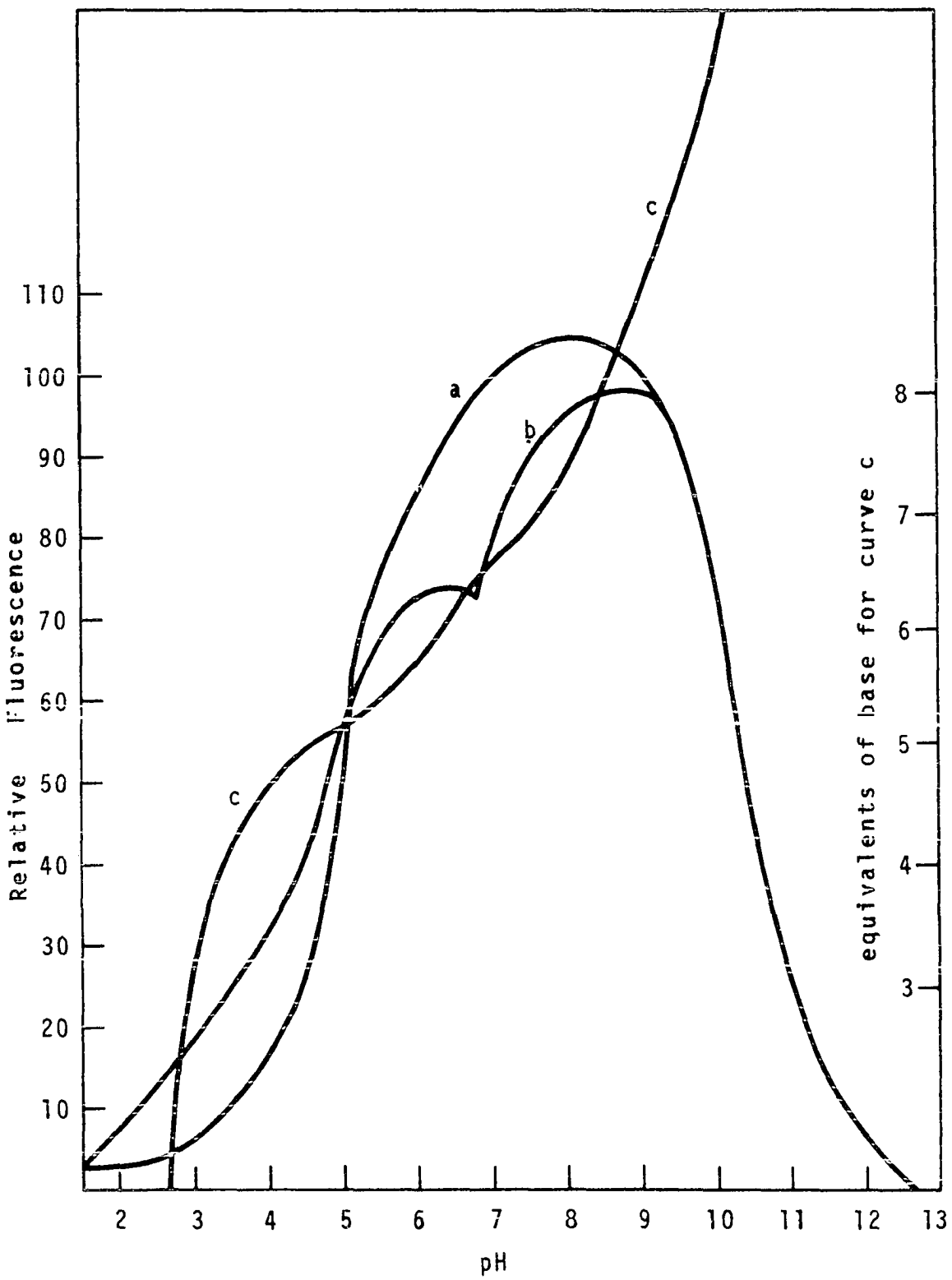

110
100
90
80
70
60
50
40
30
20
10
Relative Fluorescence
a
b
2
3
4
5
6
7
8
9
10
11
12
13
pH

Figure 48. Fluorescence of Calcein and of Calcein plus aluminum superimposed on the curve of the titration of Calcein plus aluminum as a function of pH

Curve a. Same as Figure 47

Curve b. Same as Figure 47

Curve c. Same as Figure 46


Relative Fluorescence
110
100
90
80
70
60
50
40
30
20
10
a
b
c
c
2
3
4
5
6
7
8
9
10
11
12
13
pH
8
7
6
5
4
3
equivalents of base for curve c

the Calcein-aluminum compound with Structure XLI (p. 275) is maximum. The second end-point, occurring at pH 7.55, coincides fairly close to the point at which the fluorescence curve of the aluminum compound is overtaken by the fluorescence curve of Calcein. At the pH of the faint third end-point, pH 9.8, the two fluorescence curves are totally indistinguishable, indicating that all the aluminum has been extracted from the Calcein-aluminum compound and the fluorescence of the mixture behaves essentially as that of free Calcein.

The fluorescence of Calcein as function of the amount of aluminum added is presented in Figure 49. The curve (Curve a) is nonlinear, in contradiction to the observations made by Wallach and Steck.[150] In addition, the curve shows significant decay in fluorescence with time. After 5 hours (Curve b), the fluorescence intensity was reduced to less than one-half of the original value. After fifty hours (Curve c), the solutions were characterized only by background fluorescence, indicating complete degradation of the fluorescing species.

Because the ratio of aluminum combining with Calcein could not be determined at pH 1.5 (Figure 49), an experiment was designed to plot the fluorescence at a pH at which the Calcein-aluminum compound fluoresces to a maximum degree. At the same pH, 6.4, the free Calcein fluoresces significantly

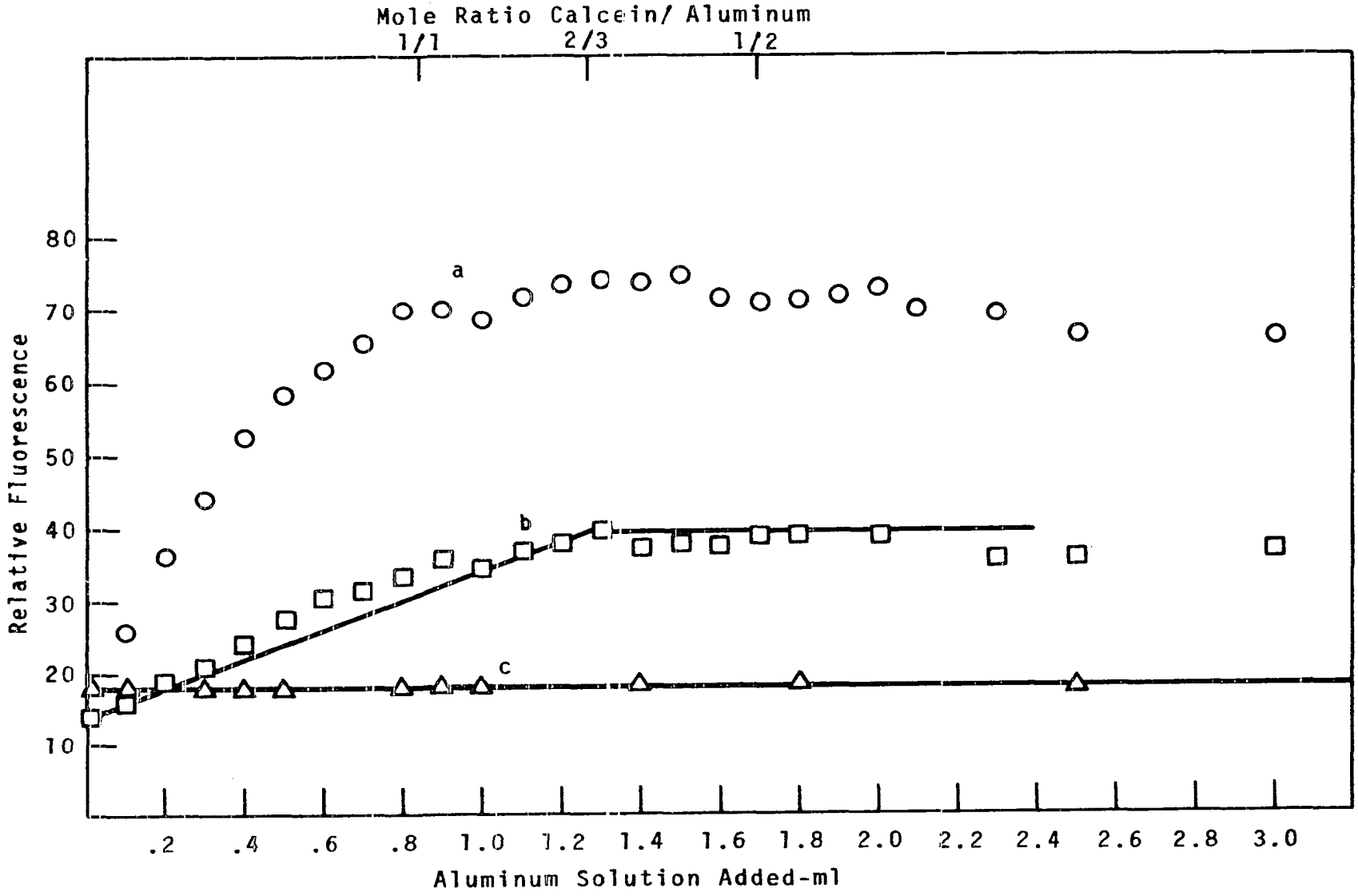
Figure 49. Fluorescence of Calcein plus aluminum as a function of the concentration of aluminum and as a function of time

One ml of 10^{-4} M solution of Calcein plus increments of 1.183×10^{-4} solution of aluminum diluted to 25.00 ml with a buffer of pH 2.5

Curve a. Immediately after mixing

Curve b. Five hours after mixing

Curve c. Fifty hours after mixing


Mole Ratio Calcein/ Aluminum
1/1
2/3
1/2
Relative Fluorescence
80
70
60
50
40
30
20
10
a
b
c
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
Aluminum Solution Added-ml

more intensely. The fluorescence at pH 6.4, plotted as a function of the increasing concentration of Calcein, is presented in Figure 50. The plot consists of two straight-line portions drawn by means of the least-squares method. The point of intersection corresponds to a 2:3 ratio of Calcein to aluminum.

3. Conclusions

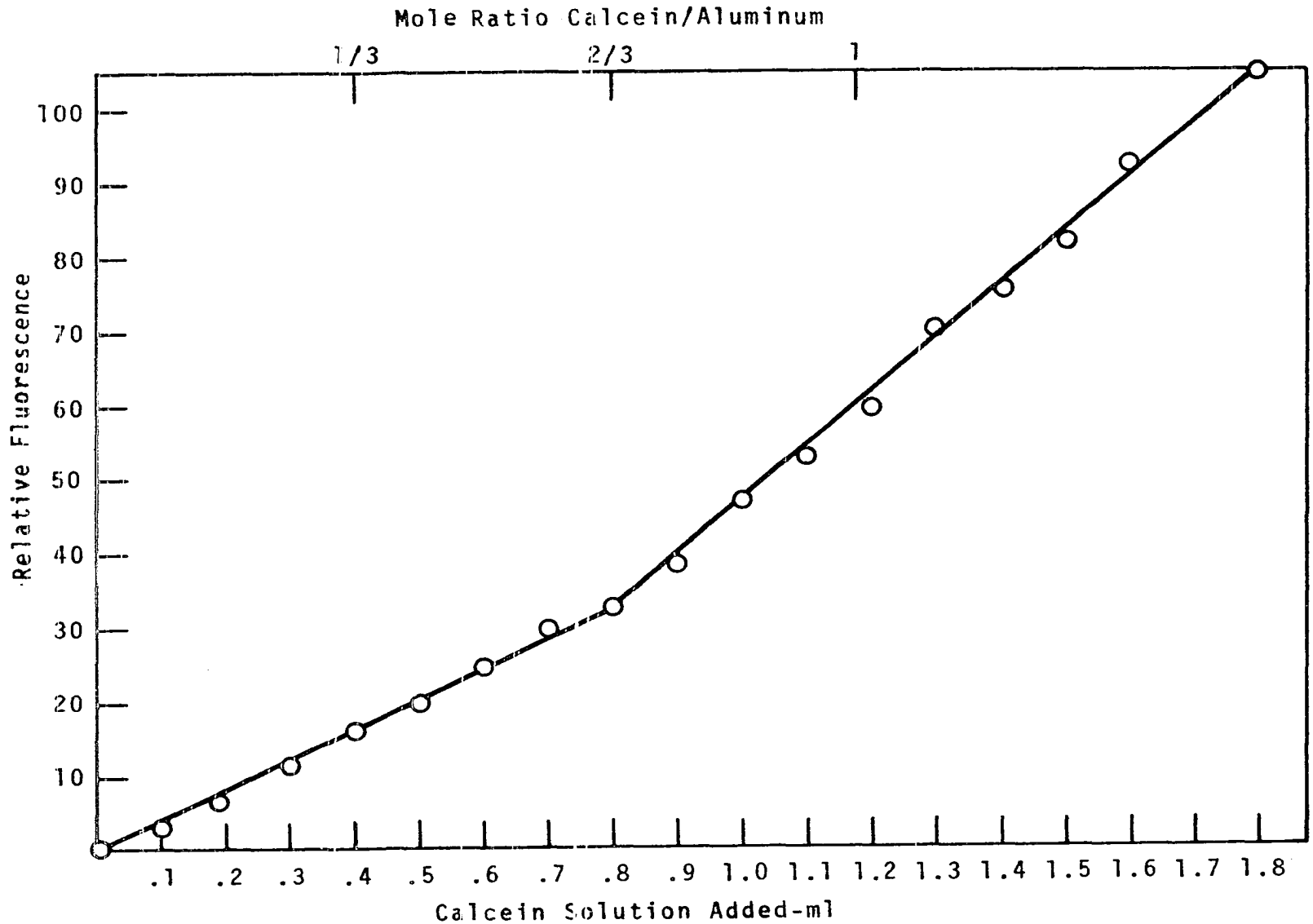
The fluorescence of the Calcein-aluminum compound is slightly different from that of free Calcein. While it is true that the fluorescence of Calcein is increased at low pH by the addition of aluminum ions, the increase is not very great and the plot of fluorescence vs. concentration of aluminum added is not linear. Furthermore, the fluorescence decays rapidly with time.

The fluorescence of the Calcein-aluminum compound as a function of pH does have some relation to the nature of the species present at a particular pH. Presumably, the Calcein-aluminum compound with Structure XLI (p. 275) existing at about pH 6.0-6.5, is the most intensely fluorescing species.

A plot of fluorescence vs. increasing concentration of Calcein in the presence of a fixed amount of aluminum resulted in two straight lines which intersect at a point corresponding to a 2:3 Calcein-to-aluminum ratio. The combining ratio of Calcein with aluminum is the same as

Figure 50. Fluorescence of Calcein plus aluminum as a function of the concentration of Calcein

One ml of 1.183×10^{-4} M aluminum chloride solution plus increments of 10^{-4} M Calcein solution diluted to 25.00 ml with a buffer of pH 6.4


Mole Ratio Calcein/Aluminum
1/3
2/3
1
Relative Fluorescence
100
90
80
70
60
50
40
30
20
10
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
Calcein Solution Added-ml

that obtained by the analysis of potentiometric titration data (Section III.C.3.b.).

E. Reaction of Calcein with Mercury

The method of potentiometric titrations of Calcein with alkali in the presence of varying mole ratios of a metal ion has been shown to be successful in establishing the combining ratio of Calcein to the metal, even when highly complicated equilibria make the reaction quite different from the expected one. Such was the case in the reaction of Calcein with aluminum, discussed in Section III.C. The application of the method was not only fruitful in establishing the combining ratio but also in estimating the formation constant.

It was thought interesting to apply the same method to a simpler case, namely the reaction of Calcein with mercury. Mercury is known to react with Calcein to produce a fluorescent metal chelate. In that respect, mercury is an exception from the usual behavior of transition metals, which quench the fluorescence of Calcein at neutral pH, and for that reason can be classified more closely with the alkaline earth metals.

As yet no data on the reaction of mercury with Calcein are available. The experiments performed in this section are only of an exploratory nature, yet the results are interesting enough to warrant some discussion.

1. Potentiometric titrations

a. Experimental The potentiometric titration of Calcein in the presence of varying mole ratios of mercury(II) has been carried out by a procedure similar to the one used in the study of the reaction of Calcein with aluminum.

A stock solution of Calcein was prepared by dissolving 0.2611 g of the highly pure Calcein in 50 per cent ethyl alcohol and diluting the solution to exactly 250 ml. A 50-ml aliquot of this solution, containing 8.15×10^{-5} moles of Calcein, was diluted with 50 ml of water, thus making the alcohol concentration in the final titration mixture 25 per cent.

A stock solution of mercury(II) was prepared by dissolving 2.715 g of mercuric chloride in deionized water and diluting to exactly 1 l. The appropriate number of ml of this stock solution, being 0.0100 M in Hg(II), was added to the mixture to be titrated to provide the desired mole ratio of Calcein to mercury.

A 10-ml aliquot of the mercury solution, without any Calcein, was also titrated with the 0.1218 N sodium hydroxide in order to establish the acidic nature of mercury(II) itself.

The pH of the titration was followed with a Corning Model 10 pH meter, equipped with a glass electrode and a saturated calomel electrode for reference.

b. Results and discussion The titration of mercury(II) by sodium hydroxide is represented by the curve in Figure 51. It is evident that mercury(II) chloride is an acidic substance that can be titrated with alkali, requiring two equivalents of alkali at the equivalence point.

The titration curves of Calcein and of Calcein with 1:1, 1:2, and 1:3 mole ratios of Calcein to mercury are presented in Figure 52, Curves a,b,c, and d, respectively. Curve a, representing the titration of Calcein, displays the typical double break, discussed in Section III.B; the two breaks correspond to two and to four equivalents of base, respectively.

Curve b represents the titration of Calcein in the presence of 1:1 mole ratio of mercury(II). There is a drawn-out end-point at 5 equivalents and a sharper end-point at 6 equivalents of base. Evidently one molecule of Calcein has combined with one mercury ion to form the compound $HgHCal^{3-}$. By analogy to the reaction of Calcein with 1 calcium ion, the reaction can be considered to take place between H_2Cal^{4-} and Hg^{2+} to form $HgHCal^{3-}$, with the accompanying release of one additional hydrogen ion. The neutralization of this hydrogen ion, together with the four hydrogen ions neutralized to produce H_2Cal^{4-}, accounts for the end-point at 5 equivalent.

A remarkable change in the acid strength of the hydrogen atom remaining in $HgHCal^{3-}$ is seen in the end-point at 6

Figure 51. Potentiometric titration of mercury(II) chloride

Mercury(II) chloride: 10 ml of a 0.0100 M solution

Titrant: 0.1218 N sodium hydroxide

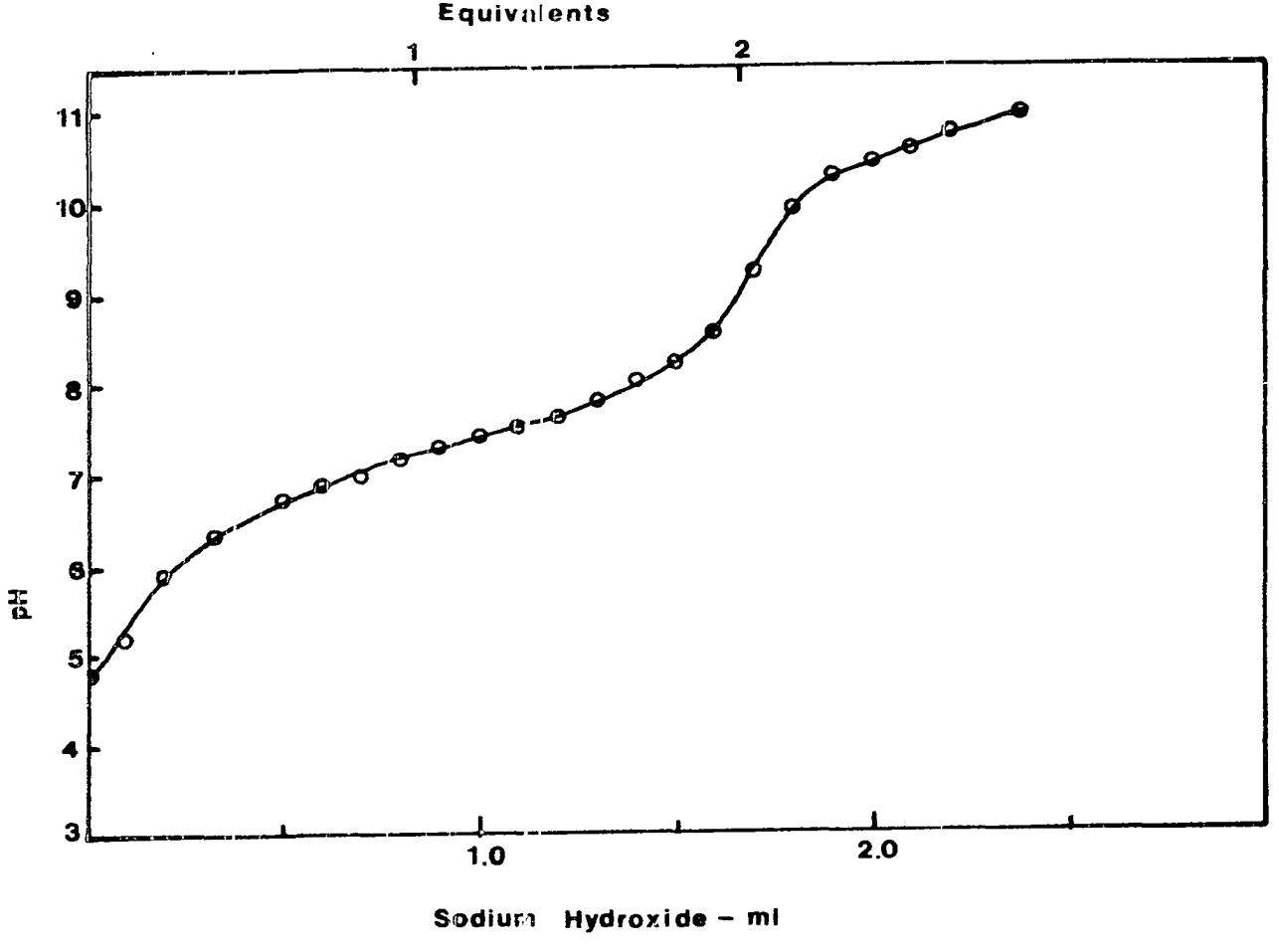

Equivalents
1
2
11
10
9
8
7
6
5
4
3
pH
1.0
2.0
Sodium Hydroxide – ml

Figure 52. Potentiometric titration of Calcein in the presence of varying amounts of mercury(II) chloride

Curve a. Calcein alone (8.15×10^{-5} moles)

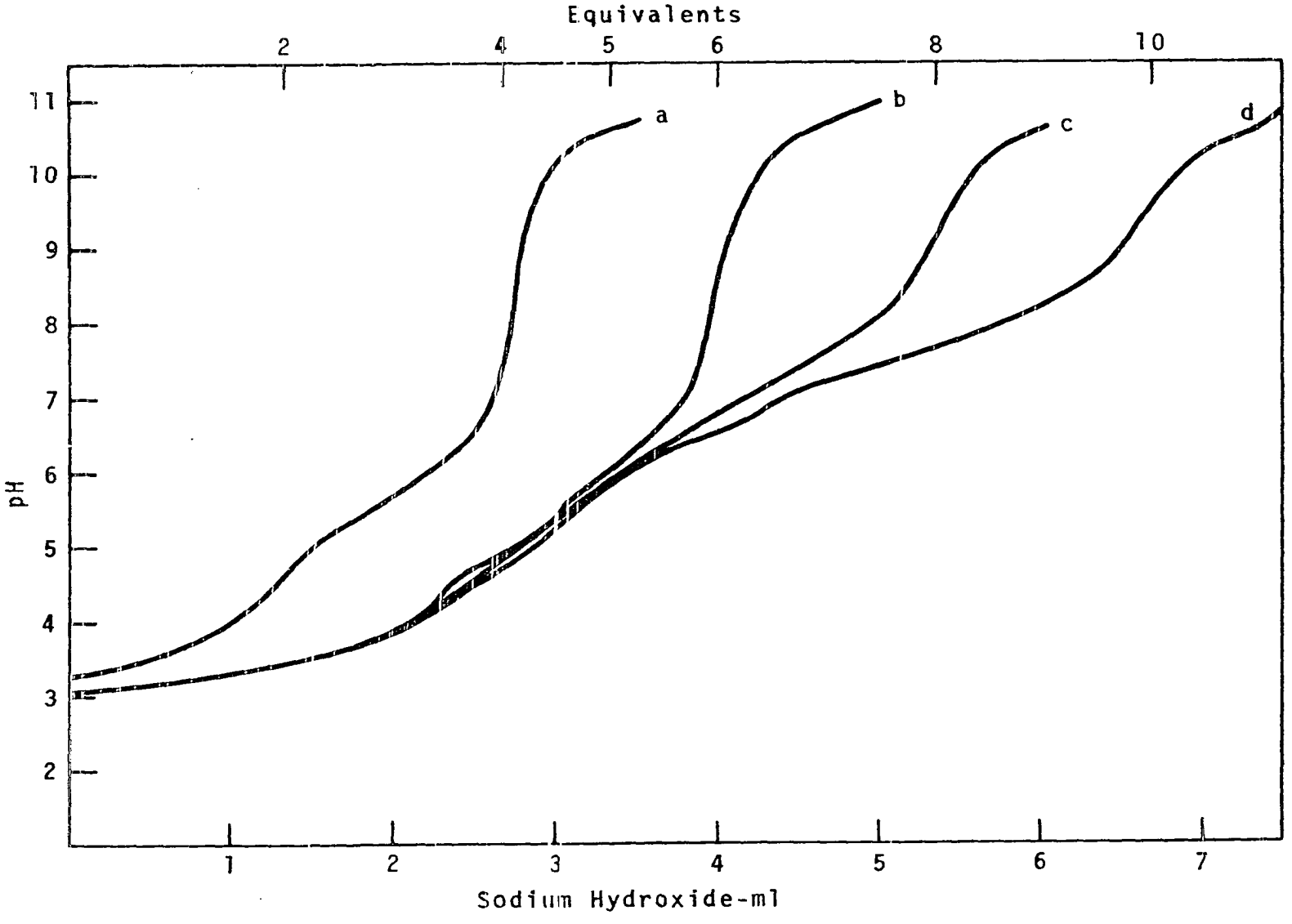
Curve b. Calcein plus $HgCl_2$ in 1:1 mole ratio

Curve c. Calcein plus $HgCl_2$ in 1:2 mole ratio

Curve d. Calcein plus $HgCl_2$ in 1:3 mole ratio

Solvent: 25 per cent ethyl alcohol

Titrant: 0.1218 N sodium hydroxide


Equivalents
2
4
5
6
8
10
11
10
9
8
7
6
5
4
3
2
pH
a
b
c
d
1
2
3
4
5
6
7
Sodium Hydroxide-ml

equivalents of base. Presumably, the hydrogen atom is still associated with the ammonium-type nitrogen from the iminodiacetic group which has not participated in the chelation. Normally, such a hydrogen atom of Calcein is not titratable. But in this case, upon union of Calcein with one doubly charged mercury ion, the positive charge incorporated into the metal chelate structure provides sufficient influence to help the titration of the remaining hydrogen atom. In other words, incorporation of a double charge into the metal chelate makes the hydrogen atom from the ammonium-type group more acidic. From the pH in the mid-point between 5 and 6 equivalents, the estimated pK for this hydrogen is now about 6.7 (in 25 per cent ethyl alcohol).

Curves c and d, representing titrations of Calcein in the presence of 1:2 and 1:3 mole ratios of mercury(II), indicate that additional mercury(II) is not bound by the Calcein. Only a 1:1 Calcein-mercury compound is formed. The excess mercury is merely titrated by the base, requiring two additional equivalents of base per mole of excess mercury(II). In Curves b, c, and d, the end-point at 5 equivalents remains invariant. The shifting of the second end-point to 8 and 10 equivalents in Curves c and d, respectively, is accounted for by the titration of excess mercury(II).

The following equations are given to summarize the neutralization reactions for each titration curve:

Curve a - Calcein alone:

$$H_6Cal + 2\ OH^- = H_4Cal^{2-} + 2\ H_2O \qquad \text{2 equivalents}$$

$$H_4Cal^{2-} + 2\ OH^- = H_2Cal^{4-} + 2\ H_2O \qquad \text{2 equivalents}$$

Curve b - Calcein plus 1:1 mercury(II):

$$H_6Cal + 4\ OH^- = H_2Cal^{4-} + 4\ H_2O$$
$$H_2Cal^{4-} + Hg^{2+} = HgHCal^{3-} + H^+ \qquad \text{5 equivalents}$$

$$HgHCal^{3-} + OH^- = HgCal^{4-} + H_2O \qquad \text{1 equivalent}$$

Curve c - Calcein plus 1:2 mercury(II):

$$H_6Cal + 4\ OH^- = H_2Cal^{4-} + 4\ H_2O$$
$$H_2Cal^{4-} + Hg^{2+} = HgHCal^{3-} + H^+ \qquad \text{5 equivalents}$$

$$HgHCal^{3-} + OH^- = HgCal^{4-} + H_2O$$
$$Hg^{2+} + 2\ OH^- = Hg(OH)_2 \qquad \text{3 equivalents}$$

Curve d - Calcein plus 1:3 mercury(II):

$$H_6Cal + 4\ OH^- = H_2Cal^{4-} + 4\ H_2O$$
$$H_2Cal^{4-} + Hg^{2+} = HgHCal^{3-} + H^+ \qquad \text{5 equivalents}$$

$$HgHCal^{3-} + OH^- = HgCal^{4-} + H_2O$$
$$2\ Hg^{2+} + 4\ OH^- = 2\ Hg(OH)_2 \qquad \text{5 equivalents}$$

2. Estimation of the formation constant

The titration Curve b in Figure 52 can be used to evaluate an approximate formation constant of the 1:1 Calcein-mercury compound. The derivation and assumptions are the same as those discussed for the determination of the formation constant of the 2:3 Calcein-aluminum compound (Section III.C.6). The same method is used. The salient equations, for Calcein, from p. 212, are:

$$H_2Cal^{4-} = HCal^{5-} + H^+ \qquad K_5 = \frac{[H^+][HCal^{5-}]}{[H_2Cal^{4-}]}$$

For the metal chelate formation, the assumed reaction is:

$$HCal^{5-} + Hg^{2+} = HgHCal^{3-}$$

and the formation constant is defined as

$$K_f = \frac{[HgHCal^{3-}]}{[Hg^{2+}][HCal^{5-}]} .$$

$$\text{Since } [HCal^{5-}] = \frac{K_5[H_2Cal^{4-}]}{[H^+]} ,$$

$$K_f = \frac{[HgHCal^{3-}][H^+]}{K_5[Hg^{2+}][H_2Cal^{4-}]} .$$

At the mid-point, i.e., at 4.5 equivalents of base added, the pH is 5.4 and $[H^+] = 4 \times 10^{-6}$. Following the assumption

made previously,

$$K_f = \frac{[H^+]}{K_5 \times \frac{1}{2}[H_2Cal^{4-}]}$$

And substituting the calculated values at the mid-point,

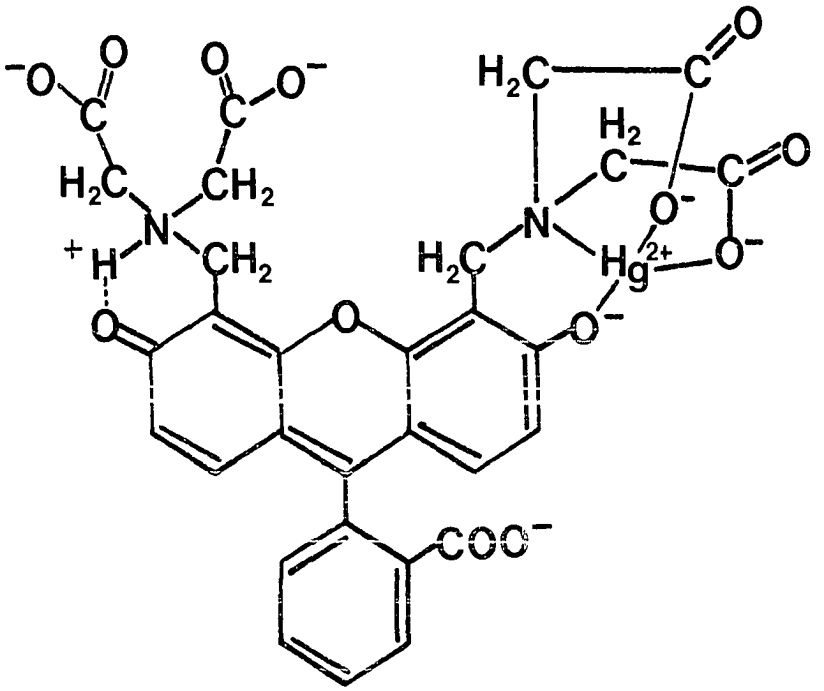
$$K_f = \frac{4 \times 10^{-6}}{(1.3 \times 10^{-10})(7.4 \times 10^{-4})} = 8.3 \times 10^7$$

3. Structure of the Calcein-mercury compound

The reaction of Hg^{2+} with Calcein in the H_2Cal^{4-} form produces only a 1:1 Calcein-mercury compound with the general formula $HgHCal^{3-}$. Since Calcein in the form H_2Cal^{4-} has the two carboxylic acid groups and the two phenolic-type groups neutralized and since the addition of Hg^{2+} releases one additional hydrogen ion, presumably from the ammonium-type group, Structure XLIII is consistent with the observed facts.

In Structure XLIII, the two phenolic-type oxygen atoms of Calcein are equivalent because of resonance; they alternate between the quinone and the phenolate ion form. The two coordination sites of the Calcein molecule are occupied by different atoms. In one site, the chelated mercury(II) ion is located. The other site is occupied by the remaining hydrogen atom, presumably involving some hydrogen bonding, as described previously by Hefley.[58] The remaining hydrogen

atom is a stronger acid than normally expected for an ammonium-type group. From Figure 52, Curve b, the apparent pK is approximately 6.7 (in 25 per cent ethyl alcohol). The increased acid strength has been discussed above (p. 306).



XLIII. $HgHCal^{3-}$, the 1:1 Calcein-mercury compound, fluorescent

4. Conclusions

It has been demonstrated, by potentiometric titrations in 25 per cent ethyl alcohol, that Calcein reacts with mercury(II) to form only a 1:1 Calcein-mercury compound; even in the presence of three-fold excess mercury(II), there is no evidence for the formation of a 1:2 Calcein-mercury compound. Qualitative observations indicate that the compound is fluorescent.

In the 1:1 Calcein-mercury compound, $HgHCal^{3-}$, the remaining hydrogen atom is sufficiently acidic to be titratable by sodium hydroxide. The increased acid strength is presumably caused by the additional positive charge of Hg^{2+} incorporated in the metal chelate, thus facilitating the leaving of a hydrogen ion.

The potentiometric titration curve has been used to evaluate the formation constant for the reaction $HCal^{5-} + Hg^{2+} = HgHCal^{3-}$. The formation constant is estimated to be 8.3×10^{7}. The value must be considered only an approximation because the reaction was carried out in 25 per cent ethyl alcohol, a solvent mixture in which the K_5 value of Calcein is not known.

F. Reaction of Calcein with Copper

The application of potentiometric titrations of Calcein in the presence of varying mole ratios of metal ions has now been successfully used in determining the combining ratio of

Calcein with aluminum and Calcein with mercury ions. In addition, the formation constants of the 3:2 Calcein-aluminum and the 1:1 Calcein-mercury compounds have been estimated.

Copper(II) solutions have been used extensively as back-titrants in chelometric titrations based on Calcein as a metallofluorochromic indicator. Specifically, copper(II) solutions have been used in the back-titration of metal ions which react with EDTA to form highly colored compounds, thus making the use of conventional metallochromic indicators impossible. The application of the quenching of fluorescence of Calcein by the first excess of copper(II) in solution proved an ideal method of end-point indication for such colored systems.

Despite these uses of the reaction of copper(II) with Calcein, the nature of the reaction has until now not been studied. The experiments described here, although only exploratory, provide a springboard for possible future studies of the reaction of Calcein with copper(II).

1. Potentiometric titrations

a. Experimental The potentiometric titrations of Calcein in the presence of 1:1, 1:2, and 1:4 mole ratios of copper(II) have been carried out by a method similar to the one used previously in studying the reaction of Calcein with aluminum and with mercury ions.

A stock solution of Calcein was prepared by dissolving 0.4028 g of the pure Calcein in 250 ml of 50 per cent ethyl alcohol. A 25-ml aliquot of this solution, containing 6.29×10^{-5} moles of Calcein, was diluted with 25 ml of 50 per cent ethyl alcohol for titration.

A stock solution of copper(II) was prepared by dissolving 1.7049 g of cupric chloride dihydrate in 1 l. of deionized water. The volume of this solution of 0.0100 M copper(II) which corresponded to one 25-ml aliquot of the Calcein solution in a 1:1 mole ratio was 6.29 ml. In addition to titrating solutions of various mole ratios of Calcein to copper, a 6.29-ml portion of the copper(II) solution was also titrated by sodium hydroxide in order to demonstrate the reaction of copper(II) itself with alkali.

The course of the titration was followed by measuring the pH after each increment of titrant (0.1211 N sodium hydroxide) was added. A Corning Model 10 pH meter with a glass electrode was used. The pH was measured against a saturated calomel reference electrode.

b. Results and discussion The potentiometric titration curves of Calcein in the presence of varying amounts of copper(II) are presented in Figure 53. The reaction of copper(II) with sodium hydroxide requiring two equivalents of alkali at the end-point, is depicted by Curve a.

Figure 53. Potentiometric titration of copper(II) chloride and of Calcein (6.29×10^{-5} moles) in the presence of varying amounts of copper(II) chloride

Curve a. Copper(II) chloride alone: 6.29 ml of 0.0100 M $CuCl_2$ in water (6.29×10^{-5} moles)

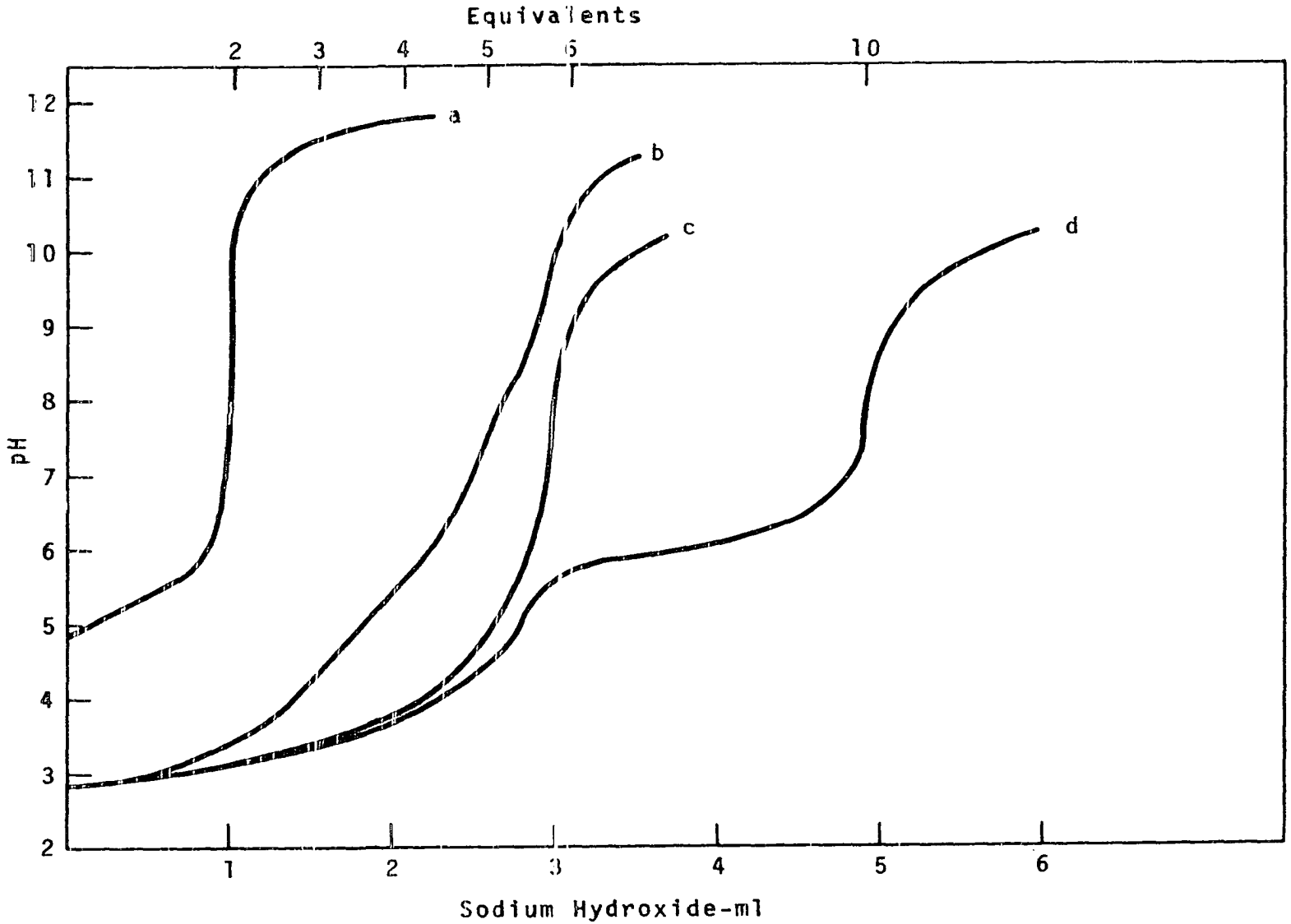
Curve b. Calcein plus $CuCl_2$ in 1:1 mole ratio

Curve c. Calcein plus $CuCl_2$ in 1:2 mole ratio

Curve d. Calcein plus $CuCl_2$ in 1:4 mole ratio

Solvent in Curves b-d: 50 per cent ethyl alcohol

Titrant: 0.1211 N sodium hydroxide


Equivalents
2
3
4
5
6
10
12
11
10
9
8
7
6
5
4
3
2
pH
a
b
c
d
1
2
3
4
5
6
Sodium Hydroxide-ml

Curve b represents the titration of Calcein in the presence of 1:1 mole ratio of copper(II). Comparison with the titration of Calcein by itself (Figure 52, Curve a) shows that a reaction has taken place between Calcein and copper. However, from the general shape of the curve and the drawn-out nature of the end-points, it is not clear in what ratio Calcein and copper appear to have combined nor what the resulting species is.

More information can be gained from Curve c, representing the titration of Calcein in the presence of 1:2 mole ratio of copper. It is evident that one molecule of Calcein has combined with two copper ions; the 6 hydrogen ions released by this process are titrated by sodium hydroxide to a sharp, well-defined end-point at 6 equivalents. The overall reaction can be expressed mathematically as

$$H_6Cal + 2\ Cu^{2+} = Cu_2Cal^{2-} + 6\ H^+$$

or as

$$H_2Cal^{4-} + 2\ Cu^{2+} = Cu_2Cal^{2-} + 2\ H^+ ,$$

depending on which form of Calcein is considered as the reactant. Either reaction accounts for the titration of 6 equivalents of hydrogen ions at the end-point.

Further evidence for the reaction of the one molecule of Calcein with two copper ions can be obtained from Curve d, which represents the titration of Calcein in the presence of excess copper(II), 1:4 mole ratio of Calcein to copper(II).

Since copper is in a two-fold excess over the required amount for the production of the Cu_2Cal^{2-} compound, the excess copper should be titrated by the base to yield an additional end-point beyond the end-point at 6 equivalents. This is indeed so; there are two breaks in Curve d. The first, occurring at 6 equivalents of base, corresponds to the titration of the 6 hydrogen ions released upon union of Calcein with two copper ions. The second, at 10 equivalents, corresponds to the titration of 2 moles of excess copper(II) that require an additional 4 equivalents of base.

The reactions described by titration Curves a, c, and d can be expressed in the following mathematical form:

Curve a - copper alone:

$$Cu^{2+} + 2\ OH^- = Cu(OH)_2 \qquad 2 \text{ equivalents}$$

Curve c - Calcein plus 1:2 copper(II):

$$H_6Cal + 2\ Cu^{2+} = Cu_2Cal^{2-} + 6\ H^+ \qquad 6 \text{ equivalents}$$

Curve d - Calcein plus 1:4 copper(II):

$$H_6Cal + 2\ Cu^{2+} = Cu_2Cal^{2-} + 6\ H^+ \qquad 6 \text{ equivalents}$$

$$2\ Cu^{2+} + 4\ OH^- = 2\ Cu(OH)_2 \qquad 4 \text{ equivalents}$$

2. Estimation of the formation constant

From the potentiometric titration data depicted in Curve c, the formation constant of the 1:2 Calcein-copper compound can be calculated. The formation of Cu_2Cal^{2-} can be expressed by the reaction

$$Cal^{6-} + 2\ Cu^{2+} = Cu_2Cal^{2-} \ .$$

The overall formation constant is expressed as

$$K = \frac{[Cu_2Cal^{6-}]}{[Cal^{6-}][Cu^{2+}]^2} \ .$$

Using the same assumptions made before, in the determination of the formation constant of the Calcein-aluminum and the Calcein-mercury compounds, and using the method of derivation based on that of Schwarzenbach et al.[124] and developed by Hefley,[58] the overall formation constant for the 1:2 Calcein-copper compound is expressed as

$$K = \frac{[H^+]^2}{[K_5K_6][Cu^{2+}]^2}$$

Substitution of the various values:

$$K_5 = \frac{[H^+][HCal^{5-}]}{[H_2Cal^{4-}]} = 1.32 \times 10^{-10}$$

$$K_6 = \frac{[H^+][Cal^{6-}]}{[HCal^{5-}]} = 2.29 \times 10^{-12}$$

pH at mid-point is 3.4, thus $[H^+] = 4 \times 10^{-4}$, $[Cu^{2+}]$ at mid-point is 1.258×10^{-3}, corrected for dilution, yields

$$K = \frac{(4 \times 10^{-4})^2}{(1.32 \times 10^{-10})(2.29 \times 10^{-12})(1.258 \times 10^{-3})}$$

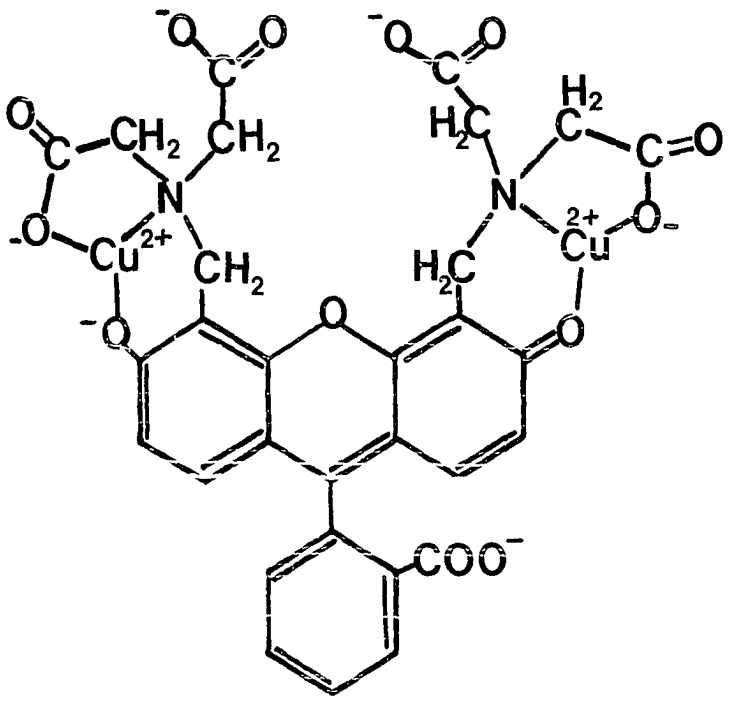
$$K = 6.6 \times 10^{20}$$

Again it must be mentioned that the formation constant of 6.6×10^{20} for the 1:2 Calcein-copper compound is only an approximate value, because the potentiometric data were obtained in 50 per cent ethyl alcohol, whereas the data for K_5 and K_6 values of Calcein were obtained by He ley[58] in aqueous solution. In addition, such a high relative concentration of ethyl alcohol may also have an effect on the kinetics with which the equilibrium is established.

3. Structure of the Calcein-copper compound

The structure of the 1:2 Calcein-copper compound, Cu_2Cal^{2-}, is depicted below (Structure XLIV). It is not certain whether both carboxylates or only one carboxylate of each chelating portion of the molecule is coordinated to the copper ion. Since copper is sexadentate, the remaining coordination sites are occupied presumably by water molecules. It must be noted that Structure XLIV is only one possible resonance form of the metal chelate. The two oxygen atoms, one in the quinone form and the other in the phenolate form,

are equivalent and resonate from one form to the other. In order to simplify the graphic requirements, the structure is drawn with only one of the carboxylate groups in each chelating function coordinated to the copper atoms.



XLIV. Cu_2Cal^{2-}, the 1:2 Calcein-copper compound, nonfluorescent

4. Conclusions

By means of potentiometric titrations of Calcein in the presence of varying mole ratios of copper(II), it has been shown that Calcein reacts with copper(II) ions to form a 1:2 compound, Cu_2Cal^{2-}. A reaction has also been observed between Calcein and copper(II) at a 1:1 mole ratio, but the nature of the reaction or of the compound formed has not been established.

The overall formation constant for the reaction

$$Cal^{6-} + 2\ Cu^{2+} = Cu_2Cal^{2-}$$

has been established. The value of 6.6×10^{20} has been obtained. The value is only an approximation because the titrations were performed in 50 per cent ethyl alcohol.

It is not understood why mercury should form only a 1:1 compound with Calcein, even in the presence of a large excess of mercury, whereas copper readily forms the 1:2 Calcein-copper compound. Qualitatively, the formation constant of the copper chelate seems larger and may be partially responsible for this tendency to form the 1:2 chelate. The reaction between Calcein and copper in a 1:1 mole ratio needs more study to elucidate the nature of the product formed.

Another unexplained phenomenon in the chemistry of the reactions of Calcein with metal ions is the fluorescence quenching caused by copper(II), and indeed by most other

transition metals, whereas the alkaline earth metals and cadmium, mercury, and zinc ions form fluorescent compounds with Calcein. Although qualitative fluorescence studies have been done with many metal ions, the nature of the difference in the fluorescence behavior has not as yet been satisfactorily explained. D. H. Wilkins[156,159] has proposed two different structures for fluorescent and nonfluorescent metal chelates in order to account for the opposite fluorescence properties. His explanation, however, is not consistent with other data and has been rightly criticized by Körbl and Svoboda.[82] Their own explanation, in turn, was also incomplete.

On first glance, it is apparent that it is not necessarily the structure of the metal chelate but the nature of the metal ion itself which is responsible for the fluorescence or nonfluorescence of the Calcein-metal chelates. In general, it seems that paramagnetic metal ions usually quench fluorescence, whereas diamagnetic metal ions restore fluorescence. Copper(II) and most of the transition metals certainly fit the description of the first group, while the alkaline earths, mercury, zinc, and cadmium belong to second category. A similar view was put forth by Eggers[37] who stated that metal ions with filled outer valence shells form fluorescent metal chelates. An interesting experiment to test this theory would be to study the fluorescence of

Calcein in the presence of Cu(II) and Cu(I); the former is paramagnetic and the latter is diamagnetic. The system also provides an interesting oxidation-reduction study coupled with fluorescence behavior.

IV. SUMMARY

The evolution of our concepts of the structure of fluorescein has been traced. Following the discovery in 1871 of fluorescein by Adolf Baeyer and the subsequent early work by Baeyer and others establishing the composition and basic features of the structure, two "landmark papers" mark the history of fluorescein; these are the papers of Orndorff and Hemmer,[106] 1927, and of Zanker and Peter,[163] 1958. The structures assigned the yellow solid form of fluorescein (the lactone structure) and the red solid form of fluorescein (the para-quinone structure) by Orndorff and Hemmer are the structures found in the textbooks to this day. The work of Zanker and Peter deals principally with the nature and structure of the various forms of fluorescein in aqueous solutions of varying pH ("prototropic forms") and with a colorless form of fluorescein in solution in dioxane; the assignments of structure of Zanker and Peter have been followed by all recent workers. The history of fluorescein has been one of faulty and incomplete experimental work, discordant observations, and improper interpretations. The nature of these discrepancies has been investigated and the specific deficiencies in the Orndorff and Hemmer and Zanker and Peter assignments of structure pinpointed. In the present work an attempt has been made to draw into one

coherent package evidence from a number of lines of investigation bearing on the structures of the various forms of fluorescein and to use the results as a basis for establishing the structure of Calcein, a derivative of fluorescein.

Commercial fluorescein has been purified by conversion to diacetylfluorescein, purification of the latter, and hydrolysis back to fluorescein; a metal-free fluorescein has been thus obtained. The yellow and red solid forms of fluorescein have been prepared and characterized. A colorless, solid form of fluorescein has been isolated and characterized.

All three solid forms of fluorescein, yellow, red, and colorless, have been studied by the techniques of X-ray diffraction spectroscopy, mass spectroscopy, and infrared spectroscopy.

The X-ray powder diffraction patterns of yellow, red, and colorless fluorescein have been found to be sharp and each distinctly different from the others. Crystallographically, all three forms are thus individual species and explanations that the difference between the forms are caused by amorphous character, impurities, or particle size are ruled out.

The mass spectra of yellow and red fluorescein have been found to be identical, a not unexpected finding inasmuch as yellow fluorescein is converted to red fluorescein when heated. The mass spectrum of red fluorescein has been

explained in terms of two concurrent but different degradation sequences. The mass spectrum of colorless fluorescein closely resembles that of red fluorescein but has additional peaks which reveal the presence in the molecule of dioxane (of crystallization) and of a lactone ring.

The infrared spectra of the yellow, red, and colorless solid forms of fluorescein have been obtained on solid films. The spectra differ sharply from each other in the region of absorption of the carbonyl group (carbon-oxygen stretching frequency) and a new absorption band has been found in the spectrum of yellow fluorescein which relates it to the pyrylium salts. Interpretation of these spectra has furnished proof of the presence in red fluorescein of a free (non-dissociated) carboxylic acid group, in colorless fluorescein of a lactone ring, and in yellow fluorescein of a pyrylium type cation and carboxylate anion.

Drawing on evidence from the general chemistry of fluorescein and related compounds, from mass spectra, and from infrared spectra, structures have been postulated or confirmed for the three solid forms of fluorescein: for colorless fluorescein, the lactone structure, in conformity with the 1958 assignment of Zanker and Peter of this structure to fluorescein in dioxane solution; to red fluorescein, the para-quinone structure, in conformity with the 1927 assignment of Orndorff and Hemmer; and for yellow fluorescein, a zwitter

ion structure involving a central, six-membered, oxygen-containing, aromatic ring bearing a positive charge and a balancing carboxylate group. The various features of these assignments of structure, the points at which they correct or confirm earlier assignments, and the aspects which are new have been discussed in detail.

The chemistry and structure of fluorescein in solution have been studied by measuring the solubility of yellow and red fluorescein in water as a function of pH, by titrating the acidic groups of various fluoresceins and related compounds in aqueous and in aqueous-ethanolic media, by measuring the fluorescence of fluorescein as a function of pH, and by obtaining the nuclear magnetic resonance spectra of yellow, red, and colorless fluorescein in dioxane solution. In addition, the oxidation-reduction properties of fluorescein have been studied by quantitative bromination, by reduction with titanous chloride, and by preparation of fluorescin, the reduced form of fluorescein.

It has been shown that the solubility curve of yellow fluorescein and of red fluorescein as a function of pH displays a minimum; the shape of the curve is typical for amphoteric substances. Although red fluorescein is slightly less soluble than yellow fluorescein, both forms have minimum solubility at about pH 3.4. The point of minimum solubility coincides with the value of the isoelectric point observed by other workers, confirming the existence of cationic,

neutral, and anionic forms of fluorescein. The acid dissociation constants have been determined by mathematical treatment of the solubility data. Their values are the same for red and yellow fluorescein, indicating that upon dissolution both forms give identical solutions.

The yellow and red forms of fluorescein have been also characterized as dibasic acids by potentiometric titrations with alkali and by back-titrations with acid in water and in 50 per cent ethyl alcohol. Most valuable data have been obtained by back-titration of yellow and red fluorescein in water. The titration curves, characterized by three end-points corresponding to the titration of excess base, of the dianion to the monoanion, and of monoanion to the neutral form, have been used to determine the acid dissociation constants. Again, the acid dissociation constants have been shown to be the same, irrespective of whether yellow or red fluorescein has been titrated, thus indicating that in solution fluorescein exists in one form.

The nature of fluorescein in solution has been established by evaluation of the acidic properties of the various possible forms, by comparison of the titration curves with those of the related compounds 3,6-dihydroxy-9,9-dimethylxanthene and phenolphthalein, and by analogy with other compounds having the pyrylium-type structure. The structure of the neutral form of fluorescein in water has been shown to be the zwitter ion structure, identical to that of yellow

fluorescein in the solid state. The structures of the cation, neutral, monoanion, and dianion forms have been discussed on the basis of the acidic properties of fluorescein. Their relative distribution has been calculated as a function of pH and plotted over the pH range from 1 to 9. The unusual acid strength of the two replaceable hydrogen atoms of fluorescein has been emphasized.

The fluorescence of fluorescein has been measured as a function of pH. Preliminary studies have indicated that the fluorescence is identical, irrespective of which form of fluorescein has been used to prepare the solution. It has been established that the neutral and the monoanion form of fluorescein are nonfluorescent. From the curve of fluorescence vs. pH and the distribution of the prototropic forms as a function of pH, it has been shown that the fluorescence is derived entirely from the dianion. The nonfluorescence of the monoanion has been used as additional support for the structure of the monoanion assigned previously on the basis of the acid properties. The fluorescence data have been used to determine the acid dissociation constants of fluorescein.

On the basis of solubility data, potentiometric acid-base titrations, and fluorescence measurements as a function of pH, it has been established that, regardless of the starting form of fluorescein, in aqueous solution fluorescein exists not in the closed lactone form nor in the open

para-quinone form with the carboxylic acid group but in the yellow zwitter ion form. The structures, the properties, and the relative distribution of the prototropic forms of fluorescein as a function of pH have been discussed.

It has been shown by NMR spectroscopy that in dioxane solution the yellow, red, and colorless forms of fluorescein all exist in the colorless form with the closed lactone structure. The decreased acidity of the resultant phenolic functional groups has been indicated.

A method for the quantitative bromination of fluorescein has been developed. The product has been identified as eosin. An attempt to titrate fluorescein directly by reduction with titanous chloride has been unsuccessful.

Fluorescin, the reduced form of fluorescein, has been prepared by reduction with zinc dust and characterized by potentiometric titration in aqueous-organic media. The acidic nature of the carboxylic group of fluorescin has been described and used as an aid in the assignment of structure for fluorescein in solution.

Calcein has been prepared by the Mannich condensation of very pure, metal-free fluorescein, iminodiacetic acid, and formaldehyde. The purity, acid-base reactions, and fluorescence properties of Calcein have been re-examined, and the chelating reactions of Calcein, aluminum, mercury, and copper have been studied; the reaction with aluminum has been investigated in detail. A new structure has been

proposed for Calcein which disposes the shortcomings and misjudgements of previous assignments and which puts the chemistry of Calcein on a firm foundation.

The previously developed synthesis of Calcein has been modified by introducing the process of freeze-drying during the isolation step in order to decrease the chance of decomposition of Calcein caused by heat. The results of elemental analysis, determination of the equivalent weight by alkalimetric titration, and weight loss on drying and ignition indicate that Calcein is best described as bearing one molecule of water of crystallization. The mass spectrum of Calcein has little diagnostic value except to demonstrate the decomposition of Calcein by high heat input.

A method for the quantitative bromination of Calcein has been developed. Surprisingly, the bromination product has been identified as eosin, the same as that for the bromination of fluorescein, indicating that the substituent groups in Calcein are easily oxidized away by the bromination reagent.

The knowledge of the acid properties of Calcein, previously established by Hefley, has been expanded by titration of Calcein in 50 per cent ethyl alcohol, by correlation of the acid properties of Calcein with those of its components, that is, of fluorescein and iminodiacetic acid, and by putting the assignment of the acid dissociation constants to the various functional groups on a firmer basis because of the corrected structure assignment.

The direct potentiometric titration of Calcein as an acid in water has never been very useful because of the low solubility of Calcein in water. By performing the titration in 50 per cent ethyl alcohol, the low solubility of Calcein has been overcome and, in addition, the four titratable hydrogen atoms have been differentiated into two classes, each with two hydrogen atoms, of different acid strength. By comparison with the titration curve of the free iminodiacetic acid itself, the first group of two replaceable hydrogen atoms has been confirmed as the two carboxylic acid functions of the methyleneiminodiacetic acid substituents. The acid dissociation constants of the third and fourth replaceable hydrogen atom of Calcein, for which the unusual acid strength has not been satisfactorily explained in the past, have been correlated with those of fluorescein itself. The acid strength of the third and fourth hydrogen atoms in Calcein has been used as a major line of evidence in relating the structure of Calcein with that of fluorescein.

The structure of Calcein, as proposed by Hefley, has been modified on the basis of the structure of fluorescein, established in the first part of this work. The novel aspect of the proposed structure of Calcein is the incorporation of the zwitter ion structure of fluorescein, the parent compound. NMR spectra of Calcein and of 4',5'-dimethylfluorescein have been used to confirm that Calcein is fluorescein substituted by two methyleneiminodiacetic acid groups in the 4' and 5'

positions. The combined structures of the ionic forms of fluorescein and of iminodiacetic acid have been used as the basis for assigning structures to the six ionic forms of Calcein obtained by the stepwise ionization of the six replaceable hydrogen atoms.

The reaction of Calcein with aluminum has been studied in detail. The nature of the complicated reaction has been elucidated by potentiometric titrations of Calcein in the presence of varying amounts of aluminum, by analogy to the reaction of ethylenediaminetetraacetic acid with aluminum, and by fluorescence measurements on solutions containing Calcein and aluminum.

Calcein reacts with aluminum in an acid medium to form a 2:3 Calcein-aluminum compound. The accompanying release of hydrogen ions is indicated by an end-point at five equivalents of base in the titration curve. The Calcein-aluminum compound itself is sufficiently acidic to react with additional base; one equivalent per mole of Calcein is used up for the neutralization of the remaining hydrogen atom on the Calcein ligands, and another equivalent of base is used up in the formation of a hydroxy species. The nature of the hydroxy species has been established by analogy to the reaction of the EDTA-aluminum compound with alkali.

Structures have been proposed for the different forms of the 2:3 Calcein-aluminum compound existing at different pH values. It has been shown that in highly alkaline solutions,

the compound is decomposed, and Calcein and aluminum react as individual species with the base. The overall formation constant of the 2:3 Calcein-aluminum compound has been estimated as 2.4×10^{48} on the basis of potentiometric titration data.

The combining ratio of 2:3 for Calcein reacting with aluminum has been also established from fluorescence data at pH 6.4. The fluorescence curve as a function of pH for Calcein in the presence of excess aluminum increases sharply in the acid region starting at pH of about 2, reaches a maximum at pH 6.0-6.5, and then begins to decrease. At a pH of about 7.0-7.5, it begins to increase again in a fashion analogous to that of Calcein alone. By pH of about 9.5, the fluorescence curves for Calcein plus aluminum and for Calcein alone are practically indistinguishable, indicating that the Calcein-aluminum compound is completely decomposed. The solutions of Calcein plus aluminum are also not stable with time. Within five hours after mixing, the fluorescence intensity had decayed to less than one-half of the original value. The relative fluorescence of the Calcein-aluminum compound at pH 2.5, measured as a function of the amount of aluminum added, is a nonlinear curve.

The reaction of Calcein with mercury(II) and with copper(II) has been briefly explored by the same method of potentiometric titration of Calcein with alkali in the presence of varying amounts of the metal ion. Mercury(II)

has been found to form only one, fluorescent compound with Calcein in the combing ratio of 1:1. The formation constand has been estimated as 8.3×10^7 in 25 per cent ethyl alcohol. The formation of a 1:2 Calcein-copper(II) compound has been established. The overall formation constant of the nonfluorescent Calcein-copper(II) compound has been estimated as 6.6×10^{20} in 50 per cent ethyl alcohol. Structures have been proposed for the compounds of Calcein with mercury(II) and with copper(II), and an explanation for the fluorescence and nonfluorescence of the metal chelates has been attempted.

To summarize, then, the structure of fluorescein, both in the solid form and in solution, has been determined and used as the basis for elucidating the structure Calcein. The evidence has been drawn from many sources, including acid-base and fluorescence properties, solubility as a function of pH, infrared and nuclear magnetic resonance spectroscopy, and X-ray diffraction and mass spectrometry. The acid-base and fluorescence properties of Calcein have been used in establishing the nature of the chelating reaction of Calcein with some metal ions, especially with aluminum.

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