1978

Preparation and identification of the sulfonic acids of fluorescein and the metallofluorochromic indicator Calcein

Samir George Gharfeh
Iowa State University

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Preparation and identification of the sulfonic acids of fluorescein and the metallofluorochromic indicator Calcein

by

Samir George Gharfeh

A Dissertation Submitted to the
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For the Major Department

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For the Graduate College

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Ames, Iowa

1978

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DEDICATION

To My Brother, Elia

I. INTRODUCTION

This dissertation describes the synthesis and properties of a sulfonated modification of the chemical *Calcein*, an analytical reagent widely used in the determination of calcium, and also the synthesis and properties of the various intermediate chemicals preparation and study of which were essential steps in the route followed to the compound desired. The final compound, a new analytical reagent, is called β-*Sulfocalcein*.

Calcein was first prepared by Diehl and Ellingboe (1) in 1956, by the Mannich condensation of fluorescein, iminodiacetic acid and formaldehyde in alkaline water solution. The procedure failed to yield a product of definite composition, although one which served well as an indicator to mark the end-point in the titration of calcium in the presence of magnesium with disodium dihydrogen ethylenediaminetetraacetate (EDTA).

Calcein was the first of the so-called *metallofluorochromic indicators*. The compound has the fluorescence and acid-base properties of fluorescein combined with the chelating and acid-base properties of EDTA. Fluorescein exhibits a brilliant, green-yellow fluorescence which depends on the acidity of the solution; the fluorescence begins at pH above 4, increases at pH up to 8, and remains constant at all high pH. The fluorescence of Calcein, on the other hand,

begins at pH 4, reaches a maximum at pH 8, and decreases at higher pH, disappearing completely at pH 13. In addition, the fluorescence is greatly influenced by the presence of certain metal ions, the fluorescence of the calcium compound at pH 13, for example, being about equal to that of the reagent alone at pH 8.

Calcein is used in two ways in the analytical chemistry of calcium, first as an indicator to mark the end-point in the titration of calcium with EDTA at pH 13, the fluorescence disappearing at the end-point, and second for the direct determination of calcium by measuring the intensity of the fluorescence of the calcium-Calcein compound. With respect to reaction toward Calcein, the metals fall into two classes: the alkaline earth metals, calcium, strontium and barium, and cadmium, all of which form fluorescent compounds in solutions of high pH; and second, the transition metals such as cobalt, nickel, iron, and copper, which quench the fluorescence of Calcein at pH 5 to 10.

Calcein was well-received and was adapted quickly to the determination of calcium in water, minerals, plant and biological materials, and in particular to calcium in blood serum, urine, and milk. A monograph published by Diehl (2) in 1964 listed in the bibliography some thirty papers dealing with Calcein and applications of it. By 1976, the number of papers was approaching two hundred. Markuszewski (3) and

Martin (4,5) give excellent reviews of the many papers and publications dealing with Calcein. These include papers dealing with structure, nomenclature, synthesis, and applications. In addition to the determination of calcium, the applications of Calcein in analytical chemistry include procedures for the direct fluorometric and titrimetric determination of a number of cations and the indirect determination of the anions, sulfate, bromide, iodide, and cyanide. Calcein is used also as an adsorption indicator, as a fluorescent stain for mineralized tissues, and as a marking dye for the growth of bone and dentine.

As long as Calcein was restricted to use as an indicator in EDTA titrations, the compound of Diehl and Ellingboe was satisfactory, but for the direct fluorometric determination of calcium and other cations, a pure Calcein of consistently definite composition was essential. Work on Calcein at Iowa State University was resumed, by Hefley (6), who modified the synthesis by starting with a pure, metal-free fluorescein and employing glacial acetic acid as a solvent for the Mannich condensation. This procedure resulted in a pure product which enabled Hefley (7) to establish unequivocally the structure of the compound and the nature of the reaction with calcium. The assignment of the structure was based on proton nuclear magnetic resonance spectroscopy (NMR). Calcein was found to be fluorescein-4',5'-bis(methyleneiminodiacetic

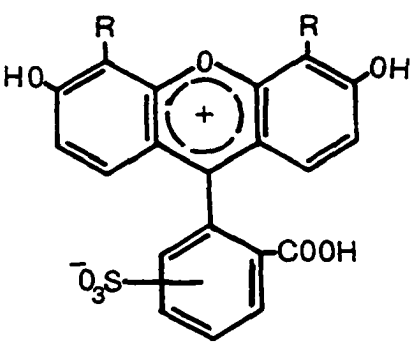
acid), or, by the more formal name 3',6'-dihydroxy-4',5'-bis[N,N'-bis(carboxymethyl)aminomethyl]fluoran. The work was confirmed later by Markuszewski (3) and finally by Martin (5) employing the carbon-13 NMR method.

Although the Hefley procedure for the synthesis gives a pure compound, unfortunately the yield is low and the procedure time-consuming, particular difficulty being encountered in the filtration step. Further work on the preparation of Calcein was carried out by Martin (4) who returned to water as the solvent for the Mannich condensation but maintained the solution essentially neutral during the reaction. He found that the addition of potassium chloride to the solution prior to the precipitate speeded up the filtration, presumably by preventing peptization during the washing process. The yield was increased to 32 per cent. The Martin procedure was adopted and modified in the present work, which involves the synthesis of a sulfonated Calcein; yields of 75 per cent of the so-called β-*Sulfocalcein* have been obtained.

The solubility of Calcein in water is low, of the order of 20 mg per 100 ml, and this low solubility has impeded the elucidation of the properties of the compound and has complicated the procedures for its use in analytical chemistry and biochemistry. The purpose of the present work is to prepare a sulfonated Calcein, a compound which would be expected to

be considerably more soluble in water than the parent compound. Not only should the increased solubility circumvent some of the problems associated with the use of the parent compound, but the differences in detail of the acid-base and chelating properties of the sulfonated compound could well lead to entirely new applications.

An initial decision was made to attempt to introduce the sulfonate group into the phthalate portion of the molecule rather than into the xanthene portion, that is, to make a Structure I rather than a Structure II. The presence of a sulfonate group in the phthalate ring was thought less likely to interfere with the introduction of the methyleneiminodiacetic acid into the xanthene portion, by the Mannich



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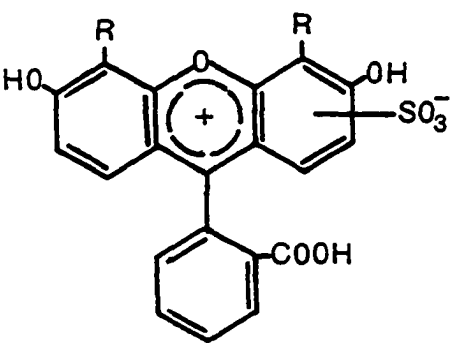

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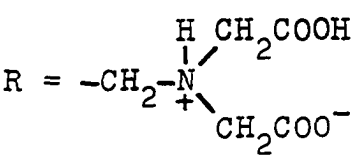
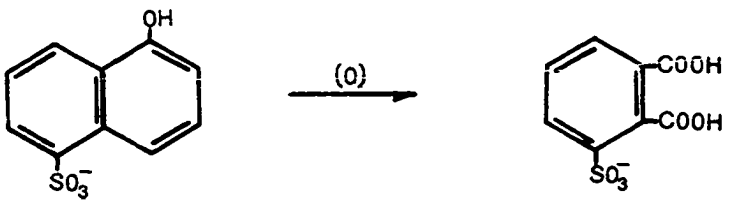


condensation. The synthetic route to a sulfonated Calcein, Structure I, attempted in the present work involved (a) the oxidation of a naphtholsulfonate to a sulfonated phthalate,

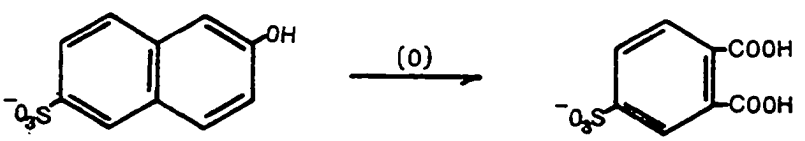
(b) reaction of the sulfonated phthalate with resorcinol to produce a sulfonated fluorescein, and (c) conversion of the latter by the Mannich condensation with formaldehyde and iminodiacetic acid to the sulfonated Calcein.

Two sulfonated phthalic acids would be expected to exist, Structures IV and VI. Although these compounds have been known since 1885, Graebe (8), the literature on them is surprisingly scant, Ree in 1886 (9) and Ioffe and Devyatova in 1962 (10). In my thesis (11) for the degree Master of Science, submitted to Iowa State University in 1977, I followed the procedures of Ioffe and Devyatova, which involve the oxidation of naphtholsulfonic acids. The Russian work is



III IV

α-Sulfophthalic acid



V VI

β-Sulfophthalic acid

sadly deficient in detail and moreover the identity of the products isolated is incorrect; the materials isolated must

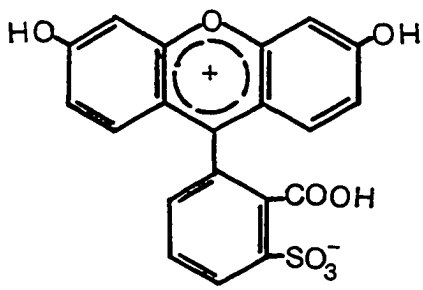
actually have been salts. A major portion of my work for the Master of Science degree was devoted to improving these syntheses, to correctly identify the salts isolated, and to determining the dissociation constants of the acids. The work for the Master of Science degree also included the conversion of these sulfonated phthalic acids to the sulfonated fluoresceins and preliminary observations on the separation and properties of these compounds. The present dissertation picks up the syntheses at this stage.

The synthesis of sulfonated fluorescein has received even less attention than that of the sulfonated phthalic acids. This seems odd in view of the very extensive literature on fluorescein. In 1885, Graebe (8) described very briefly the preparation of a sulfonic acid of fluorescein by heating β-sulfophthalic acid with resorcinol. Graebe limited himself to giving an empirical formula and did not discuss the structure of the product. Sulfonated fluorescein does not appear again in the literature until 1961, at which time Ioffe, Devyatova, and Ruskulyak (12) described the preparation of α- and β-isomers of sulfofluorescein. The route followed was the same as that for making the parent compound, fluorescein, that is, by the condensation of the sulfophthalic acid with resorcinol on heating the mixture with concentrated sulfuric acid.

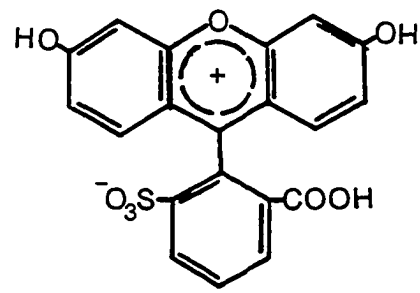
Two sulfofluoresceins are possible from the condensation of α-sulfophthalic acid with resorcinol, depending on which

carboxyl groups furnishes the 9'-carbon atom of the xanthene ring, the possible products being 4-sulfofluorescein (Structure VII), and 7-sulfofluorescein (Structure VIII). (The numbering system of Chemical Abstracts for fluorescein is followed here.) Two isomeric sulfofluoresceins are also possible from the condensation of β-sulfophthalic acid with resorcinol, 5-sulfofluorescein (Structure IX) and 6-sulfofluorescein (Structure X).

"α-Isomers"

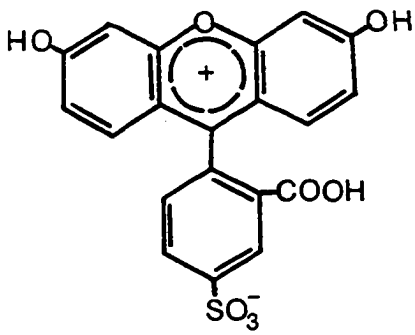


VII
4-Sulfofluorescein

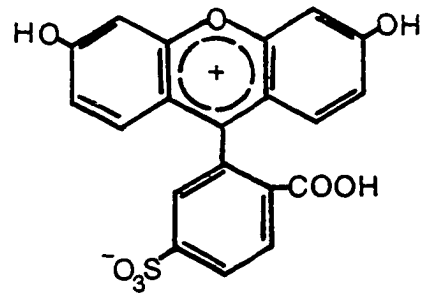


VIII
7-Sulfofluorescein

"β-Isomers"



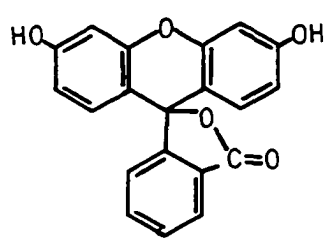
5-Sulfofluorescein



6-Sulfofluorescein

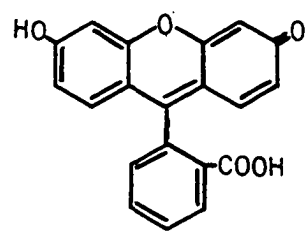
Ioffe, Devyatova, and Roskulyak reported that mixtures of sulfofluoresceins were obtained from both α- and β-sulfophthalic acids, that the mixed sulfofluoresceins were obtained as the free acids, and that only the β-isomers could be separated, by differential solubility in water at 80°. In the work reported in my thesis for the Master of Science degree (8), mixtures of ammonium and potassium salts rather than the free acids were obtained, and the β-isomers as well as the α-isomers were not easily separable by fractional crystallization from water. In the early work (11) and in the present work the condensation and separation procedures were worked out in satisfactory detail and the various sulfofluoresceins, obtained as the potassium salts, were prepared, separated, purified, and identified. A new procedure was devised for the preparation of the free acids. For purification, the potassium salts were converted to the diacetylsulfofluoresceins, the latter recrystallized from acetic anhydride, and the purified diacetyls hydrolyzed back to the sulfofluoresceins. In the course of this work on purification, the procedures were modified and in the end the intermediate steps were eliminated, that is, the diacetylation and recrystallization from acetic anhydride were circumvented and the accompanying losses of material avoided. The new procedure for the separation of the isomers yielded materials chromatographically pure.

During 1976, Markuszewski (3) presented an elegant study in which the basic chemistry of fluorescein was clarified. On the basis of X-ray diffraction powder patterns and mass spectrometry, he proved that colorless, yellow, and red fluorescein are isomeric but distinctly different in structure and that the differences are not just a result of particle size or aggregation of crystalline masses. Largely on the basis of infrared spectrophotometry, Markuszewski assigned the closed lactone structure to the colorless form (Structure XI), the quinone-carboxylic acid structure to the red form (Structure XII), and the zwitter ion structure containing a pyrylium-type ring to the yellow form (Structure XIII).



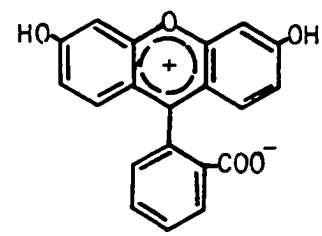
XI

Colorless fluorescein lactone form



XII

Red fluorescein quinone form



XIII

Yellow fluorescein zwitter ion form

Markuszewski (3) established, by measurements of solubility as a function of pH, by potentiometric acid-base titration, by measurement of fluorescence as a function of

pH, and by color analogies, that fluorescein in solution in the acid range was present in the zwitter ion (yellow) form only, and in the alkaline range present as the quinone-carboxylate (red) form. He did not consider the possibility that all three forms might coexist in solution and he excluded completely the possibility that some lactone might be present in aqueous solution.

When dissolved in dioxane or acetone, fluorescein is colorless but changes to the yellow form when water is added. In the present work, evidence is presented that the lactone form is actually present in aqueous solutions of fluorescein, the evidence coming from the characteristics of ethanol-water solutions of fluorescein. Attention as directed this way because of the properties of certain of the sulfofluoresceins which indicate that the lactone form is the predominant form in water solution.

In the early work (11) and in the present work, it was observed: (1) that the sulfofluoresceins are slightly soluble in acetone and dioxane and that such solutions are yellow in color rather than colorless; (2) that colorless diacetylsulfofluoresceins when left exposed to the air or dissolved in water gradually becomes yellow; (3) that when a sulfofluorescein is precipitated from ice cold water or from boiling water-ethanol solution by the addition of strong acid, conditions under which fluorescein is precipitated in the red form, only a yellow precipitate is formed.

From these observations it was concluded that the sulfofluoresceins cannot be prepared in the solid colorless form nor in the solid red form. These observations are confined to the solid forms because other evidence was obtained that in solution the colorless and red forms are present in addition to the yellow form.

The various isomeric and ionic forms of sulfofluorescein are presented in Figure 1; reference will be made to these various structures and forms as the discussion progresses throughout the dissertation.

Because so little was known about sulfonated fluorescein, it was necessary to devote a considerable portion of the work being here reported to a study of the properties and behavior of the isomeric sulfofluoresceins before proceeding to the synthesis of a sulfocalcein.

This dissertation consists of two parts, the first dealing with the sulfofluoresceins, the identification of the three isomers obtained in significant amounts, 4-, 5-, and 6-sulfofluorescein, the determination of the structure of each in the solid state and in solution, and the determination of the acid-base properties of each. These studies involve chromatography, proton nuclear magnetic resonance, infrared and visible spectrophotometry, acid-base titration in water and in 50 per cent ethanol, and measurements of solubility, absorbance and fluorescence as functions of pH.

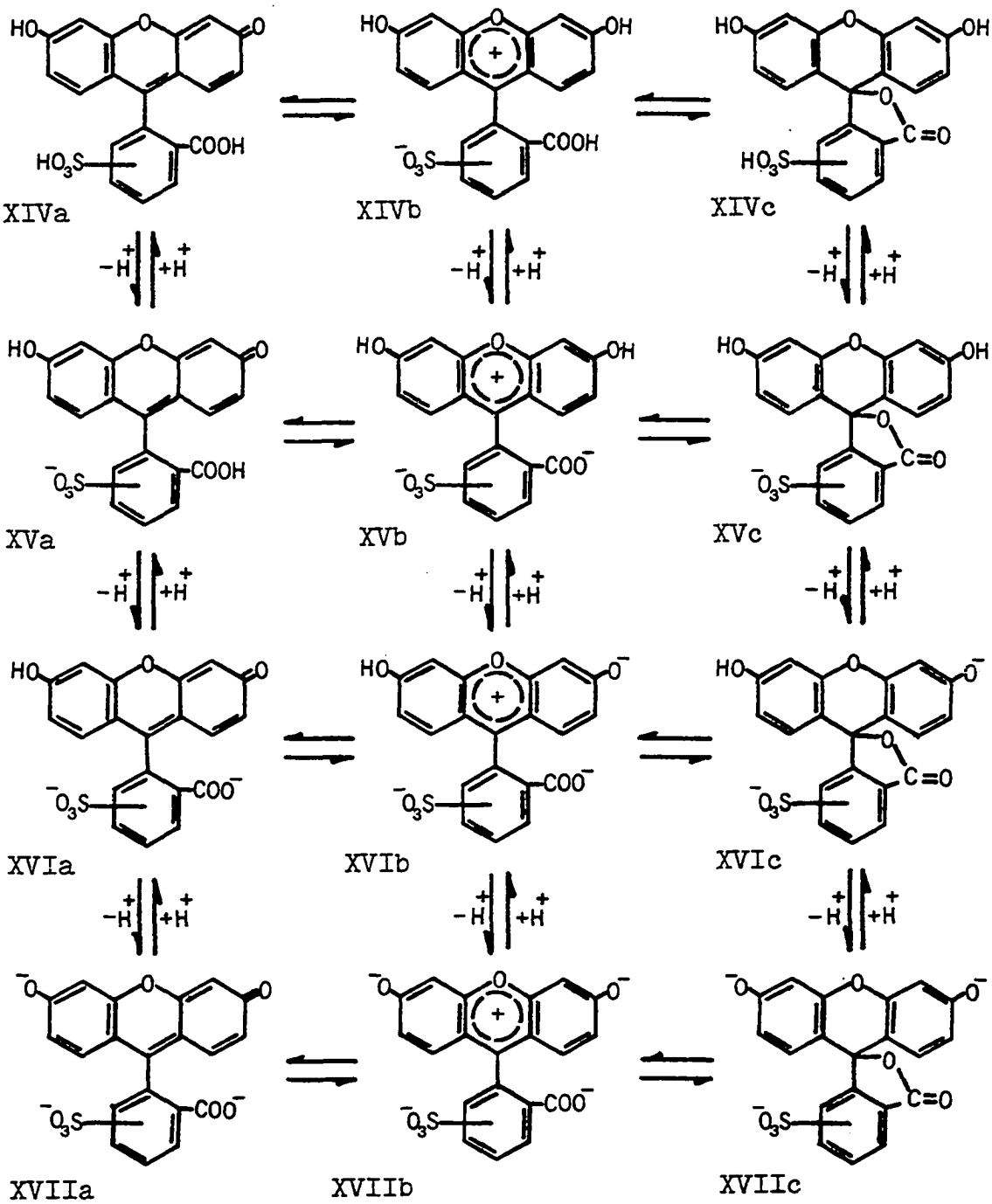
Figure 1. The isomers and the prototropic forms of sulfofluorescein, H_3Sfl.

Structure XIV. Neutral molecule (H_3Sfl)

Structure XV. Monoanion (H_2Sfl^-)

Structure XVI. Dianion ($HSfl^{2-}$)

Structure XVII. Trianion (Sfl^{3-})


XIVa
XIVb
XIVc
XVa
XVb
XVc
XVIa
XVIb
XVIc
XVIIa
XVIIb
XVIIc

The second part of the dissertation deals with the preparation of a sulfocalcein, the determination of its acid-base properties, and finally an application of this new metallofluorochromic reagent to the determination of calcium.

II. PREPARATION AND SEPARATION OF SULFONIC ACIDS OF FLUORESCEIN

A. Introduction

The synthesis of a sulfofluorescein with the sulfonic acid group attached to the phthalate ring of the molecule was first accomplished by Ioffe, Devyatova, and Roskulyak (12); the route followed was the condensation of a sulfophthalic acid with resorcinol by heating the mixture with concentrated sulfuric acid. In my earlier work (11), following the same procedure, a mixture of ammonium and potassium salts was obtained rather than the free acid reported by the Russian workers. By modification of the procedure, I was able to obtain the various sulfonated fluoresceins in pure form as the monopotassium salts and to establish the identity of each.

Inasmuch as a metal-free sulfofluorescein was desired for the work at hand, the potassium salt was further purified by conversion to the diacetylsulfofluorescein, recrystallization of the latter from acetic anhydride, and hydrolysis back to sulfofluorescein. The hydrolysis was effected by passing an aqueous solution of the diacetyl compound through a strong acid cation exchange resin in the hydrogen form. Owing to the fact that some of the sulfofluorescein was lost during the recrystallization, and the

unpleasant nature of acetic anhydride, the method of preparing the metal-free acid of sulfofluorescein was subjected to considerable study and finally made free of objection.

The Russian workers (12) reported that both of the two isomers of sulfofluorescein expected were produced by the condensation of 3-sulfophthalic acid with resorcinol, that is, 4-sulfofluorescein, Structure VII, and 7-sulfofluorescein, Structure VIII; the mixture is designated as the α-isomers. The Russian workers reported that they were unable to separate these isomers. My work confirmed their findings except that one isomer was obtained as a major product, and the only one which I was able to isolate in chromatographically pure state. On the other hand, the condensation of 4-sulfophthalic acid with resorcinol gave the two isomers expected in about equal yield; 5-sulfofluorescein, Structure IX, and 6-sulfofluorescein, Structure X; the mixture is designated as the β-isomers. These isomers were obtained and separated by a new method. In my hands the separation procedure of Ioffe, Devyatova, and Roskulyak (12), based on the differential solubility of the β-isomers in water at 80°, failed. The new procedure is based on the fractional crystallization of the isomers from ethanol-water mixtures.

B. Preparation of α-Sulfofluorescein (4-Sulfofluorescein and 7-Sulfofluorescein) as Free Acids

A weighed amount of the monopotassium salt of α-sulfofluorescein, 10 g, was added to about one liter of 50 per cent ethanol, the mixture was heated to promote dissolution, then the solution was cooled to room temperature and passed through a column containing about 200 ml of a strong-acid cation exchange resin, Dowex 50-X8, in the hydrogen form. The column was washed with 200 ml of 50 per cent ethanol. The eluate was then evaporated on a hot plate until crystallization began, and was then cooled which caused a yellow, amorphous precipitate to form. The precipitate was collected by filtration and washed with a few portions of deionized water. The free acid was first air-dried and then dried at 100° for about three hours. Yield of purified, metal-free α-sulfofluorescein, free acid: 8.4 g.

A few milligrams of α-sulfofluorescein was dissolved in about 5 ml of 0.1 N sodium hydroxide. A spot of this solution was applied to a strip of filter paper, Whatman No. 3, 10 cm wide by 35 cm long. This length of the strip was necessary to obtain a good resolution of the two isomers. The driving solvent was water buffered at pH 4.0 by dissolving 10.2 g of potassium hydrogen phthalate in one liter of deionized water. On elution two spots were observed,

a dark spot, Rf 0.41, and a very faint spot, Rf 0.73. This observation indicated that one of the isomers is formed only in small amount. This is not surprising for the formation of one of the isomers, 7-fluorescein, requires the participation of the carboxyl group lying between the other carboxyl group and the sulfonic acid group and the reaction might well be impeded by steric hindrance effects.

The free acid α-sulfofluorescein after three crystallizations from ethanol-water solution, gave only one spot, Rf 0.41, when examined by chromatography as described above.

The equivalent weight of the acid, obtained by potentiometric titration in water with 0.1 N sodium hydroxide, was 137.7; the theoretical equivalent weight for sulfofluorescein with three replaceable hydrogen atoms (protons) is 137.5. Expressed alternatively, the purity of the acid was 99.9 per cent.

The acid did not melt below 300°, but did change gradually to a light orange above 150°; when cooled, the material reverted to the yellow color.

C. Preparation of β-Sulfofluorescein (5-Sulfofluorescein and 6-Sulfofluorescein) as Free Acids

The procedure followed was identical with that developed for the preparation of α-sulfofluorescein, Chapter II, Section B, above. An amount, 10 g, of the monopotassium salt

of β-sulfofluorescein was dissolved in one liter of 50 per cent ethanol and the solution passed through a strong-acid cation exchange column in the hydrogen form. Yield of β-sulfofluorescein, free acid, 8.5 g.

The product was examined by paper chromatography as described above. On elution two dark spots of about equal intensity were observed, Rf values 0.51 and 0.64. Apparently, the isomers, 5-sulfofluorescein and 6-sulfofluorescein, are formed and in about equal amounts.

D. Separation of the β-Isomers. 5-Sulfofluorescein and 6-Sulfofluorescein

The method developed for the separation of β-isomers depends on the fact that the two isomers differ in solubility in water-ethanol solution and that this difference depends on the amount of ethanol in the mixture.

A weighed amount of β-sulfofluorescein, 10 g, was added to one liter of deionized water, the mixture was heated on a hot plate with continuous stirring and ethanol was added until all the compound was dissolved. The solution was filtered and then evaporated on a hot plate with continuous stirring until the volume of the solution was reduced to about 1000 ml. The solution was cooled while being stirred; the yellow precipitate which formed was collected by filtration. This precipitate, called B_I, was examined by

ascending paper chromatography as in Chapter II, Section B. A spot with Rf 0.51 was slightly darker than the spot with Rf 0.64. The filtrate was evaporated further to reduce the volume to about 600 ml, then cooled; the yellow precipitate (B_{II}) which formed was collected by filtration and examined by paper chromatography. The spot with Rf 0.64 was darker than the spot with Rf 0.51. The filtrate was evaporated further to about 200 ml, cooled and the yellow precipitate (B_{III}) was collected; this mixture on chromatographic examination showed two spots of equal intensity. The filtrate and B_{III} were saved.

The precipitate B_I was treated again as above, by adding the precipitate to dionized water, 1 g in 100 ml. The mixture heated and enough ethanol added until all the compound had dissolved. The evaporation procedure was repeated and the first batch collected; this material was richer in the isomer giving the spot Rf 0.51 and contained less of the isomer giving the spot Rf 0.64. The second batch contents were just the opposite, darker spot Rf 0.64. The third batch containing about equal amounts of the two isomers. Again, the final filtrate was saved.

The precipitate, called B_{II}, was added to water using the same preparation mentioned above, 1 g in 100 ml, then ethanol added and the solution evaporated and the first batch collected; this material showed a dark spot Rf 0.64

and a very faint spot Rf 0.51. Further evaporation gave a second batch containing about equal amounts of the two isomers.

The crystallization outlined above was repeated until the precipitate collected, when examined by paper chromatography showed only one spot, either with Rf 0.64 or with Rf 0.51. The process required fifteen to twenty crystallizations but proved effective. The two isomers 5-sulfofluorescein and 6-sulfofluorescein were obtained in a chromatographically pure state.

With careful handling and saving the final filtrate, the loss of compound during the process was minimal. Starting with 10 g of the isomeric mixture, I was able to obtain 3.8 g of 5-sulfofluorescein, 2.7 g of 6-sulfofluorescein, and 2.3 g of the isomeric mixture.

The isomer with Rf 0.51 was yellow and when examined under a magnifying lens found to be crystalline. Potentiometric titration with 0.1 N sodium hydroxide gave for the equivalent weight 137.8, the theoretical equivalent weight for sulfofluorescein with three protons is 137.5. Expressed alternatively, the purity of the acid was 99.7 per cent. The impurity was probably water. The acid did not melt when heated up to 300°. A slight change in color from yellow to orange was observed at 160°.

The isomer with Rf 0.64 was yellow and crystalline. When titrated potentiometrically with 0.1 N sodium hydroxide,

the equivalent weight was found to be 143.2; the theoretical equivalent weight of sulfofluorescein with three protons and one molecule of water of crystallization is 143.5. When heated up to 300°, this acid did not melt; loss of water of crystallization began about 110° and a color change to orange occurred at 160°. When cooled, the color of both isomers changed back to yellow.

E. Discussion

The separation of β-isomers of sulfofluorescein proved tedious and time consuming, but it was only after such separation had been accomplished and each isomer identified that a study of the acid-base behavior and of the physical properties of the isomers was begun. In the end, the study of the properties of the individual isomers may indicate that for the purposes of analytical chemistry, a mixture would be satisfactory and the separation unnecessary.

The yellow color of the three isomers, 4-, 5-, and 6-sulfofluorescein, and the fact these free acids did not melt when heated to 300° suggests that free acids are in the zwitter ion form (Structure XIVb) in the solid state. Although the color of the free acids begins to change from yellow to orange above 150°, which may be interpreted as an indication of conversion to the quinone form (Structure XIVa), yet when cooled the color changed back to yellow.

Such behavior is not observed with fluorescein, for when yellow fluorescein (zwitter ion) is heated to 300°, although no melting occurs, the color changes to red (quinone form); no reversion to the yellow color occurs on cooling.

III. IDENTIFICATION OF THE ISOMERIC SULFOFLUORESCEINS BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

A. Introduction

Infrared spectroscopy was used by Roskulyak and Zelenin (13) to determine the positions of the sulfonate groups in the β-isomers of sulfofluorescein (Structures IX and X) formed by the condensation of resorcinol with 4-sulfophthalic acid (Structure IV). They compared the infrared spectra of the β-isomers with those of m- and p-sulfobenzoic acids, in the region 700-900 cm^{-1} in which the out-of-plane carbon-hydrogen bending bands occur. Since the out-of-plane bending of a ring hydrogen atom is strongly coupled to adjacent hydrogen atoms, the position of absorption of the out-of-plane bending band is characteristic of the number of adjacent hydrogen atoms in the ring. The bands are usually reliable for alkyl substituted benzene and some polynuclear aromatic compounds, but caution must be exercised in the interpretation of spectra when polar groups are attached directly to the ring (14). This explains why the Russian workers used the comparative method to arrive at their conclusion, that is, the more water soluble sulfofluorescein is 5-sulfofluorescein (Structure IX) and the less water soluble sulfofluorescein is 6-sulfofluorescein (Structure X). Their conclusion was based on the fact that in the spectrum

of the readily water-soluble isomer were bands at frequencies of 740 and 821 cm^{-1}, and these bands were also found in the spectrum of m-sulfobenzoic acid, but were absent from the spectrum of the difficulty water-soluble isomer and that of p-sulfobenzoic acid.

Roskulyak and Zelenin used mineral oil as a medium for their infrared studies. When I repeated their work using potassium bromide pellets, I obtained better resolution, and a comparison of the infrared spectra of the β-isomers of sulfofluorescein with those of m- and p-sulfobenzoic acids showed very small difference. In view of the large number of frequencies in the spectra of the compounds under investigation, the conclusions drawn by the Russian workers appears questionable. For the further identification of the α- and β- isomers I used proton nuclear magnetic resonance (NMR); this proved fruitful and positive in making the identifications.

Hefley (7) by studying the NMR of fluorescein and fluorescein derivatives, in dimethylsulfoxide (DMSO), was able to assign the chemical shifts for the protons on the xanthene and on the phthalate groups of fluorescein. Her work was confirmed later by Markuszewski (3), who used dioxane as a solvent. In the present work, dimethylsulfoxide (DMSO) was used; this proved an excellent solvent for the sulfonated fluoresceins. The NMR spectrum of yellow

fluorescein was also obtained using DMSO as a solvent, so that comparisons with the sulfofluoresceins could be made.

B. Experimental Work

1. Apparatus

The Varian model A-60 Nuclear Magnetic Resonance Spectrometer was used. This instrument has a magnet which generates a magnetic field in the region of 14,000 gause, and a radio frequency oscillator set at 60 megacycles per second. The offset for all spectra obtained was set at 120 cps which is equivalent to a shift of 2 ppm.

2. Materials

The yellow fluorescein used was prepared by purification of commercial fluorescein through diacetylation and hydrolysis of the latter in basic alcoholic solution. The separated α- and β-isomers of sulfofluorescein described previously, were used. The solutions were prepared in reagent-grade DMSO. The reference used to calibrate the spectrometer, that is, to set δ at zero, was tetramethylsilane (TMS).

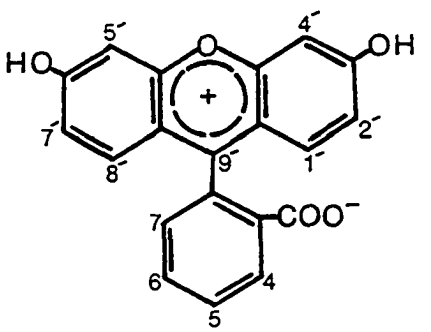
C. Results and Discussion

1. The NMR spectrum of fluorescein

Since the aromatic protons of the compounds under investigation appear beyond $\delta = 6$ ppm only the region between 5 and 10 ppm was recorded. Above $\delta = 5$, the very strong NMR

signal of the solvent was expected to swamp the spectrum in that region.

It is appropriate to reproduce here the zwitter ion form of fluorescein (Structure XIII) with the positions of the protons numbered following the practice of Chemical Abstracts:



XIII

The NMR spectrum of fluorescein in DMSO, with an offset of 120 cps, Figure 2, is characterized by six groups of peaks: a singlet (A) at about 6.64 ppm integrates to 4 protons and is assigned to the protons 1', 2', 7', 8' on the xanthene ring; a singlet (B) at about 6.78 ppm integrates to 2 protons and is assigned to the protons 4', 5' on the xanthene ring; a multiplet (C) at about 7.34 ppm integrate to one proton and is assigned to proton 7 on the phthalate ring; a quintet (D) at about 7.78 ppm integrates to two protons and is assigned to protons 6 and 5 on the phthalate ring; and multiplet (E) at about 8.10 ppm and is assigned to proton 4

Figure 2. NMR spectrum of fluorescein in dimethylsulfoxide. Offset 120 cps.

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

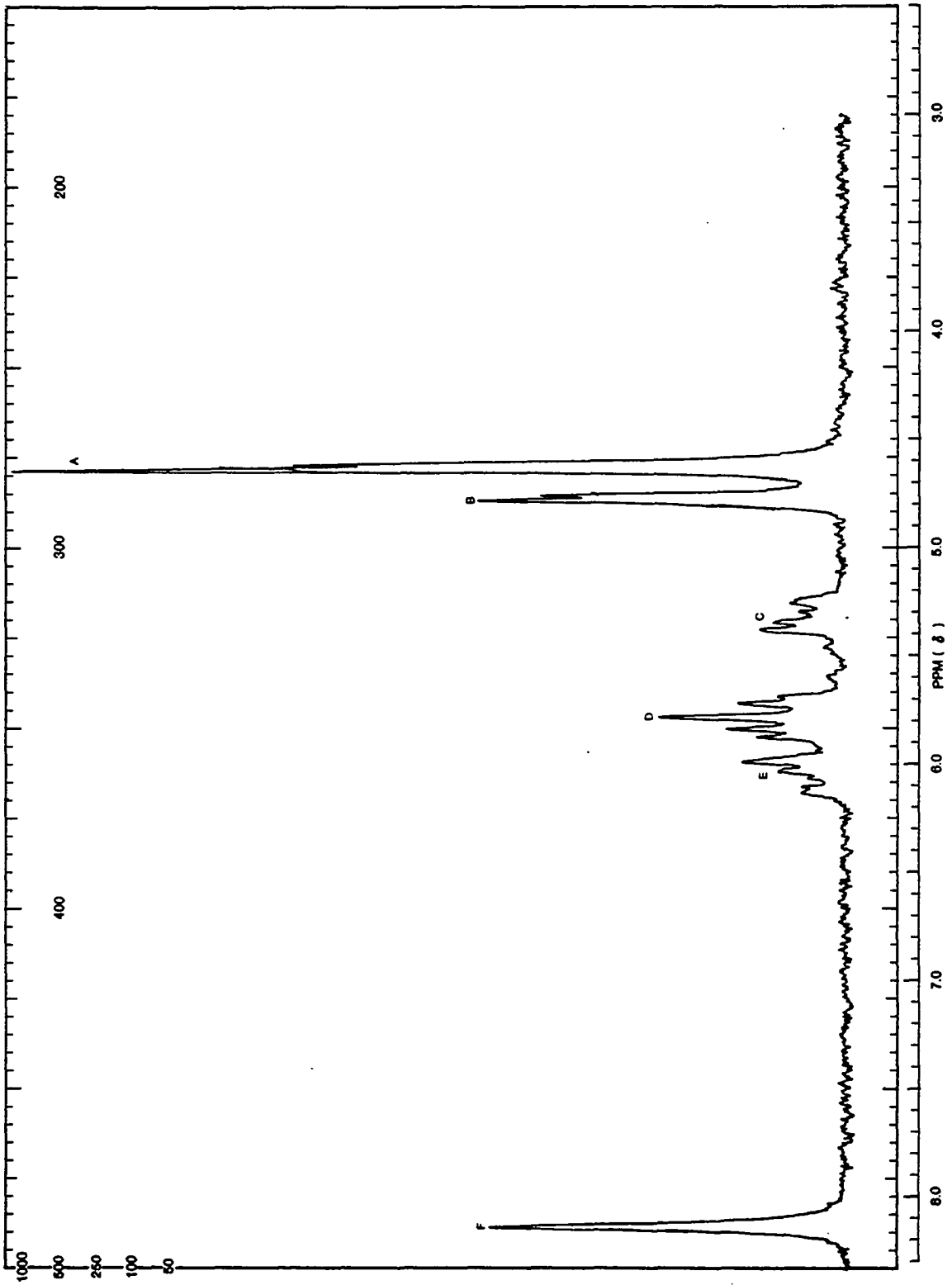
B. Protons (2) at positions 4' and 5' of the xanthene ring.

C. Protons (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.

F. Protons (2) (replaceable).


1000
500
250
100
50
200
300
400
A
B
C
D
E
F
8.0
7.0
6.0
5.0
4.0
3.0
PPM (δ)

on the phthalate ring; a singlet (F) at 10.15 ppm, integrates to 2 protons and is assigned to the phenolic hydrogen atoms on the xanthene ring. The singlet (F) collapses, Figure 3, when deuterium oxide (D_2O) is added the solution of fluorescein in DMSO, this collapse being caused by exchange between the phenolic hydrogen atoms and deuterium. These assignments are in full agreement with those of Hefley.

With respect to the phenolic hydrogen atoms, the chemical shift is very susceptible to hydrogen bonding, the shift depending on concentration, solvent, and temperature. The hydrogen bonding decreases the electron density around the proton and thus moves the proton absorption to lower field. The extent of intermolecular hydrogen bonding is decreased by dilution with a nonpolar solvent and with increased temperature. Polar solvents like DMSO introduce the additional complication of hydrogen bonding between the phenolic protons and the solvent. Keeping this in mind, it is not surprising to find the band of the phenolic protons of fluorescein down field at δ 10, while the phenolic protons are generally observed at δ 7.5 to 4.0. In this work, I observed that the peak for phenolic and carboxylic protons of the compounds under investigation range from δ 6 to 10 ppm depending on the concentration and mainly on the content of water in the solution. Three spectra are reported for 5-sulfofluorescein, Figures 6, 7 and 8, with increasing amount of water respectively, to illustrate the point.

Figure 3. NMR spectrum of fluorescein in dimethylsulfoxide and deuterium oxide. Offset 120 cps.

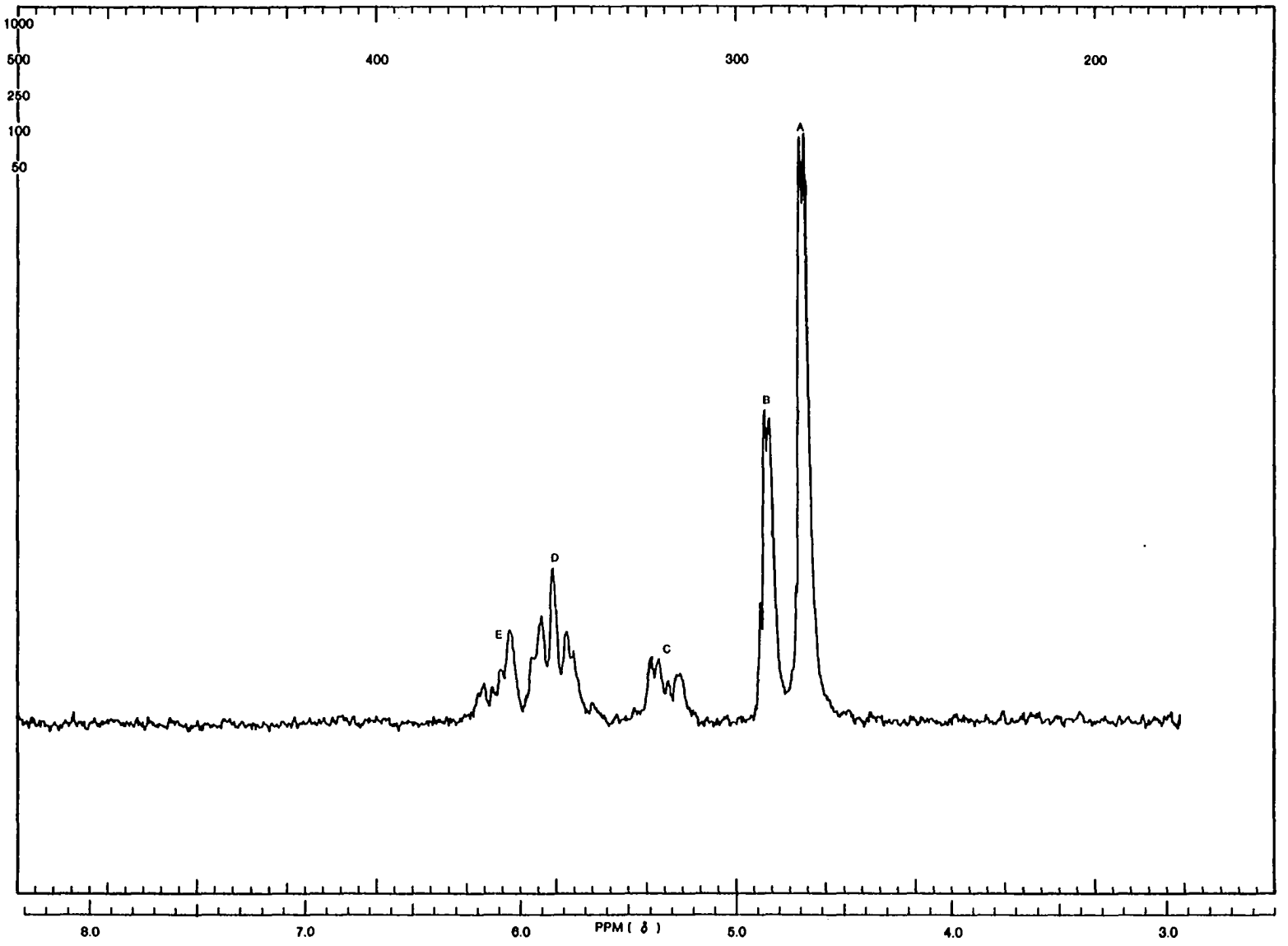
A. Protons (4) at positions 1', 2', 7', and 8' of the xanthene ring.

B. Protons (2) at positions 4' and 5' of the xanthene ring.

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.


1000
500
400
300
200
250
100
50
A
B
C
D
E
8.0
7.0
6.0
PPM (δ)
5.0
4.0
3.0

Hefley observed the two phenolic protons at $\delta = 9.83$ using DMSO as a solvent, while Markuszewski observed the same protons at $\delta = 7.86$. Markuszewski suggested that the difference could be due to the fact that fluorescein in DMSO exists as the zwitter ion (Structure XIII) or as the quinone form (Structure XII). The two replaceable protons of both of these structures are much more acidic than the two replaceable protons of the lactone form (Structure XI), the form which fluorescein assumes in dioxane. Such explanation is not very sound especially as the water content increases in a solution of fluorescein in DMSO the chemical shift for the replaceable hydrogen atoms moves upfield, yet the zwitter ion is supposed to increase in concentration as the content of water increases. Many factors affect the chemical shift of the replaceable protons and this shift cannot be used to explain the form of fluorescein either in DMSO or in dioxane.

The positions and assignments of peaks in the NMR spectra of fluorescein and the three isomers of sulfofluorescein are summarized in Table 1. The identification of the position of the sulfonic acid group on the phthalate ring is based on the chemical shifts of the peaks as they compare to those of fluorescein, and on the splitting patterns.

Table 1. Nuclear magnetic resonance spectra of fluorescein and the three sulfofluoresceins in dimethylsulfoxide

Proton	Chemical Shift, ppm	Multiplicity	Integration
	Fluorescein		
1',8' 2',7'	6.67	singlet	4
4',5'	6.79	singlet	2
7	7.32	multiplet	1
5,6	7.78	quintet	2
4	8.08	multiplet	1
replaceable hydrogen atoms	10.15	singlet	2
	6-Sulfofluorescein		
1',8' 2',7'	6.77	singlet	4
4',5'	6.85	singlet	2
7	7.42	singlet	1
4,5	8.05	singlet	2
replaceable hydrogen atoms	9.27	singlet	>3
	5-Sulfofluorescein		
1',8' 2',7'	6.83	singlet	4
4',5'	6.94	singlet	2
7	7.38	doublet	1
6	8.16	doublet	1
4	8.32	singlet	1
replaceable hydrogen atoms	9.28	singlet	>3

Table 1. (Continued)

Proton	Chemical Shift, ppm	Multiplicity	Integration
	4-Sulfofluorescein		
1',8' 2',7'	6.64	singlet	4
4',5'	6.79	singlet	2
7	7.24	doublet	1
6	7.78	triplet	1
5	8.13	doublet	1
replaceable hydrogen atoms	8.92	singlet	>3

2. The NMR spectra of β-isomers

The NMR spectrum of that component of β-sulfofluorescein having Rf = 0.64 taken in DMSO, and with an offset 120 cps, Figure 4, is characterized by five groups of peaks: a singlet (A) at about 6.77 ppm integrates to 4 protons, 1',2',7',8' on the xanthene rings; a singlet (B) at about 6.85 ppm integrates to 2 protons, 4',5' on the xanthene rings. It is obvious that the chemical shift and the splittings are essentially the same as the protons on the xanthene ring of fluorescein. The peaks ascribed to the protons on the phthalate ring are considerably different in splitting

Figure 4. NMR spectrum of 6-sulfofluorescein in dimethylsulfoxide.

Offset 120 cps.

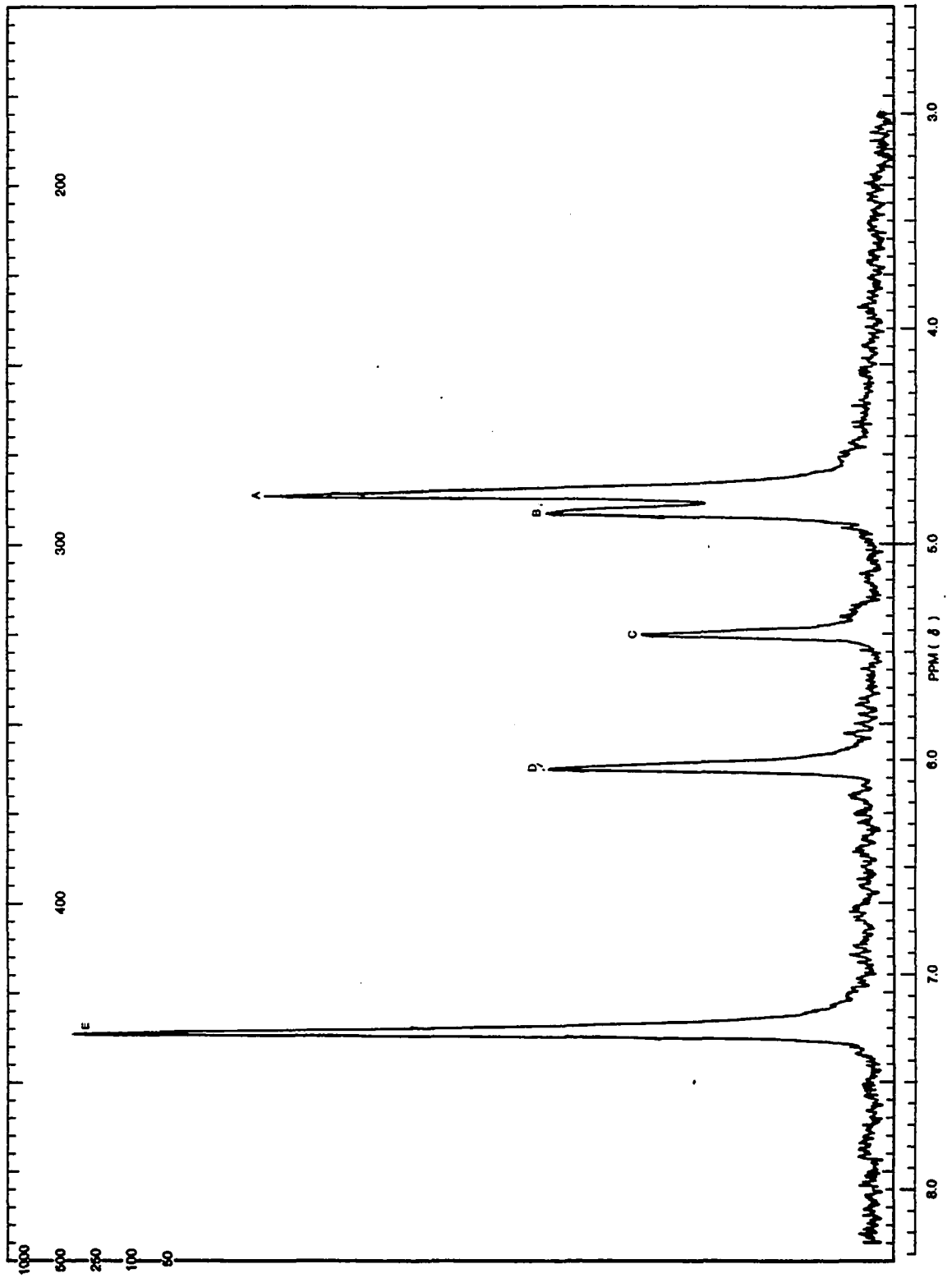
A. Protons (4) at positions 1', 2', 7', and 8' of the xanthene ring.

B. Protons (2) at positions 4' and 5' of the xanthene ring.

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

D. Protons (2) at positions 4 and 5 of the phthalate ring.

E. Protons >3 (replaceable).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3.0
PPM (δ)

pattern. A singlet (C) at about 7.42 integrates to one proton which is very close to the chemical shift value of proton 7 in fluorescein $\delta = 7.32$. The fact that it is a sharp singlet, means there is no hydrogen atom attached to the adjacent carbon, number 6, thus the logical conclusion is that the sulfonic group is attached to this carbon. This is confirmed by examining the peak (D), a singlet at about 8.05 ppm, which integrates to two protons. The chemical shift value for proton 4 in fluorescein is 8.08. Thus, the chemical shifts for protons 5 and 4 on the phthalate ring in this particular isomer of sulfluorescein are equal owing to the fact that proton 5 is in the position ortho to the sulfonic group, while proton 4 is in the position ortho to the carboxylic group, both protons 4 and 5 are experiencing the same deshielding effect. The conclusion is that this isomer is 6-sulfofluorescein. The singlet peak (F) at about 9.27 ppm is assigned to the replaceable hydrogen atoms. This peak as indicated above integrates to more than 3 protons and is variable in position according to water content of the solution. When deuterium oxide is added, the peak collapses, Figure 5, owing to the rapid exchange of hydrogen and deuterium.

The NMR spectrum of that component of β-sulfofluorescein having Rf = 0.51 in DMSO and with an offset 120 cps (Figure 6) is characterizes by six groups of peaks. The first two

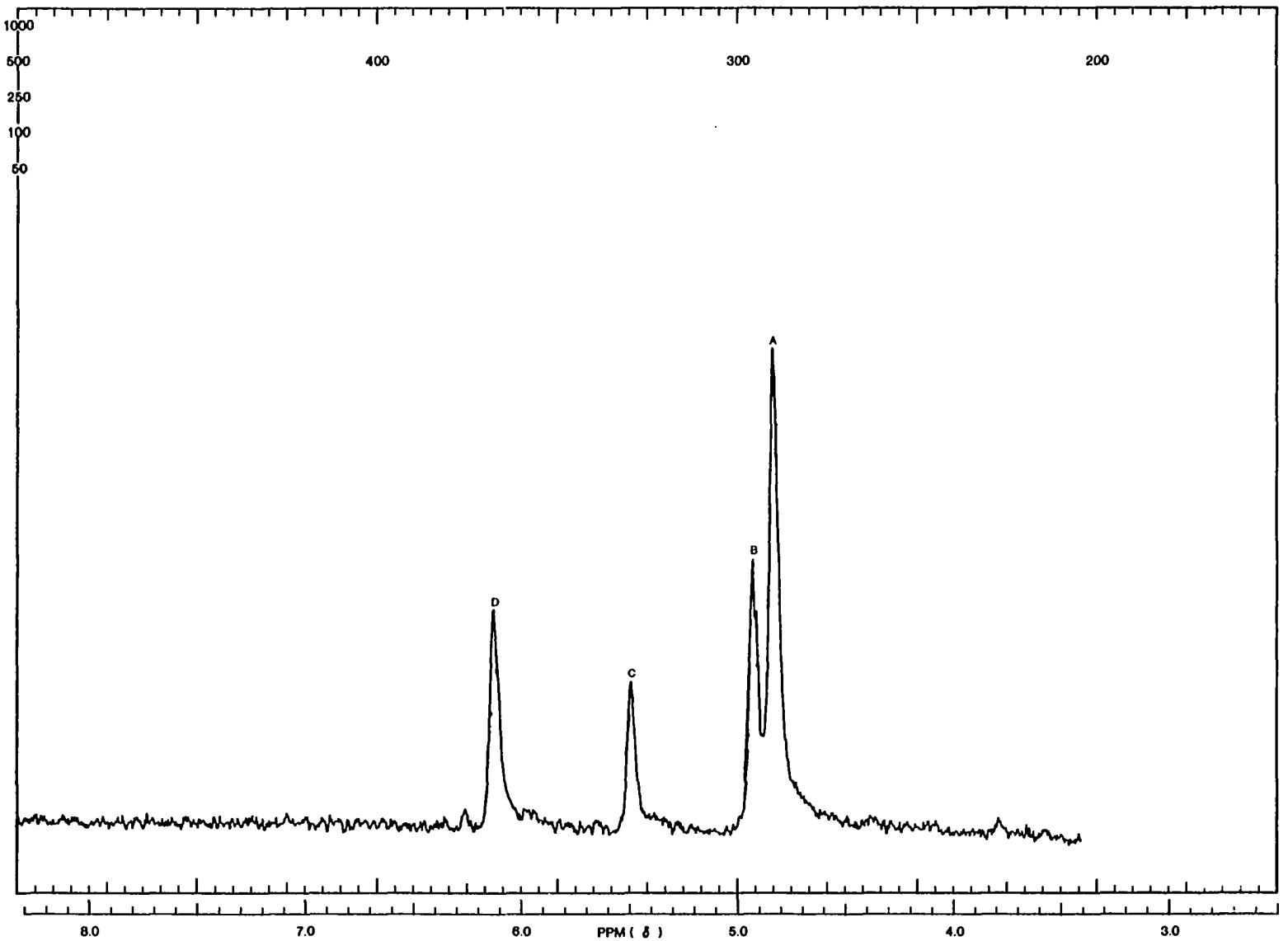
Figure 5. NMR spectrum of 6-sulfofluorescein in dimethylsulfoxide and deuterium oxide. Offset 120 cps.

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

B. Protons (2) in positions 4' and 5' of the xanthene ring.

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

D. Protons (2) at positions 4 and 5 of the phthalate ring.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PPM (δ)
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4.0
3.0

peaks (A) and (B) are at about 6.83 ppm and 6.94 ppm, respectively. They integrate to six protons and are assigned to the protons on the xanthene portion of the molecule as discussed above. The doublet (C) is about 7.38 ppm, it integrates to one proton, and the chemical shift compares well to that of proton 7 on the phthalate ring of fluorescein, 7.32 ppm. The fact that the peak is a doublet with a coupling constant, J = 7 cps, indicates that there is a proton in the ortho position with respect to proton 7, that is, a hydrogen atom on carbon number 6. The doublet (D) is at 8.16 ppm; it integrates to one proton with a coupling constant, J = 7 cps. First, the chemical shift for proton 6 of the sulfluorescein, more downfield than that of fluorescein, owing to the deshielding effect of the sulfonic acid group, second the value of the coupling constant is 7 cps, which confirms that protons 6 and 7 are ortho to each other. The singlet (E) at about 8.32 ppm integrates to one proton and is assigned to proton 4 on the phthalate ring, by reason that the peak is a singlet indicates that there is no proton on the adjacent carbon atom, and again the chemical shift for proton 4 is 0.24 ppm more downfield than proton 4 of fluorescein, owing to the deshielding effect of the sulfonic acid group. Thus, this isomer is 5-sulfofluorescein as expected. For more confirmation, an examination of the doublet (D) and the singlet (E) was made; a splitting of the

Figure 6. NMR spectrum of 5-sulfofluorescein in dimethylsulfoxide.

Offset 120 cps.

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

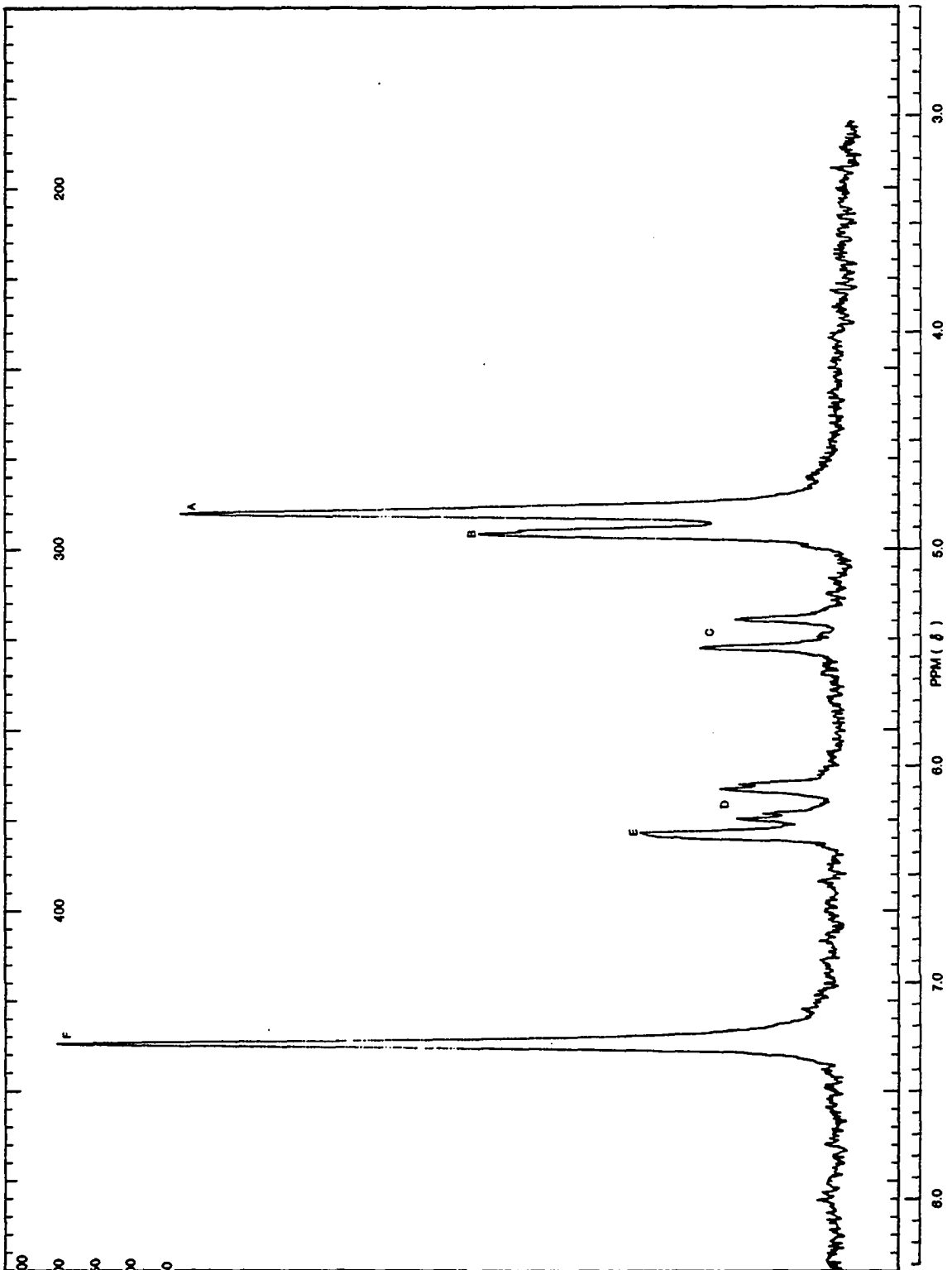
B. Protons (2) at positions 4' and 5' of the xanthene ring.

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

D. Proton (1) at position 6 of the phthalate ring.

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

F. Proton >3 (replaceable).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PPM (δ)

Figure 7. NMR spectrum of 5-sulfofluorescein in dimethylsulfoxide.

Offset 120 cps.

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

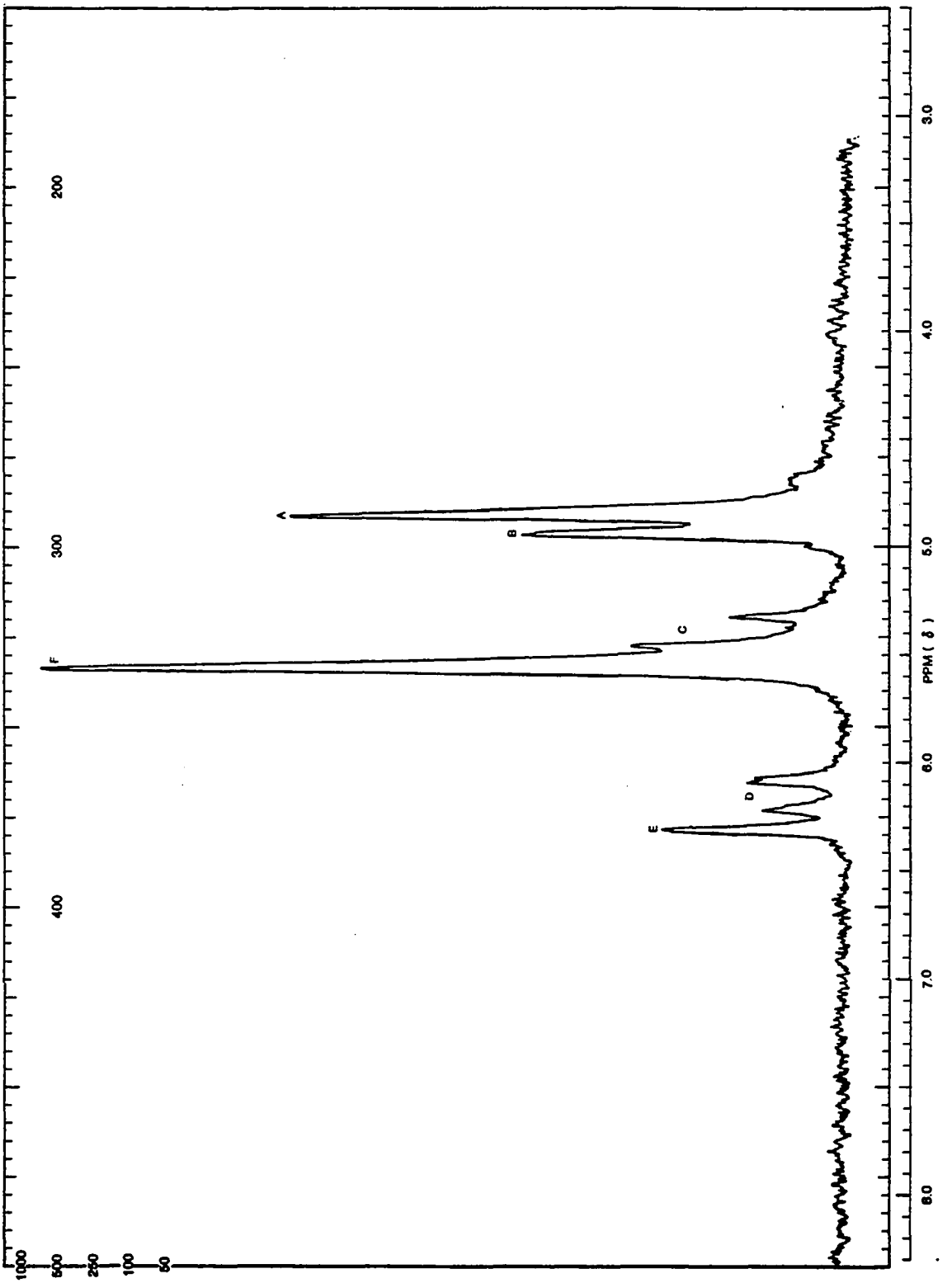
B. Protons (2) at positions 4' and 5' of the xanthene ring.

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

D. Proton (1) at position 6 of the phthalate ring.

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

F. Proton >3 (replaceable).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PPM (δ)

Figure 8. NMR spectrum of 5-sulfofluorescein in dimethylsulfoxide.

Offset 120 cps.

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

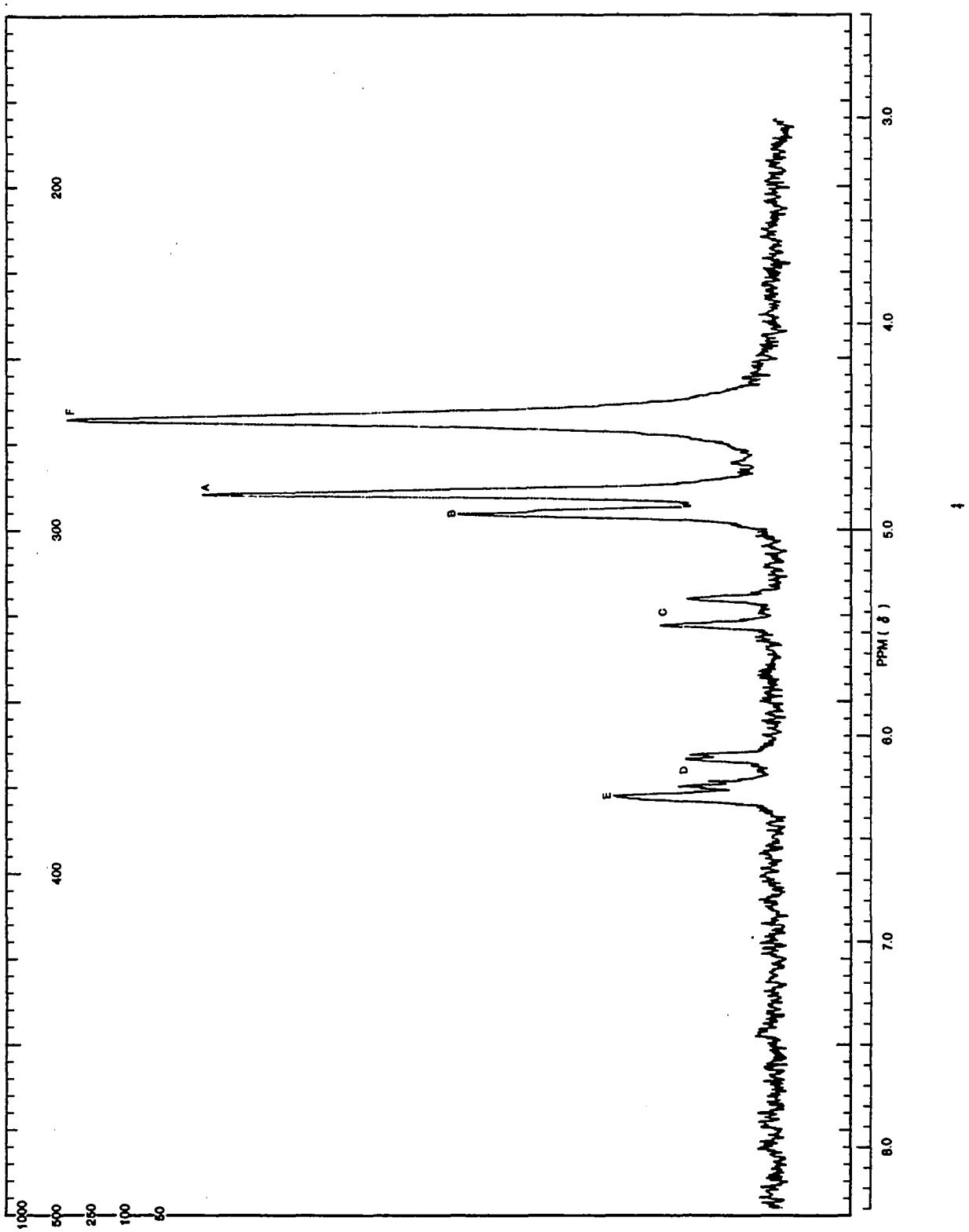
B. Protons (2) at positions 4' and 5' of the xanthene ring.

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

D. Proton (1) at position 6 of the phthalate ring.

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

F. Proton >3 (replaceable).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PPM (δ)

order 1 cps can be observed which indicates the two protons are in meta position with respect to each other, that is, on the 5- and 4-positions of the phthalate ring. The singlet (F) at about 8.28 ppm is assigned to the replaceable hydrogen atoms, keeping in mind that the position and the integration of this peak is a function of water content of the solution. Again, when deuterium oxide is added to the solution, the singlet (F) collapses, Figure 9, owing to the rapid exchange of hydrogen and deuterium.

3. The NMR spectrum of the α-isomer

The NMR spectra of the one α-isomer isolated in sufficient quantity to work with, in DMSO and with an offset of 120 cps (Figure 10), is characterized by six groups of peaks. The first two peaks (A) and (B), are at about 6.64 and 6.79 ppm, respectively. They integrate to six protons and are assigned to the protons on the xanthene portion of the molecule as discussed above. The doublet (C) is about 7.24 ppm; it integrates to one proton, the chemical shift compares well to that of proton 7 on the phthalate ring of fluorescein 7.32 ppm. The fact the peak is a doublet with a coupling constant, J = 7 cps, indicates that there is a hydrogen atom on the adjacent carbon atom, that is, in the ortho position with respect to carbon number 7. The triplet (C) integrates to one proton and at about 7.78 ppm the same region for the chemical shift of proton 6 on the phthalate

Figure 9. NMR spectrum of 5-sulfofluorescein in dimethylsulfoxide and deuterium oxide. Offset 120 cps.

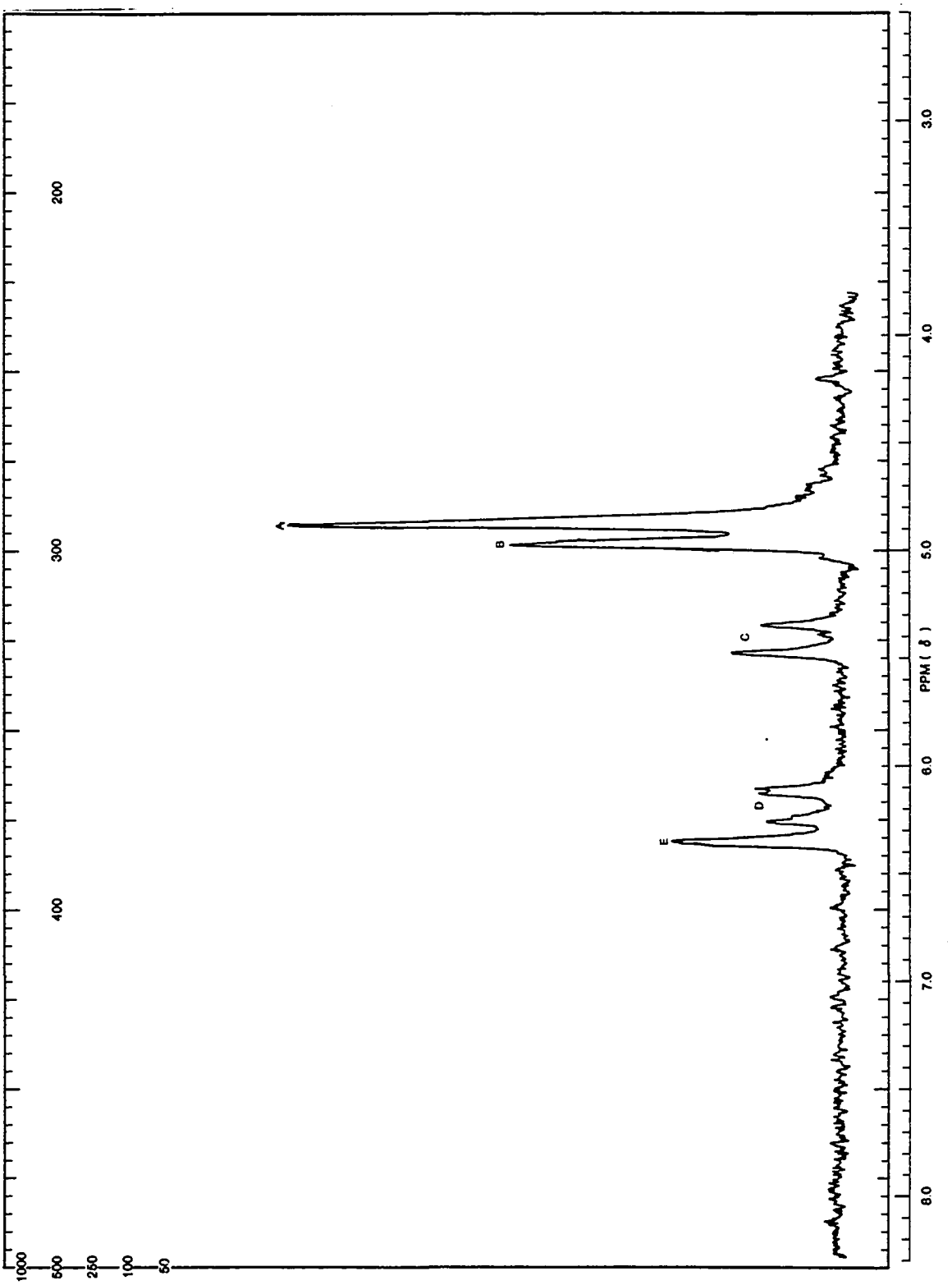
A. Protons (4) at positions 1', 2', 7', and 8' of the xanthene ring.

B. Protons (2) at positions 4' and 5' of the xanthene ring.

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

D. Proton (1) at position 6 of the phthalate ring.

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

Figure 10. NMR spectrum of 4-sulfofluorescein in dimethylsulfoxide.

Offset 120 cps.

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

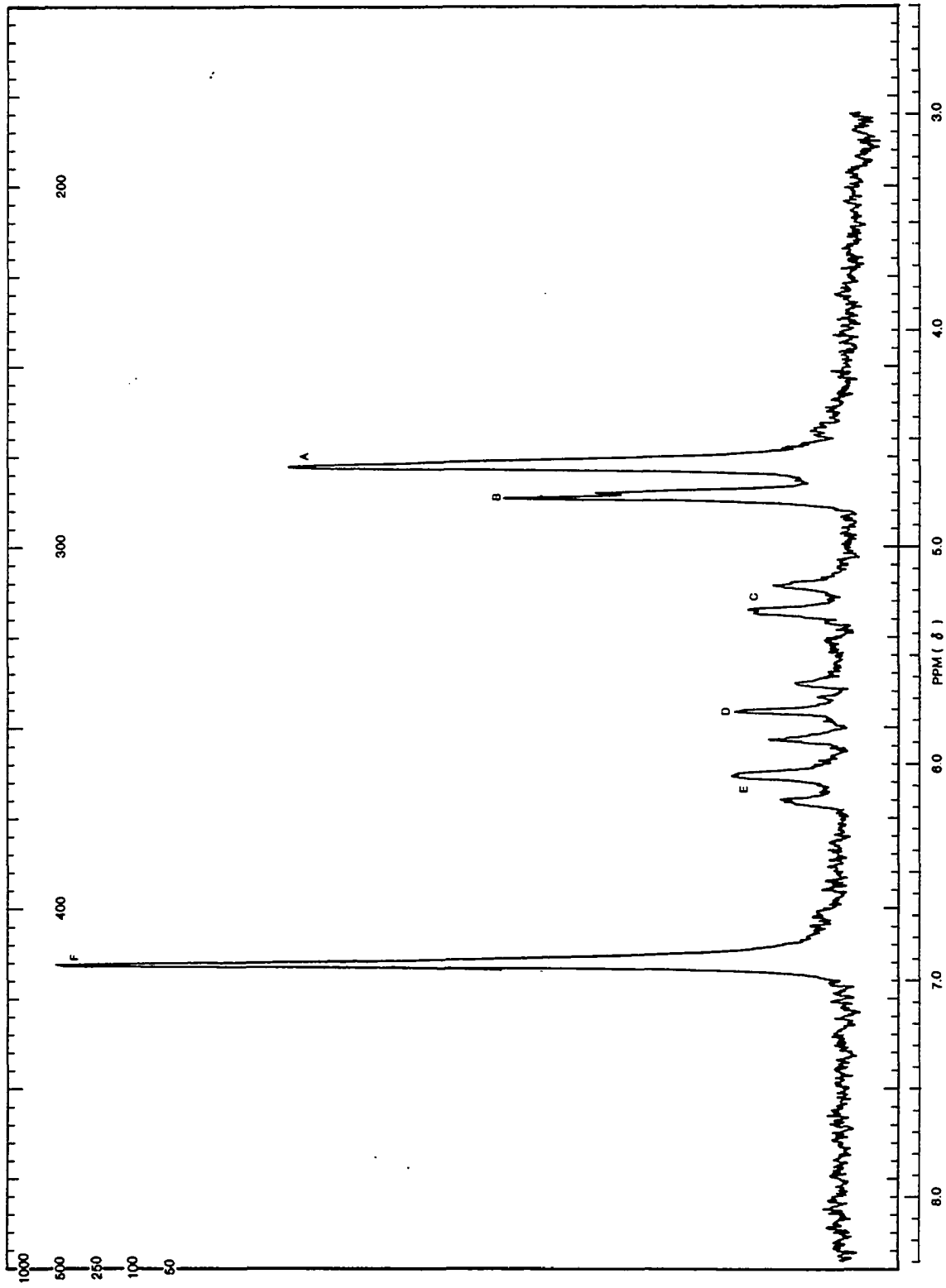
B. Protons (2) at positions 4' and 5' of the xanthene ring.

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

D. Proton (1) at position 6 of the phthalate ring.

E. Proton (1) at position 5 of the phthalate ring.

F. Protons >3 (replaceable).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PPM (δ)

of fluorescein. The fact peak (D) is a triplet means, either proton 6 is being coupled by two equivalent protons on an adjacent carbon, which could not be the case here, or by two unequivalent protons with the same coupling constants. The quartet merges into a triplet, which is the case here. Proton 6 is being coupled by two protons 5 and 7 and both are in the ortho position with coupling constants of 7 cps. The doublet (E) integrates to one proton, at about 8.13 ppm, and is coupled by proton 6, with a coupling constant, $J = 7$ cps. The chemical shift for proton 5 more downfield than the same proton on the phthalate ring of fluorescein owing to the deshielding effect of the sulfonic acid group in the ortho position.

The argument that the peak at 8.13 ppm, in the NMR spectrum of the α-isomer, corresponds to proton 4 on the phthalate ring of fluorescein, $\delta = 8.08$ ppm, could lead to a conclusion that the isolated isomer is 7-sulfofluorescein rather than 4-sulfofluorescein. This argement can be refuted; in the case of 7-sulfofluorescein (Structure VIII) the protons 4 and 6 on the phthalate ring are expected to be more downfield than proton 5, owing to the effect of the carboxylic and sulfonic acid groups, respectively. Thus, a semiquartet or a triplet assigned to proton 5 would be expected, and this multiplet would be upfield with respect to the two doublets assigned to the protons 5 and 6.

Obviously, this was not the case in the spectra obtained, Figures 10 and 11) of the α-isomer. The chemical shifts and multiplicities of the peaks confirm that the isolated α-isomer is 4-sulfofluorescein.

Again, the singlet (F) is about 8.92 ppm and is assigned to the replaceable hydrogen atoms. The chemical shift and the integration of this singlet are functions of water content of the solution. And again, the peak collapses, Figure 11, when deuterium oxide is added to the solution owing to the rapid exchange of hydrogen and deuterium.

D. Conclusion

The β-isomers of sulfofluorescein have been identified by proton nuclear magnetic resonance spectroscopy and found to be 5-sulfofluorescein and 6-sulfofluorescein. The single α-isomer of sulfofluorescein isolated in sufficient quantity to study has been shown to be 4-sulfofluorescein.

Figure 11. NMR spectrum of 4-sulfofluorescein in dimethylsulfoxide and deuterium oxide. Offset 120 cps.

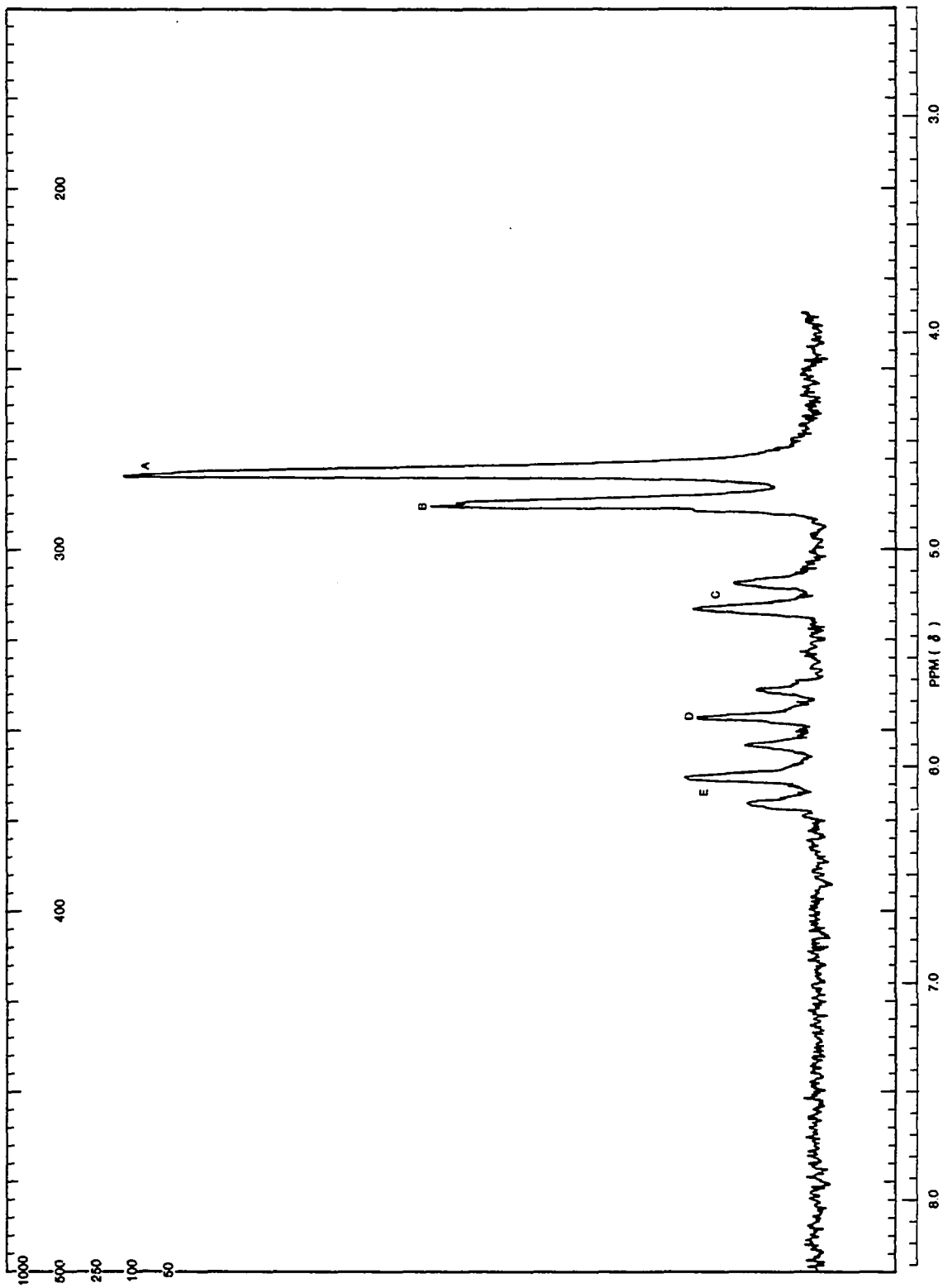
A. Protons (4) at positions 1', 2', 7', and 8' of the xanthene ring.

B. Protons (2) at positions 4' and 5' of the xanthene ring.

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

D. Proton (1) at position 6 of the phthalate ring.

E. Proton (1) at position 5 of the phthalate ring.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IV. INFRARED SPECTROSCOPY OF FLUORESCEIN AND THREE SULFOFLUORESCEINS

A. Introduction

Although fluorescein was first prepared by Baeyer (15) over one hundred years ago, a definitive assignment of structure to the yellow, red and newly discovered colorless forms of the compound and to the various species of the compound in solution was not made until the work of Markuszewski (3) in 1976. By correlating information from a number of lines of study, Markuszewski clarified the entire field. In the opening chapter of his dissertation, Markuszewski has an excellent review of the history of the problems connected with establishing the structures of fluorescein and of the related compound Calcein.

The early confusion on the structure of fluorescein stems from the existence of three forms of the compound, yellow, red and colorless, both in the solid form and in solution, and the fact that these forms are interconvertible depending on the nature of the solvent, the presence of water, the pH, and the temperature. Mixtures of the yellow and red solids are obtained unless the conditions during preparation are rigidly controlled. Further, commercial fluorescein is usually badly contaminated with heavy metals, such as mercury, iron, zinc and aluminum (Hefley (6)) which alter the chemistry and physical properties of the compound.

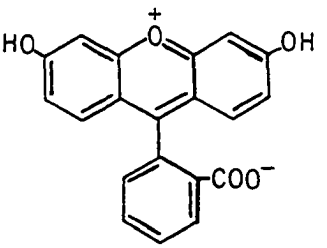
In the infrared spectra of the various forms of fluorescein, curiously, no absorption bands appear at wave numbers greater than 1800 cm^{-1} if the spectra are obtained on suspensions (mulls) in mineral oil; that is, the characteristic broad bands of carboxylic acid and phenol groups are missing. Such bands are present if the spectra are obtained by the potassium bromide pellet technique. As will be reported later in this chapter, conversions of one form to another occur during the preparation of the potassium bromide-fluorescein pellets, a fact which was not appreciated by the early workers.

An assignment of a structure to fluorescein by interpretation of the infrared spectrum was attempted first by Davies and Jones (16), who related the spectra of fluorescein and its sodium salt to the spectra of phenol, sodium phenoxide, sodium benzoate, phenolphthalein, and the sodium salt of phenolphthalein. On the basis of the absence of a band in the region of absorption by the carboxyl group, 3300 cm^{-1} to 2500 cm^{-1} and the absence of the quinone absorption band in the region of 1680 cm^{-1} they ruled out the quinone structure for fluorescein, Structure XII. On the basis of the absence of bands corresponding to absorption by the carboxylate ion, usually in the region 1560 cm^{-1} and 1400 cm^{-1}, they also ruled out the zwitter ion form, Structure XIII. They finally concluded that fluorescein exists in the lactone form,

Structure XI. For further support they pointed to an absorption band observed at 1729 cm^{-1}, absorption usually indicative of a five-membered lactone ring. Davies and Jones failed to mention the form of fluorescein used in their study, whether yellow or red, and they did not report how the thin films on which the spectra were obtained were prepared. Their spectra are reproduced in their paper; on modern standards the absorption bands are weak and poorly resolved.

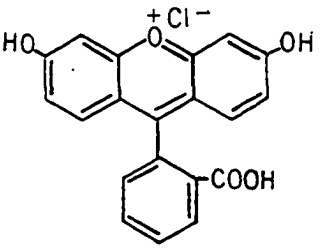
The second study of the infrared spectra of fluorescein is that of Skylar and Mikhailov (17). They obtained the spectrum of both the yellow and red forms on suspensions in petrolatum. They observed an absorption band at 1700 cm^{-1} in the spectrum of the red form and claimed that this absorption band was due to the presence of fluorescein hydrochloride, Structure XIX. They state that the absorption band at 1700 cm^{-1} was absent when red fluorescein was prepared by slow evaporation of a solution of yellow fluorescein (as opposed apparently to precipitating the yellow form by the addition of dilute hydrochloric acid to an alkaline solution of fluorescein). Skylar and Mikhailov reported that when both of the yellow and red forms were treated with ether containing hydrogen chloride, a band of medium intensity developed in the region 1730 cm^{-1}. They associated this absorption band with the carboxyl group of fluorescein hydrochloride. Although admitting that they found differences in

the 1600 cm^{-1} to 1700 cm^{-1} region between the spectra of the yellow and red solid forms, Skylar and Mikhailov assigned to both forms the zwitter ion structure, Structure XVIII. This conclusion was supported by the absence in both spectra of an absorption band characteristic of the lactone group. Skylar and Mikhailov concluded finally that the yellow and red forms of fluorescein are identical, the yellow form being amorphous and the red form crystalline.



XVIII

Zwitter ion form of fluorescein, Skylar and Mikhailov



XIX

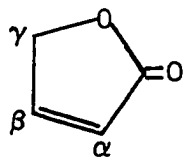
Fluorescein hydrochloride, Skylar and Mikhailov

Zanker and Peter (18), from a study of the absorption in the ultraviolet of fluorescein in dioxane-water mixtures, recognized that a colorless form of fluorescein exists in nonpolar solvents such as dioxane and attributed this form to the lactone, Structure XI.

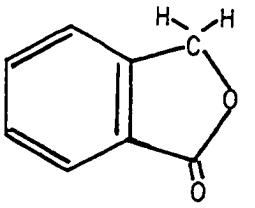
Markuszewski (3) was the first to prepare the colorless fluorescein in the solid state, by freeze-drying a solution of purified fluorescein in dioxane. Markuszewski obtained

the X-ray diffraction patterns of the yellow, red and colorless forms of fluorescein and found that each form is a definite and individual species; this disposed effectively of the argument that the difference in color is merely a physical modification of the solid, that is, the yellow solid amorphous, the red form crystalline. Markuszewski then went on to obtain the infrared spectrum on films of each of the three solid forms and was finally able to make definitive assignments of structure to each form: to the colorless form, the lactone structure (XI); to the yellow form, the zwitter ion structure (XIII); to the red form, the para-quinone structure (XII).

Markuszewski observed that the infrared spectrum of colorless, solid fluorescein is marked by a prominent absorption band with a maximum at 1730 and a shoulder at 1760 cm^{-1}, a band which is absent in the spectra of the red and yellow forms. The carbonyl stretching band for unsaturated five-membered ring (14) γ-lactone, Structure XX, occurs at 1750 cm^{-1}, while saturated γ-lactones absorb at a higher frequency, 1795 - 1760 cm^{-1}. Markuszewski explained the shift to 1730 cm^{-1} in the case of fluorescein, by considering the compound to be a phthalide, Structure XXI, substituted in the γ-position, for which the absorption band falls in the region, 1745 - 1715 cm^{-1}. Markuszewski supported the proposed assignment, that is, the lactone



XX. γ-Lactone



XXI. Phthalide

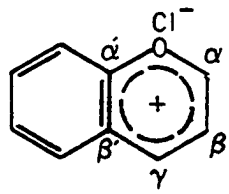
structure to colorless fluorescein by drawing analogy to phenolphthalein, Structure XXV, p. 265, a compound known to contain the lactone ring and similar in all aspects to fluorescein except that the bridging oxygen atom is absent and which is colorless.

Markuszewski assigned an absorption band at 1711 cm^{-1} occurring in the spectrum of red fluorescein to the carbon-oxygen stretching of a carboxylic acid group. It is known that the band corresponding to the carbonyl stretch of a conjugated aryl acid falls (14) in the region 1710 - 1680 cm^{-1}. Markuszewski did not observe the carbonyl absorption band of the quinone, which normally falls in the region, 1655 - 1635 cm^{-1} for a highly conjucated compound, he explained that this absorption could have merged with the absorption band at 1600 - 1585 cm^{-1} corresponding to the skeletal vibration of the aromatic carbon-carbon bond, and attributed this shift to lower frequency to conjugation. Markuszewski assigned the para-quinone structure (XII) to red fluorescein. This assignment was supported by the color

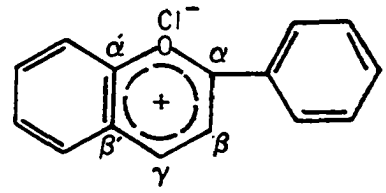
which is that of a para-quinone structure containing a highly conjugated chromophore. The same chromophore and red color is characteristic of an alkaline solution of phenolphthalein and the solid dipotassium salt of phenolphthalein.

Finally, Markuszewski assigned the zwitter ion structure (XIII) to the yellow form. The proof was based again on the infrared spectrum and by analogy to related compounds containing the pyrylium ring. This structure explains the amphoteric nature of fluorescein in solution and the highly acidic character of the two replaceable hydrogen atoms in the molecule. Two features of the infrared spectrum of the yellow form differentiate it from the colorless and red solids: 1) no absorption band appeared in the region 1800 - 1600 cm^{-1}, characteristic of the carbonyl stretching band of a carboxylic acid group or a lactone group; and 2) a prominent band appeared at 1536 cm^{-1}, not present in the spectra of the red and colorless solid fluoresceins. Although this band had been observed previously by Skylar and Mikhailov (17), it was Markuszewski (3) who pointed out that a class of compounds known as the pyrylium salts, such as benzopyrylium, Structure XXII, and flavylium salts, Structure XXIII, which have a six-membered, oxygen-containing ring carrying a positive charge, are characterized by two absorption bands (19), one at 1650 - 1615 cm^{-1} attributed to a symmetric carbon-oxygen stretching vibration, and a

second, attributed to an asymmetric stretching vibration in the region, 1540 - 1530 cm^{-1}. Although Markuszewski observed



XXII. Benzopyrylium chloride



XXIII. Flavylium chloride

the asymmetric stretching band at 1536 cm^{-1} and indicated that the symmetric stretching band is absent owing to the merging with the aromatic carbon-carbon skeletal vibration at 1595 - 1572 cm^{-1}, my work will prove that a good, well-resolved spectrum contains both of the asymmetric and the symmetric bands. This confirms Markuszewski's proposal that yellow fluorescein is analogous to the pyrylium-type compounds and has the zwitter ion structure. Finally, because the zwitter ion structure (XIII) contains a carboxylate group on the phthalate ring of the molecule one would expect to observe the two absorption bands characteristic of the carboxylate anion, 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. The absence of these two bands should not pose a problem, first the band due to the symmetrical stretching is usually weak and less likely to be observed in a complex molecule like fluorescein for which

other strong bands lie in the same region. Second, the asymmetric stretching band is merged with the aromatic carbon-carbon stretching band, such phenomenon was observed and explained by Markuszewski (3) and by Davies and Jones (16).

Different locations have been suggested for the positive charge of the zwitter ion of fluorescein. One location is on the bridging oxygen, the other on carbon 9' of the xanthene ring. Markuszewski adopted the representation for which the positive charge exists in an aromatic-like distribution over the entire ring. This representation was based on the conclusion of Martensson and Warren (20) who performed molecular orbitals calculations on the pyrylium ring and stated that although the α- and γ-atoms have partially increased concentration of the positive charge, this charge cannot be assigned to any particular atom in the ring. This representation will be adopted in the present work on the sulfofluoresceins.

Unfortunately, in none of the studies mentioned above has the whole spectrum of the various fluoresceins been given, that is over the range 4000 - 675 cm^{-1}. All are restricted to the region 1800 - 675 cm^{-1}. Some of the spectra are characterized by broad and poorly resolved bands, especially those reported by Davies and Jones, and by Markuszewski. Thus, I assumed the task of obtaining

well-resolved spectra for the yellow and red forms of fluorescein, hoping to confirm further the work of Markuszewski and to have these spectra for reference in the work on the sulfofluoresceins.

B. Infrared Spectra of the Various Forms of Fluorescein

1. Preparation of materials

Commercial fluorescein was purified by conversion to diacetylfluorescein and hydrolysis of the latter back to fluorescein. This procedure freed the fluorescein of the heavy metals present in commercial fluorescein and from other organic impurities. The acetylation procedure used was that described by Dolinsky and Jones (21) as modified by Markuszewski (3) and further improved in the present work.

a. Diacetylfluorescein An amount of 20 g of crude fluorescein was mixed with 10 g of anhydrous sodium acetate and added to 100 ml of acetic anhydride. The mixture was refluxed at about 100° for approximately 4 hours. Then the hot mixture was poured into approximately 500 ml of deionized water. The precipitate was allowed to settle for 2 hours, then collected by filtration and washed with small amounts of ethyl alcohol. This material had a yellow tinge but after the three more recrystallizations from benzene was just slightly off-white in color.

b. Fluorescein Diacetylfluorescein was hydrolyzed by suspending 10 g of the compound in about 200 ml of ethanol containing 4 g of sodium hydroxide. The mixture was refluxed on a steam bath for about 30 minutes. The hot solution was filtered and the filtrate was allowed to cool to room temperature, then diluted to about 1500 ml with ice cold deionized water to prevent any formation of the red fluorescein. The solution was acidified by adding glacial acetic acid dropwise with stirring. The yellow precipitate which formed was collected by filtration and washed generously with deionized water. The material was then dried at 110-120° for 3 hours.

Red fluorescein was prepared by suspending 5 g of yellow fluorescein in about 500 ml of 80 per cent ethyl alcohol. The mixture was heated on a hot plate with continuous stirring until all was dissolved. The hot solution was filtered and the filtrate was evaporated on a steam bath until a deep red precipitate formed. The red fluorescein was collected by filtration, while the solution was still hot, precaution being taken not to wash the compound with water, but only with 95 per cent ethanol. This prevented the formation of yellow fluorescein. The compound was dried at 110-120° for 3 hours.

2. Apparatus and procedure

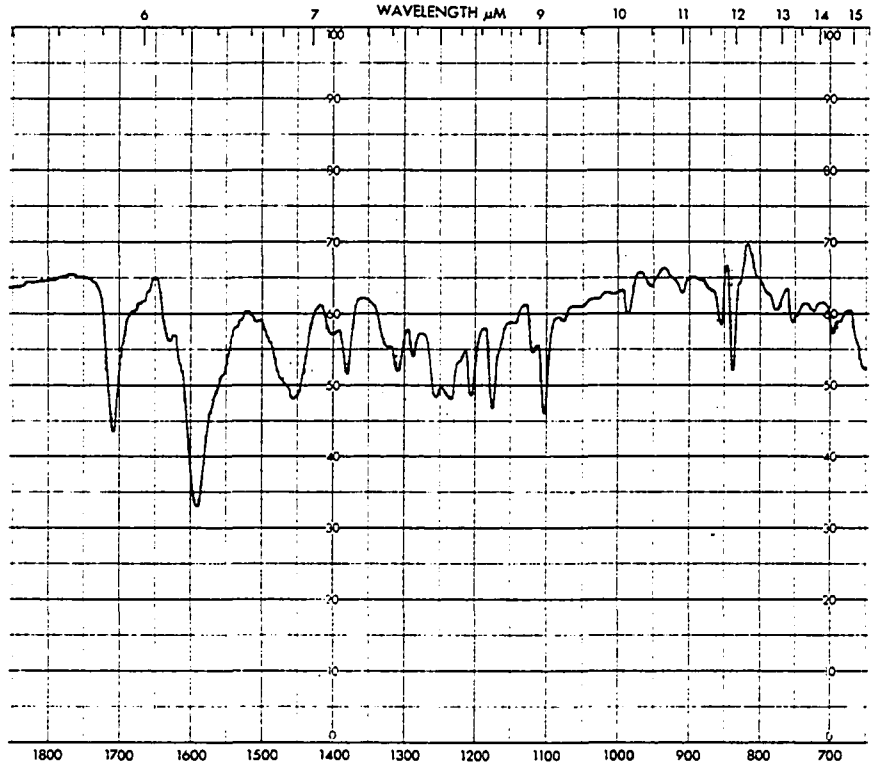
The infrared spectra of the yellow and red forms of fluorescein were obtained on solid films and on potassium bromide pellets using the Beckman IR 4250 Spectrophotometer. The solid films were prepared by suspending a few milligrams of the solid form of fluorescein in a minimum amount of anhydrous ether, mixing and transferring the suspension to the surface of a sodium chloride plate, and allowing the solvent to evaporate. The thin film remaining adhered to the plate. The plate was mounted in the spectrophotometer and the spectrum was obtained.

The potassium bromide pellets were prepared by mixing a few milligrams of the solid form of fluorescein with dry, powdered potassium bromide. The materials were mixed and ground well, then pressed in vacuum to form transparent discs. The pressure applied was in the order of 5000 pounds per square inch. The disc was mounted in the spectrophotometer and the spectrum was obtained. The nujol mulling technique was tried but found unsatisfactory. The spectra were poorly resolved and the absorption bands were all weak.

3. Results and discussion

a. General observations The infrared spectra of the red form of fluorescein obtained on the thin film and on the potassium bromide pellet are presented in Figures 12a and 12b, respectively, and the corresponding spectra

Figure 12a. Infrared spectrum of red fluorescein, thin film.


WAVELENGTH μM
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50
40
30
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10
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1800
1700
1600
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1400
1300
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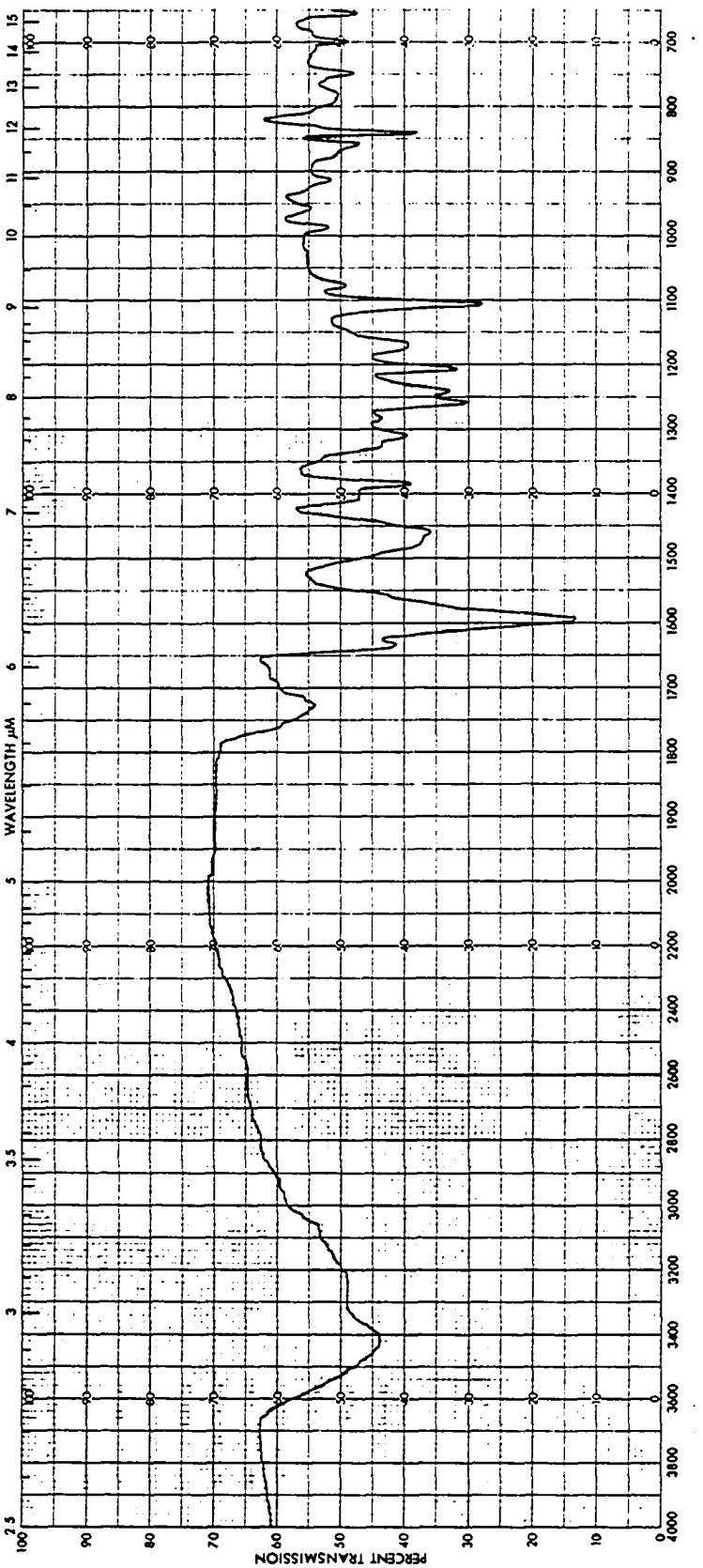
1100
1000
900
800
700

Figure 12b. Infrared spectrum of red fluorescein, potassium bromide pellet.


WAVELENGTH μM
PERCENT TRANSMISSION

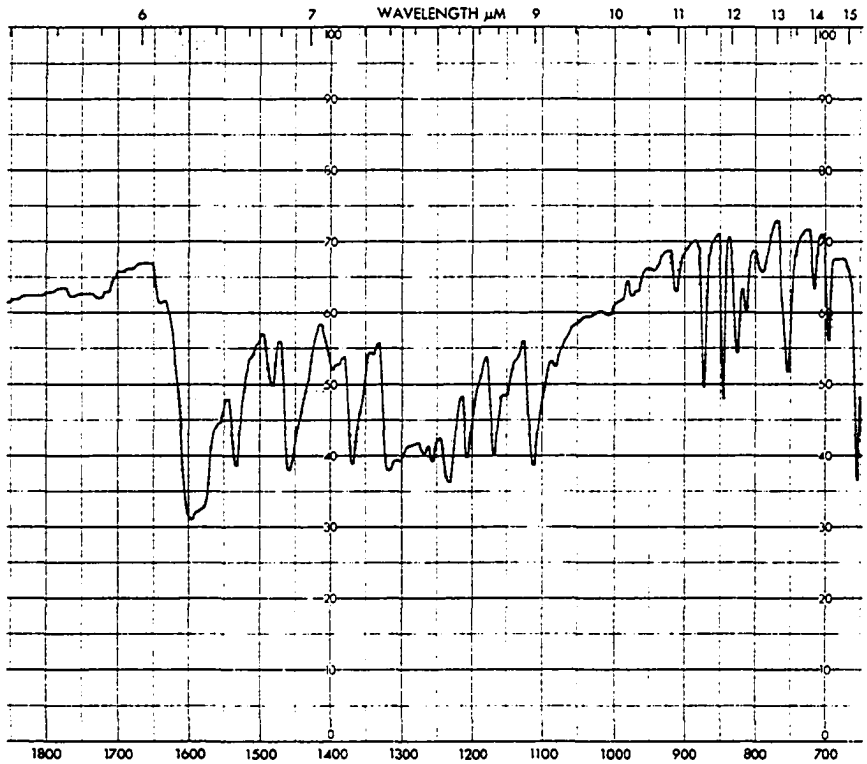
of the yellow form are presented in Figures 13a and 13b. The infrared spectra of both forms obtained on thin films, in which it is believed no conversion from one form to another has occurred, are presented in Table 2. The bands are grouped into four regions: 1800 - 1600 cm^{-1}, associated with the carbonyl stretching vibration; 1600 - 1400 cm^{-1}, associated with the aromatic skeletal carbon-carbon stretch; 1300 - 1000 cm^{-1}, associated with the aromatic carbon-hydrogen in-plane bending with carbon-oxygen stretch; and 900 - 675 cm^{-1}, associated with the aromatic carbon-hydrogen out-of-plane bending.

The spectra obtained on the thin films of the red and yellow forms were well-resolved in the region 1800 - 675 cm^{-1}. In the region 4000 - 1800 cm^{-1} no significant bands were present. The spectra obtained on the potassium bromide pellets covered the entire range 4000 - 675 cm^{-1}.

The spectra of the yellow and red forms obtained on the potassium bromide pellete were identical to those obtained on the thin films in the region 1700 - 675 cm^{-1}, any difference being a matter of intensity of absorption only. Significant differences appeared in the region 1800 - 1700 cm^{-1}, the region of absorption of the carbonyl group.

b. <u>The red form</u> The para-quinone structure assigned by Markuszewski to red fluorescein is characterized

Figure 13a. Infrared spectrum of yellow fluorescein, thin film.


WAVELENGTH μM
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7
9
10
11
12
13
14
15
100
90
80
70
60
50
40
30
20
10
0
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700

Figure 13b. Infrared spectrum of yellow fluorescein, potassium bromide pellet.

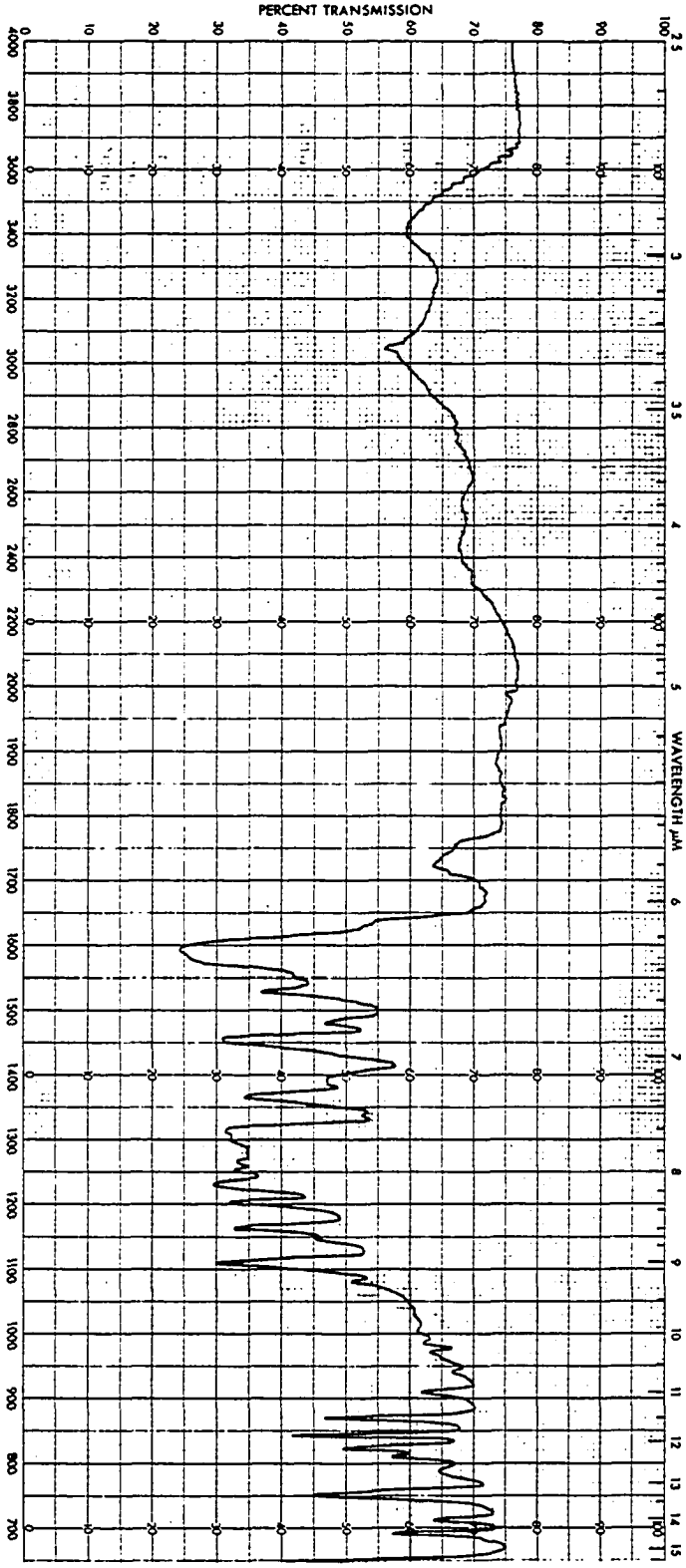
PERCENT TRANSMISSION
WAVELENGTH μM

Table 2. Infrared absorption bands of the yellow and red solid forms of fluorescein obtained on thin films

Form	Frequency in Wavenumber, cm^{-1}				
	Carbon-oxygen stretch, carboxyl or carbonyl				
	Lactone	Carboxylic acid	Carboxylate	Quinone	Pyrylium type
Red	None	1708	None	1632	None
Yellow	1770-1720[a]	None	1598-1578[b]	None	1537 and 1640

Form	Aromatic skeletal carbon-carbon stretch			
Red	1590	--	1460 broad	1400 shoulder plus 1380
Yellow	1589-1578[c]	1483	1458[d]	1398 shoulder plus 1370

Form	Carbon-oxygen stretch, and aromatic carbon-hydrogen in-plane bending					
Red	1320 shoulder plus 1310	1290	1255, 1235 doublet	1207	1175	1120 shoulder plus 1104
Yellow	1315 plus 1310 shoulder	1270, 1255 doublet	1232	1208	1170 plus 1150 shoulder	1107

Form	Aromatic carbon-hydrogen out-of-plane bending					
Red	870 shoulder plus 855	838, 800 shoulder	780	755	724	698 plus 690 shoulder
Yellow	873, 845	825, 812	790	754	716	695

[a]Very weak band attributed to the presence of some colorless fluorescein.

[b]Contains aromatic carbon-carbon stretching band.

[c]Contains asymmetric carboxylate stretching band.

[d]Contains symmetric carboxylate stretching band.

by the presence of three functional groups, a carboxyl group on the phthalate ring, and a carbonyl group and a phenolic group on the xanthene portion of the molecule. Each of these groups is characterized by absorption bands in specific regions of the infrared spectrum.

The oxygen-hydrogen stretching vibration band in a carboxylic acid group in a solid compound, is characterized by a broad band in the region 3300 - 2500 cm^{-1}, centered about 3000 cm^{-1}. An aryl-conjugated carboxylic acid is characterized by the carbon-oxygen stretching band in the region 1710 - 1680 cm^{-1} (14). In the spectrum of red fluorescein obtained on the potassium bromide pellet, Figure 12b, a broad band is present and extending to about 2600 cm^{-1}. Admittedly, this absorption band for the carboxylic acid group is weak, but this should not be surprising because the bands in this region 4000 - 1800 cm^{-1} of fluorescein, seem to be greatly influenced by the medium used in obtaining the spectrum. In the spectrum of red fluorescein obtained on the thin film, Figure 12a, a band is present at 1708 cm^{-1}, confirming further the presence of a carboxylic acid group. In the spectrum of red fluorescein obtained on the potassium bromide pellet, Figure 12b, a broad band is present with a maximum absorption at 1730 cm^{-1} and two shoulders, one at about 1760 cm^{-1} and the other at about 1700 cm^{-1}. The band at 1730 cm^{-1}

and the shoulder at 1760 cm^{-1} are exactly at the frequencies at which bands in the spectrum of thin films of colorless fluorescein were observed by Markuszewski. Thus, it is evident that some conversion of the red solid to the colorless solid took place during the preparation of the potassium bromide pellet, either because of the pressure applied or because of the anhydrous nature of the potassium bromide.

In both spectra of red fluorescein, that is, obtained on the thin film and on the potassium bromide pellet, a band is present at 1632 cm^{-1}; this band is in frequency range characteristic of the carbon-oxygen stretching vibration of a highly conjugated quinone, 1655 - 1626 cm^{-1} (14).

Finally, the oxygen-hydrogen stretching vibration band in a phenol with intermolecular hydrogen bonding normally is broad and absorbs in the region 3550 - 3200 cm^{-1}. In the spectrum of red fluorescein obtained on the potassium bromide pellet, there is a definite, broad band centered at about 3410 cm^{-1}. This is attributed to the phenolic oxygen-hydrogen stretch merged with the stretching vibration band of the carboxylic acid group.

c. <u>The yellow form</u> The zwitter ion structure assigned by Markuszewski to the yellow solid form of fluorescein is characterized by the presence of three functional groups, a carboxylate anion on the phthalate ring, two phenolic groups on the xanthene portion of the molecule, and

a pyrylium-type, six-membered, oxygen-bearing ring carrying a positive charge.

The carboxylate anion gives rise to two bands, a strong band near 1650 - 1550 cm^{-1} attributed to the asymmetrical stretching vibration (14), and a weaker band near 1430 - 1400 cm^{-1}, attributed to the symmetrical stretching vibration. Conjugation with a phenyl group results in delocalization of the π-electrons which reduces the double bond character of the carbon-oxygen bond, shifting the absorption to lower frequency. The frequency of the asymmetric stretching vibration is close to that of the aromatic carbon-carbon skeletal vibration at 1600 cm^{-1}. There is present in the spectra of solid yellow fluorescein, Figures 13a and 13b, a strong, broad band at 1598 - 1578 cm^{-1} which was interpreted by Davies and Jones (16) and by Markuszewski (3) as a merging of the two bands; the corresponding skeletal vibration in the spectrum of red fluorescein, at 1590 cm^{-1}, is sharp, lending additional support to the probable presence in the spectrum of the yellow solid of the asymmetric vibration of a carboxylate group. The symmetric stretching vibration of the carboxylate anion, normally at 1430 - 1400 cm^{-1}, is weak and sometimes not observed at all, especially in a complex molecule for which other strong absorption bands lie nearby. Another aromatic skeletal vibration has a frequency in this range and the strong,

rather broad band present at 1458 cm^{-1} in the spectrum of yellow fluorescein is very probably a merging of the two bands.

In the spectrum of yellow fluorescein, Figure 13b, obtained on potassium bromide pellet, a definite broad band centered at 3410 cm^{-1} is present. This is attributed to the phenolic oxygen-hydrogen stretching vibration band.

Pyrylium-type compounds give rise to two bands, a symmetric oxygen-carbon stretching vibration band which falls in the region 1540 - 1530 cm^{-1} and an asymmetric oxygen-carbon stretching vibration in the region 1650 - 1615 cm^{-1} (19). In the infrared spectra of yellow fluorescein, Figures 13a and 13b, a prominent band at 1537 cm^{-1} is present which does not appear in the spectra of red fluorescein. This is the band which led Markuszewski (3) to assign the pyrylium-ring structure to yellow fluorescein. Markuszewski failed to find the symmetrical stretching band and thought it might be merged with the skeletal, carbon-carbon stretching vibration at 1595 - 1572 cm^{-1}. In the present work, in the spectra of yellow fluorescein a shoulder is present at 1640 cm^{-1}, most prominently in the spectrum obtained on the thin film; this confirms further the presence of the pyrylium ring in the yellow form.

Carboxylic acids in the solid form tend to exist as dimers and give rise to a broad band, 3300 - 2500 cm^{-1}, centered near 3000 cm^{-1}. This band is not expected in the

spectrum of a structure such as that assigned to yellow fluorescein by Markuszewski; however, such a band does appear, although with low absorption. The appearance of this band in the spectrum of yellow fluorescein obtained on potassium bromide pellet, Figure 13b, is attributed to the conversion of the yellow form to the red form during the preparation of the potassium bromide pellet.

The weak band, 1770 - 1720 cm^{-1}, in the spectrum of yellow fluorescein obtained on the thin film, Figure 13a, indicates that some lactone co-exists with the yellow form. This becomes even more apparent later in this dissertation when the absorbance, solubility, and acid-base properties of yellow fluorescein are discussed.

In the spectrum of yellow fluorescein obtained on the potassium bromide pellet, Figure 13b, the lactone band is intensified with a maximum at 1730 cm^{-1} and two shoulders, one at about 1760 cm^{-1} and the other at about 1710 cm^{-1}. This is an indication again that some conversion of form is taking place, that is, of the yellow form to the colorless form and probably also to the red form.

4. Conclusion

The assignment of the para-quinone structure (XII) to red fluorescein, proposed first by Orndorff and Hemmer (22) and proven by Markuszewski (3) has been confirmed further

in the present work. First, the frequency of the carbon-oxygen stretching vibration band at 1632 cm^{-1}, characteristic of a highly conjugated quinone has been found; this band was absent in the spectra obtained by previous workers owing probably to the poor resolution in their spectra rather than a complete merging with the strong and broad aromatic carbon-carbon absorption band centered at 1590 cm^{-1}.

Further evidence for the assignment of the zwitter ion structure (XIII) to yellow fluorescein has been found; in locating the asymmetric carbon-oxygen stretching band of the pyrylium ring, at 1640 cm^{-1} and observing the phenolic oxygen-hydrogen stretching band, centered at 3410 cm^{-1}.

A comparison between the infrared spectra of the red and of the yellow forms obtained on potassium bromide pellets and the spectra obtained on thin films indicates that in preparing the potassium bromide pellets, some conversion of the yellow form to the colorless and the red forms is taking place. The same phenomenon was observed in the case of red fluorescein, some red was being converted to the colorless form in preparing the potassium bromide pellet.

C. Infrared Spectra of the Free Acids of Sulfofluoresceins

1. Introduction

In preparing the free acids of sulfofluoresceins, it was observed that these fluorescein derivatives yield the yellow solid form whether precipitated from cold water or from hot ethanol-water solutions by acidifying with 20 per cent hydrochloric acid. Fluorescein when precipitated from ethanol-water solution by acid separates in the red form. Again, when the yellow solid form of fluorescein is heated, although it does not melt at temperatures up to 300°, it changes to the red solid form during the heating and subsequently when treated with water reverts only partially to the yellow form. The free acid of sulfofluorescein when heated acquires an orange color above 150° and does not melt at temperatures up to 300°, but on cooling reverts immediately to the original yellow color. These observations indicate that the red solid form of the sulfofluorescein is less stable than the red form of the parent compound. This same stability will be observed again in preparing the samples of sulfofluorescein by the potassium bromide pellet technique for infrared study.

By extrapolating the information about the structures of the different forms of fluorescein presented in the previous section, the yellow form of sulfofluorescein is

expected to have the zwitter ion structure (XIV). The study of the infrared spectrum now to be reported was directed to substantiating this.

It was observed that the spectra of the sulfofluoresceins obtained on nujol mulls or on thin films showed no absorption bands in the region, 4000 - 1800 cm^{-1}; on the other hand, in the spectra obtained on the potassium bromide pellets bands were present. Because some conversions of the yellow and the red forms of fluorescein were observed during the preparation of potassium bromide pellets, it appeared likely that such interconversions could also occur among the sulfofluoresceins.

2. Preparation of materials

The free acid of the sulfofluoresceins, namely, 4-, 5-, and 6-sulfofluorescein, were prepared and purified as discussed previously in Chapter II.

3. Apparatus and procedure

The infrared spectra of 4-, 5-, and 6-sulfofluorescein, free acids, were obtained on thin films and on potassium bromide pellets using the Beckman IR 4250 Spectrophotometer. The samples were prepared as outlined above.

4. Results and discussion

The spectra of 4-, 5-, and 6-sulfofluorescein obtained on thin films are presented in Figures 14a, 15a, and 16a,

respectively. No absorption bands appear in these spectra in the region 4000 - 1800 cm^{-1}, the region in which characteristic absorption bands of phenols, carboxylic acids and aromatic carbon-hydrogen stretching vibration appear. In the spectra obtained on potassium bromide pellets, Figures 14b, 15b, and 16b, these absorption bands are prominent.

A comparison between the spectra obtained on potassium bromide pellets and those obtained on thin films, through the region 1800 - 675 cm^{-1} and especially in the region, 1800 - 1600 cm^{-1}, indicated that no conversion of the yellow form to another form occurred.

a. Infrared spectra of the yellow 4-sulfofluorescein

The spectra of 4-sulfofluorescein, free acid, obtained on the thin film and on potassium bromide pellet are presented in Figures 14a and 14b, respectively; the absorption bands are summarized in Table 3.

In the region 3550 - 3200 cm^{-1} (14), the region in which phenols with intermolecular hydrogen bonding absorb, a band is present at 3390 cm^{-1}; this band is associated with the oxygen-hydrogen stretching vibration. This band seems to overlap with a broad band present at 3250 - 2200 cm^{-1} and centered about 3020. This band is indicative of the presence of a free carboxylic acid group which is normally characterized by a broad band at 3300 - 2500 cm^{-1} centered about 3000 cm^{-1} (14). This band is due to the oxygen-hydrogen

Figure 14a. Infrared spectrum of yellow 4-sulfofluorescein, thin film.

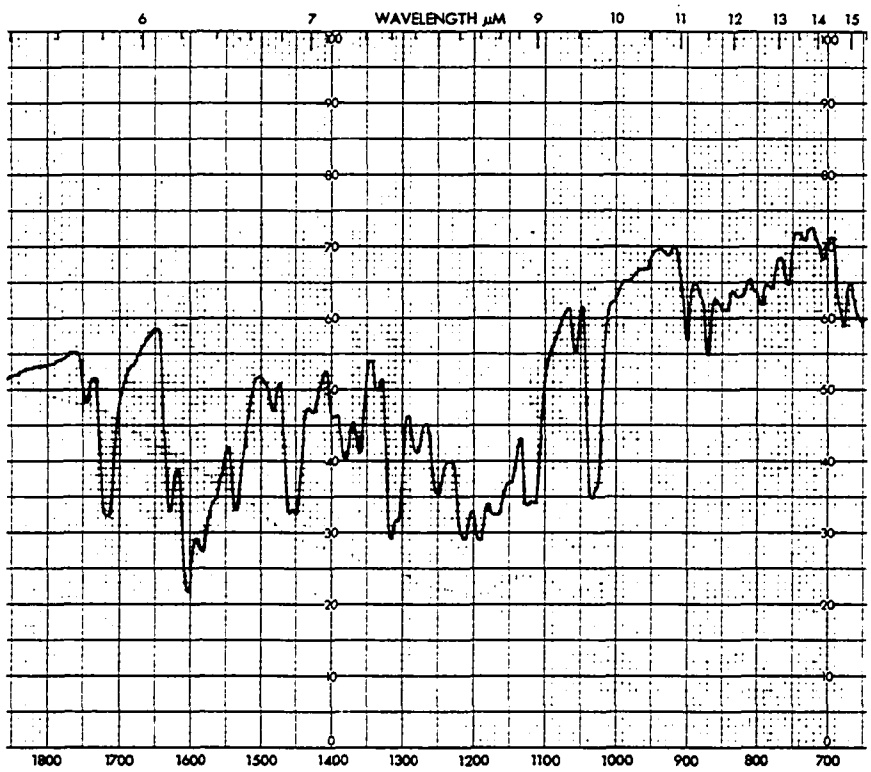
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Figure 14b. Infrared spectrum of yellow 4-sulfofluorescein, potassium bromide pellet.

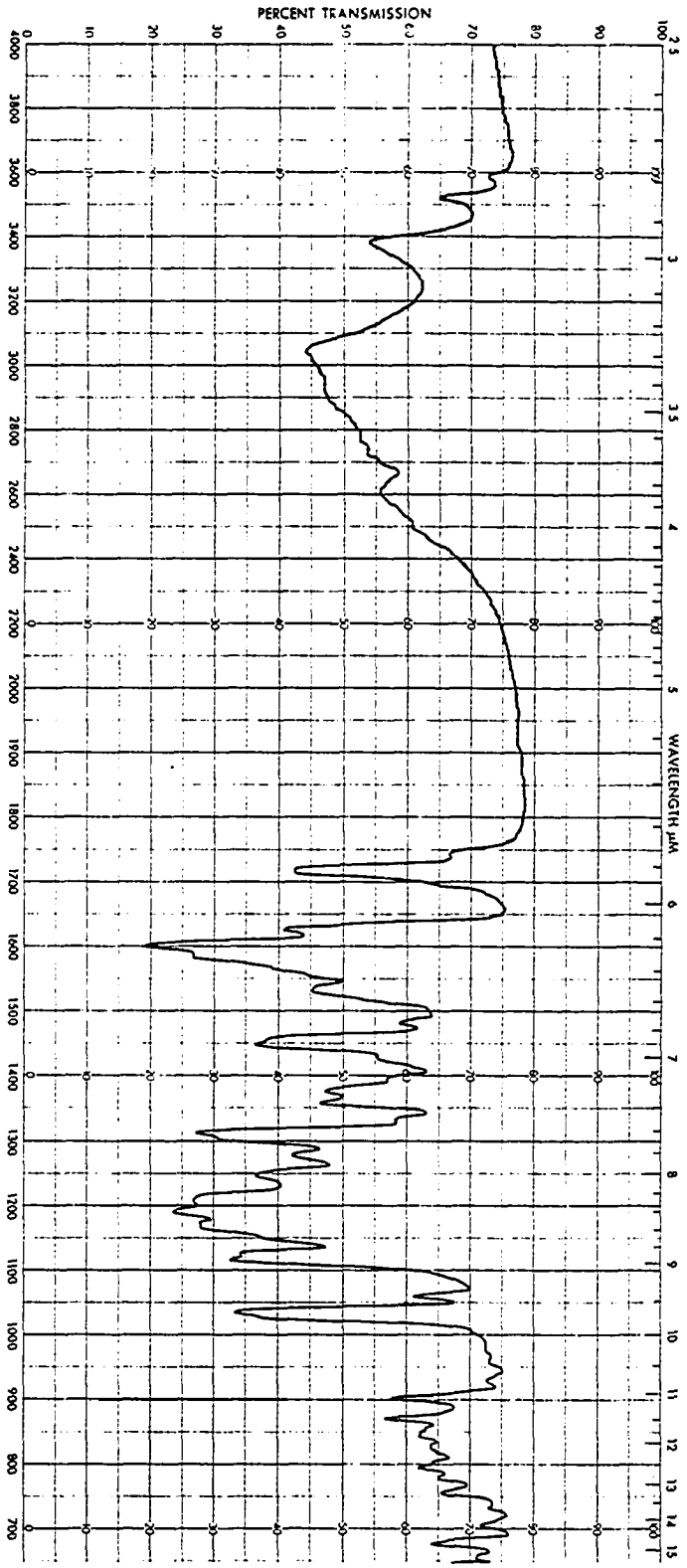
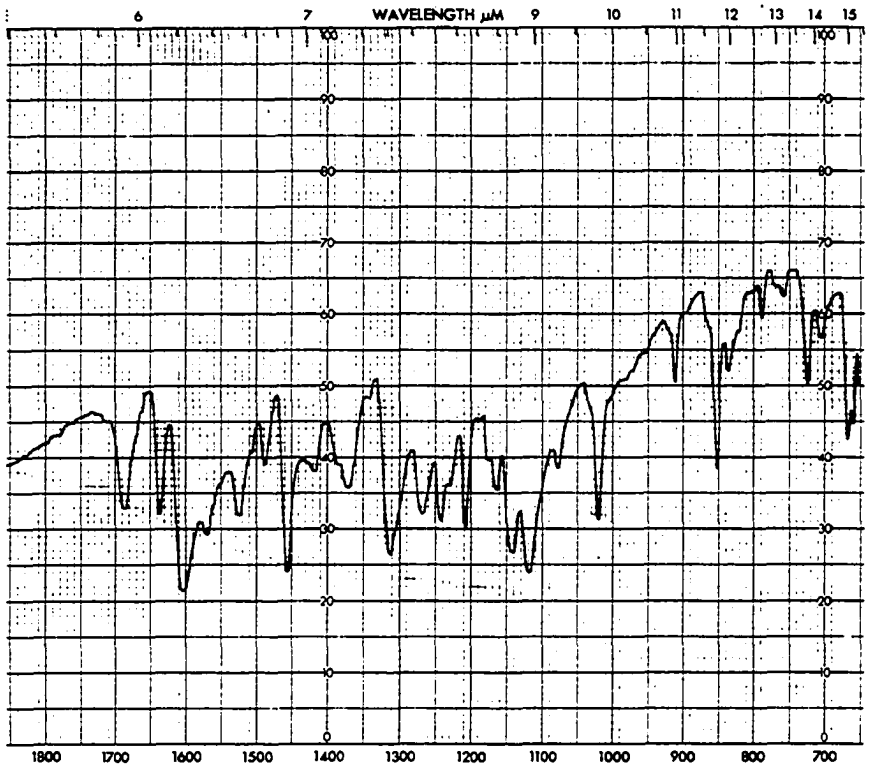

PERCENT TRANSMISSION
WAVELENGTH μM

Figure 15a. Infrared spectrum of yellow 5-sulfofluorescein, thin film.

Figure 15b. Infrared spectrum of yellow 6-sulfofluorescein, thin film.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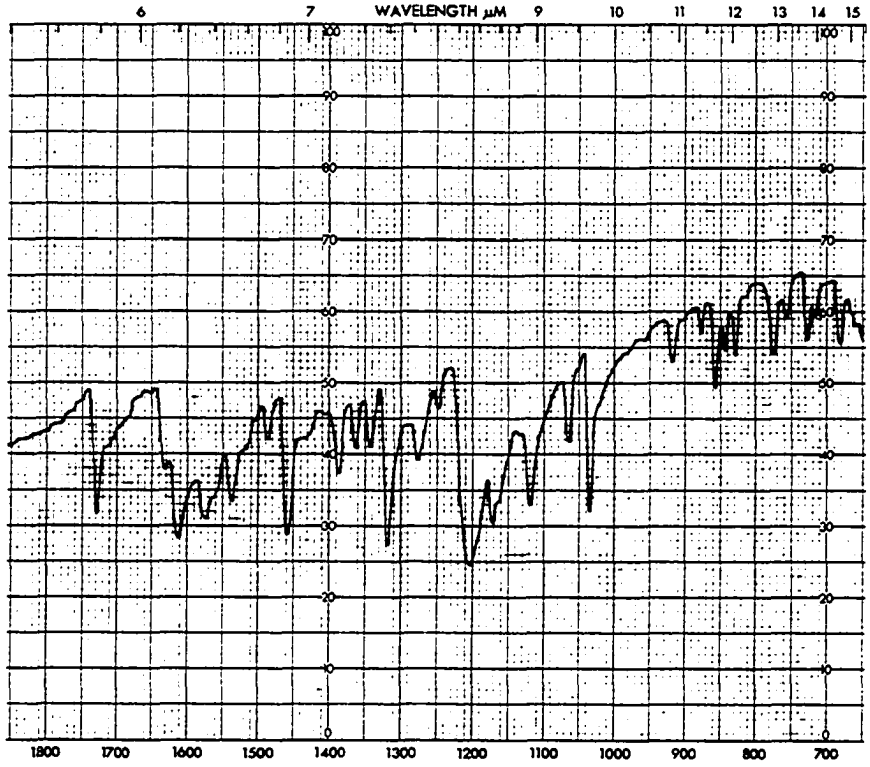
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WAVELENGTH μM
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Figure 16a. Infrared spectrum of yellow 5-sulfofluorescein, potassium bromide pellet.

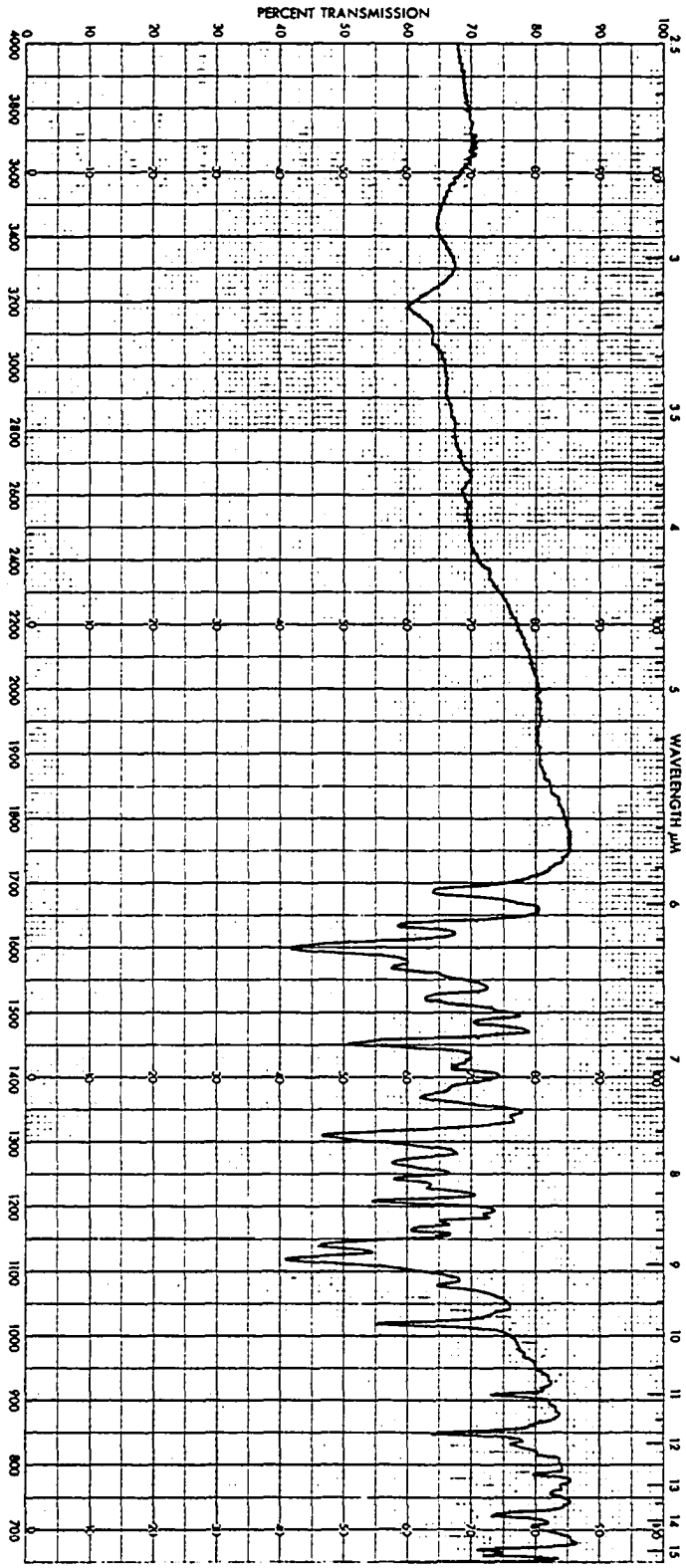

PERCENT TRANSMISSION
WAVELENGTH μM

Figure 16b. Infrared spectrum of yellow 6-sulfofluorescein, potassium bromide pellet.

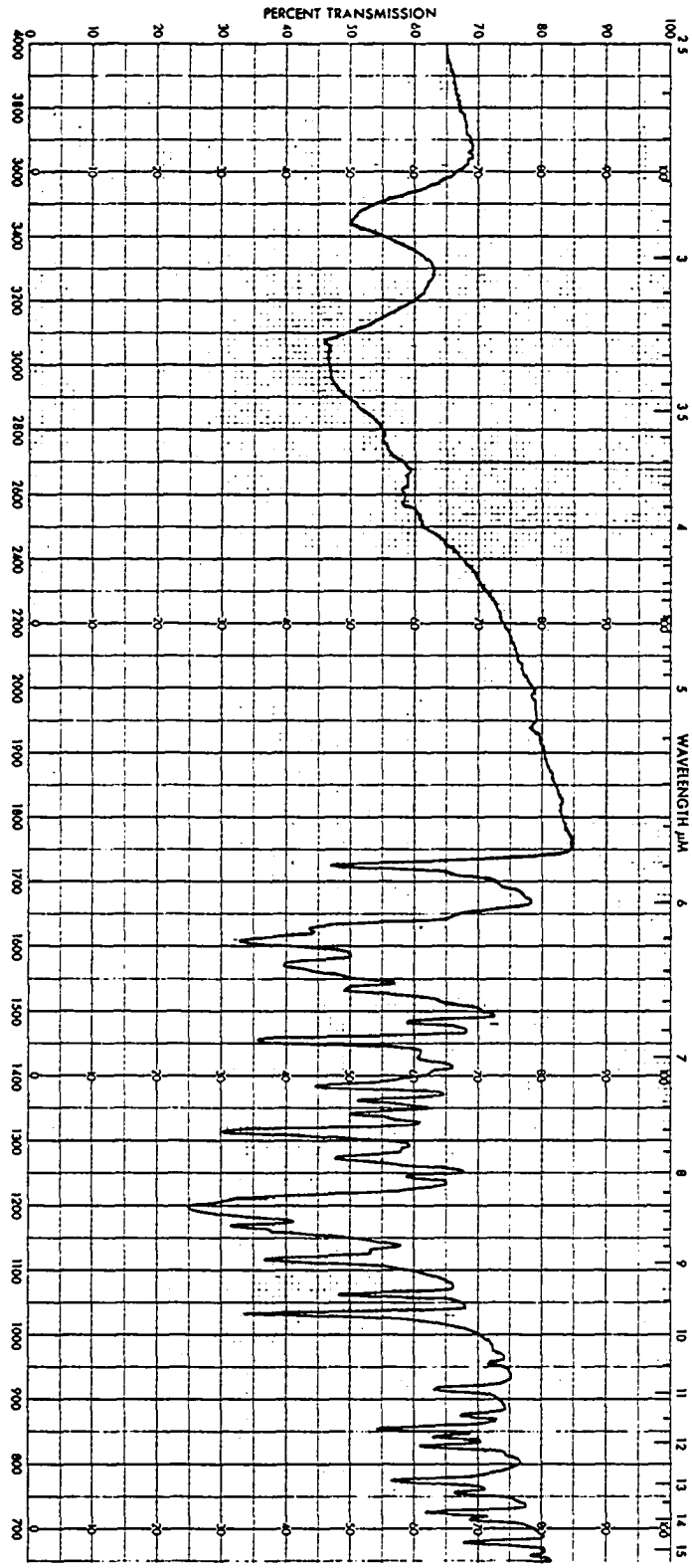

PERCENT TRANSMISSION
WAVELENGTH μM

Table 3. Absorption bands of 4-, 5-, and 6-sulfofluorescein obtained on potassium bromide pellets

Compound	Phenol	Carboxylic acid	Lactone	Pyrylium-type
	Frequency in Wavenumbers, cm^{-1}			
	Oxygen-hydrogen stretch and carbon-oxygen stretch for carboxyl or carbonyl			
4-Sulfofluorescein	3465-3250	3250-2200 1720	1745 (a)	1628, 1535
5-Sulfofluorescein	3600-3300	3300-2500 1687	none	1637, 1525
6-Sulfofluorescein	3640-3300	3300-2200 1725	none	1628, 1535

Compound					
	Aromatic skeletal carbon-carbon stretch				
4-Sulfofluorescein	1600	1580 sh	1480 sh	1460, 1450 d	1425 sh
5-Sulfofluorescein	1602	1570 sh	1485 sh	1452	1415 sh
6-Sulfofluorescein	1610	1570	1486 sh	1455	1430 sh

Table 3. (Continued)

Carbon-oxygen stretch, carbon-hydrogen, and oxygen-hydrogen in-plane bending						
4-Sulfo-fluorescein	1390 sh 1380,1360d	1335 sh 1315,1305d	1280,1250	1214 (1190,1170)s	1125,1112d	(1058,1036)s
5-Sulfo-fluorescein	1380 sh 1370	1340 sh 1310	1268 1242,1230d	1208 (1190 sh, 1178 sh, 1165,1140)s	1118	(1078,1020)s
6-Sulfo-fluorescein	1385	1340	1274	(1198, 1170, 1160sh)s	1130sh,1118	(1063,1035)s

Aromatic carbon-hydrogen out-of-plane bending, wavenumber cm^{-1}					
4-Sulfo-fluorescein	900 m 870 m	845 vw 830 vw	795 v 770 vw	755 m 730 vw	704 w 675 m
5-Sulfo-fluorescein	908 m	850 m 832 vw	788 w 770 vw	755 w 722 m	705 w 670 m
6-Sulfo-fluorescein	915 m 878 w	855 m 842 w 828 w	775 m	765 w 725 m	715 w 678 m

sh = shoulder d = doublet s = associated with sulfonate stretch

m = medium w = weak vw = very weak

stretching vibration of a carboxylic acid in the solid form. In the region of carbonyl absorption, 1800 - 1600 cm^{-1}, a band is present at 1720 cm^{-1} with a prominent shoulder at 1745 cm^{-1}. The band at 1720 cm^{-1} is associated with the carbon-oxygen stretching vibration of a carboxylic acid, the weak absorption band at 1745 cm^{-1} is indicative of the presence of some lactone, for as discussed above (Part A), a γ-lactone absorbs at 1750 cm^{-1}.

In the spectrum of 4-sulfofluorescein there are two prominent bands present, one at 1628 cm^{-1} and the other at 1535 cm^{-1}. As explained in the preceding discussion of yellow fluorescein, the infrared spectra of pyrylium-ring compounds are characterized by two absorption bands (19), one at 1650 - 1615 cm^{-1} attributed to a symmetric carbon-oxygen stretching vibration, and a second band at 1540 - 1530 cm^{-1} attributed to an asymmetric stretching vibration. Thus, the pyrylium-ring is present in 4-sulfofluorescein.

The infrared spectra of typical alkyl and aryl sulfonates are characterized by two absorption bands, a doublet around 1175 cm^{-1}, attributed to asymmetric sulfur-oxygen stretch, and a second band around 1055 cm^{-1}, attributed to symmetric stretch. In the spectrum of 4-sulfofluorescein there is a strong band present at 1036 cm^{-1}, which is not present in the spectra of yellow and of red fluorescein. Also, a weak band is present at 1058 cm^{-1}.

In the spectrum of 4-sulfofluorescein there is a doublet present at 1170 - 1190 cm^{-1}. This band lies in a region of absorption corresponding to carbon-hydrogen in-plane bending and carbon-oxygen stretch. Thus, some merging is to be expected. This is confirmed by the fact that both of the spectra in red and yellow forms of fluorescein there is a singlet band at 1175 and 1170 cm^{-1}, respectively.

The observations just recorded indicate strongly that the form assumed by the free acid of 4-sulfofluorescein is characterized by the presence of the following functional groups: free carboxylic, phenolic, sulfonate, and pyrylium-type ring. Thus, the zwitter ion structure (VII) is proposed for the yellow solid form of 4-sulfofluorescein, analogous to the structure of yellow fluorescein, Structure XIII.

The low absorption band at 1745 cm^{-1} was attributed to the presence of a lactone and the explanation of this band is that the yellow form of 4-sulfofluorescein contains some of the colorless solid form. Further evidence for this will be offered in the following sections, during a discussion of the acid-base chemistry, solubility and absorbance in the visible. The compound 4-sulfofluorescein does exist in the colorless form in acidic solutions, the amount of the colorless form being enhanced by the presence of ethyl alcohol in solution.

b. Infrared spectra of the yellow 5-sulfofluorescein

The spectra of 5-sulfofluorescein, free acid, obtained on the thin film and on the potassium bromide pellet are presented in Figures 15a and 16a, respectively; the absorption bands are also tabulated, Table 3.

The absorption bands in the region, 3600 - 2000 cm^{-1}, are weak, a phenomenon which had been encountered before, especially when the spectra were obtained on nujol mulls or on thin films. The broad and weak band present, 3600 - 3300 cm^{-1}, is attributed to the oxygen-hydrogen stretch in a phenol, while the broad weak band, 3300 - 2500 cm^{-1} is attributed to the oxygen-hydrogen stretch in a free carboxylic acid.

The band present at 1687 cm^{-1} is characteristic of the carbon-oxygen stretching vibration in a free carboxylic acid. In general, aryl conjugated acids show absorption for the dimer in the region 1710 - 1680 cm^{-1}.

The absorption bands characteristic of the pyrylium-type ring as discussed above are also present in the spectrum of 5-sulfofluorescein, one at 1637 cm^{-1} and the other at 1525 cm^{-1}.

Finally, there is a strong band present at 1020 cm^{-1}; this is assigned to the symmetric sulfur-oxygen stretch. A doublet at 1140 - 1165 cm^{-1} is also present; this is assigned to the asymmetric stretch band of the sulfonate.

Again, on the basis of the characteristic bands found, the zwitter ion structure (IX) is proposed to the yellow, solid form of 5-sulfofluorescein.

c. Infrared spectra of the yellow 6-sulfofluorescein

The spectra of 6-sulfofluorescein, free acid, obtained on the thin film and on the potassium bromide pellet are presented in Figures 15b and 16b, respectively; the absorption bands are also tabulated, Table 3.

In the spectrum of 6-sulfofluorescein obtained on the potassium bromide pellet, a broad band is present in the 3640 - 3300 cm^{-1} region; this is attributed to the oxygen-hydrogen stretch of the phenolic group. Another very broad band is present in the region 3300 to about 2200 cm^{-1} and is attributed to the oxygen-hydrogen stretch of the carboxylic acid group.

In general, carboxylic acids in the solid state exist as dimers, and aryl conjugated acids show absorption for the dimer in the 1710 - 1680 cm^{-1} region (14). In the spectrum of 6-sulfofluorescein a band is present at 1725 cm^{-1}. The shift to higher frequency is due to the inductive effect of the sulfonate group. This effect is most prominent when the sulfonate group is on the ortho or para position with respect to the carboxylic acid group. For comparison, the infrared spectra of the salts of the isomeric sulfobenzoic acids were obtained on potassium

bromide pellets; the correspondence in the position of the carboxyl group stretching vibration band was excellent: monoammonium salt of o-sulfobenzoic acid 1720 cm^{-1} and 4-sulfofluorescein 1720 cm^{-1}; monosodium salt of m-sulfobenzoic acid 1687 cm^{-1} and 5-sulfofluorescein 1687 cm^{-1}; monopotassium salt of p-sulfobenzoic acid 1720 cm^{-1} and 6-sulfofluorescein 1725 cm^{-1}.

The two characteristic bands of the pyrylium-type ring are also present, at 1628 and 1535 cm^{-1}. Finally, a strong band is present at 1035 cm^{-1} which corresponds to the symmetric sulfur-oxygen stretch of an aryl sulfonate; also the doublet at 1170 - 1198 cm^{-1} corresponding to the asymmetric stretch.

Thus, the infrared spectra of 6-sulfofluorescein strongly indicate that the yellow solid form of the compound is the zwitter ion structure (X).

5. Conclusion

The zwitter ion structure is proposed to the yellow solid form of the three free acids of sulfofluorescein. This proposal is supported by: first, the infrared study presented above in which the absorption bands characteristic of the sulfonate, carboxylic acid, phenols and pyrylium-ring appear; and second, by analogy to the yellow solid form of the parent compound. Like fluorescein, the sulfofluoresceins are yellow in color and do not melt when heated up to 300°,

a characteristic of the salt-like composition of the zwitter ion.

The lactone form of the sulfofluorescein was detected in the infrared spectra of 4-sulfofluorescein. In the following sections, especially the one dealing with absorbance in the visible region, further proof of this is offered.

Finally, it has been shown that the red, solid form of the sulfofluoresceins is not stable, and no interconversion from one form to another takes place during the preparation of the potassium bromide-sulfofluorescein sample.

V. THE PROTOTROPIC FORMS AND STRUCTURES OF FLUORESCEIN AND OF THE SULFOFLUORESCEINS IN SOLUTION

A. Introduction

As shown in Chapter IV, above, the solid forms of the sulfonic acids of fluorescein exist in the yellow, zwitter ion-sulfonate form. The acid-base behavior of these compounds in solution, mainly in water and to some extent in 50 per cent ethanol, is now reported. The primary interest was to determine the acid dissociation constant for 4-, 5-, and 6-sulfofluorescein, and at the same time to correlate the prototropic forms of the compounds with those of fluorescein.

The tools used to determine the acid dissociation constants of the compounds were potentiometric titration, measurements of solubility as a function of pH, and absorbance in the visible and fluorescence as a function of pH. These studies were of interest in themselves for they added a new dimension to the understanding of the dissolved forms of fluorescein itself.

Markuszewski (3) clarified and advanced the chemistry of fluorescein and established a foundation for further investigations in the area. He established by solubility measurement as a function of pH, by potentiometric acid-base titration of fluorescein and related compounds, and by

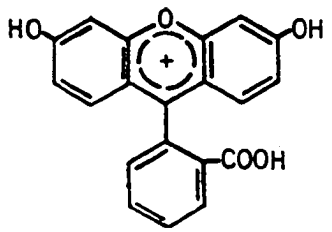
fluorescence studies as a function of pH, that all the three forms of fluorescein, colorless, yellow and red, assume the zwitter ion form once dissolved in water at pH <7. More specifically, the monocation, at pH <3, and the neutral molecule at pH 3 to 4, both exist as the zwitter ion only. The monoanion at pH 4 to 7 exists predominantly in the zwitter ion form and finally the dianion at pH >7 exists predominantly in the quinone form. Missing from the Markuszewski investigation was a study of the absorbance of fluorescein in the visible region.

The values which have been reported for the acid dissociation constants of fluorescein differ appreciably, the differences arising from the method of determination, the ionic strength of the solutions examined, and the solvent employed; this information was compiled by Markuszewski (ref. 3, Table 5, pp. 96-97). The acid dissociation constants of fluorescein in aqueous solution at ionic strength of 0.1 as determined by Markuszewski are:

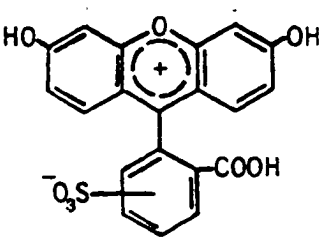
		Acidic group
$pK_1 = 2.15$	$H_3Fl^+ \rightleftharpoons H_2Fl + H^+$	carboxylic
$pK_2 = 4.73$	$H_2Fl \rightleftharpoons HFl^- + H^+$	phenolic
$pK_3 = 6.55$	$HFl^- \rightleftharpoons Fl^{2-} + H^+$	phenolic

Because of the additional sulfonic acid group, sulfofluorescein resembles the monocation of fluorescein, Structure XXIV,

but is a neutral molecule, Structure XIVa; by analogy it is designated by H_3Sfl.



XXIV. Cation of fluorescein



XIVa. Sulfofluorescein

Following the same three step dissociation

$$H_3Sfl \rightleftharpoons H_2Sfl^- + H^+$$

$$H_2Sfl^- \rightleftharpoons HSfl^{2-} + H^+$$

$$HSfl^{2-} \rightleftharpoons Sfl^{3-} + H^+$$

a comparison becomes possible between fluorescein and the isomers of sulfofluorescein with respect to acid dissociation constants, absorbance as a function of pH, and fluorescence as a function of pH. Because the sulfonic acid group is a strong acid, significant differences are to be expected.

B. Absorption Spectra of Fluorescein and of the Sulfofluorescein in Aqueous Solution at Various Values of pH

1. Introduction

Fluorescein has attracted great attention over the years and numerous studies have been made of the fluorescence of the compound. Surprisingly, not many papers deal with the absorbance, either in water or in water-ethanol solutions. Lindqvist (23) studied the absorbance in water of ionic strength 0.01 as a function of pH. Primarily, he was interested in determining the acid dissociation constants, for which he reported (expressed as the negative logarithms) $pK_1 = 2.2$, $pK_2 = 4.4$, and $pK_3 = 6.7$. Lindqvist assumed that the absorption bands in the visible at different pH values were those of the quinone form, except in the acidic region pH <2. Below pH 2, Lindqvist assumed the monocation to predominate and to be in the zwitter ion form. Lindqvist pointed out that the absorption, at pH 3.3, at which the neutral molecule, assumed to be the lactone, is low and he concluded that the neutral molecule does not exist entirely in the colorless lactone form but also is present to a certain extent in the quinone form. Lindqvist failed to notice that the monocation and the neutral form absorb at the same wavelength, 440 nm.

Ioffe, Devyatova and Roskulyak (12) reported very briefly the absorption in the visible region of the α- and β-isomers of sulfofluorescein. The continuous absorption curves reported are at only two values of pH, 9 and 3 to 4. Without further discussion, they came to the conclusion that all absorption curves are almost identical except that the α-isomers differ substantially in the height of the maximum. This conclusion is wrong with respect to the α-sulfofluorescein; in the basic region the color intensity of the α-isomer is about equal to that of the β-isomers and of fluorescein, and in the acidic region, at pH = 3, the α-isomer is practically colorless.

In the present work, the absorbance of fluorescein in water and in 50 per cent water-ethanol has been measured, primarily to secure firsthand information for comparing the absorbance of fluorescein with those of the sulfofluoresceins. Care has been taken, of course, to be certain that all of the compounds were in a pure state and that all data was obtained under the same conditions of ionic strength and composition of buffer solutions.

2. Experimental work

a. Materials The yellow fluorescein used was prepared by purification of commercial fluorescein through diacetylation and hydrolysis of the latter in basic alcoholic solution. The α- and β-sulfonic acids of fluorescein were

purified by crystallization from water-ethanol solution and the use of cation exchange column, as described in Chapter II.

b. Buffers The buffers were prepared by using 0.1 M solutions of hydrochloric acid, potassium hydrogen phthalate (KHP), potassium chloride, potassium hydroxide, and boric acid in 0.1 M potassium chloride solution. The pH of the buffer solutions was measured before and after the addition of the compound under study, using a Hach Model 8594 pH meter set on the expanded scale and supplied with a Beckman high-alkalinity glass electrode. The pH meter was calibrated by using at least two buffer solutions prepared according to specifications of the National Bureau of Standards, as outlined in Diehl (ref. 24, p. 58).

c. Apparatus and procedure The Cary 14 Recording Spectrophotometer was used to obtain a continuous spectrum and the Hach DR-2 Spectrophotometer, supplied with a flow-through cell, was used to obtain single-points absorbance measurements.

Stock solutions of 5.0×10^{-4} M were prepared by dissolving the appropriate amount of the compound under investigation in about 30 ml of 0.1 M potassium hydroxide and diluting with deionized water in a one-liter volumetric flask. Then, 2.00 ml of the stock solution were transferred to a 100-ml volumetric flask and diluted with the buffers

described above, thus maintaining an ionic strength of 0.1. The final concentrations of the different solutions were 1.0×10^{-5} M. These solutions were used to obtain the continuous absorption spectra.

In the work with Hach DR-2 Spectrophotometer, different stock solutions were prepared, the same as described above and the final concentrations of the 4-, 5-, and 6-sulfofluorescein solutions were 5.0×10^{-6} M; the concentration of the solutions of yellow fluorescein were 8.0×10^{-6} M.

3. Fluorescein

The absorption spectra of fluorescein were obtained at pH 1.03, 3.12, 5.22, and 8.11.

In the spectrum at pH 1.03, one absorption band appears, with a maximum at 438 nm. At pH 3.12, about the pH of minimum solubility and the isoelectric point according to Markuszewski (3), the maximum is much lower and only very slightly shifted, to 440 nm. A more detailed measurement of absorbance at 440 nm as a function of pH through this region appeared very suitable for obtaining the first dissociation constant, that is, for the reaction, $H_3Fl^+ = H_2Fl + H^+$.

In the absorption spectrum at pH 5.12, two absorption bands appear, of about equal height and with maxima at 455 nm and 476 nm.

In the absorption spectrum at pH 8.12, a single band appears, with greatly increased absorbance and a maximum at

491 nm. A more detailed measurement of absorbance at 491 nm as a function of pH through this region appeared suitable for obtaining the third dissociation constant, that is the constant for the reaction, $HFl^- = Fl^{2-} + H^+$.

On the basis of various lines of evidence, Markuszewski (3) concluded that the neutral form of fluorescein in water solution was the yellow, zwitter ion-carboxylate structure. That the maximum in the absorption curves of fluorescein at pH 1.03 and 3.12, Figure 17, is the same but the intensity at the lower pH much greater may be interpreted two ways.

One interpretation is that the same positively charged pyrylium ring is present in both prototropic forms, H_3Fl^+ and H_2Fl, the molar absorptivity of the protonated monocation being much the larger of the two. That is, that the same chromophore is present in both species but the intensity of absorption is altered by the removal of the negative charge on the carboxylate group by the addition of the proton.

The second explanation, supported by other lines of evidence to be offered later in this dissertation, is that the neutral species, H_2Fl, is present in large part as the colorless lactone.

At pH 8.12, the fluorescein is about completely converted to the dianion, Fl^{2-}, and the spectrum is that of the quinone-carboxylate structure, with a very intense absorption and a maximum at 491 nm.

Figure 17. Absorption spectrum of fluorescein in aqueous solution.

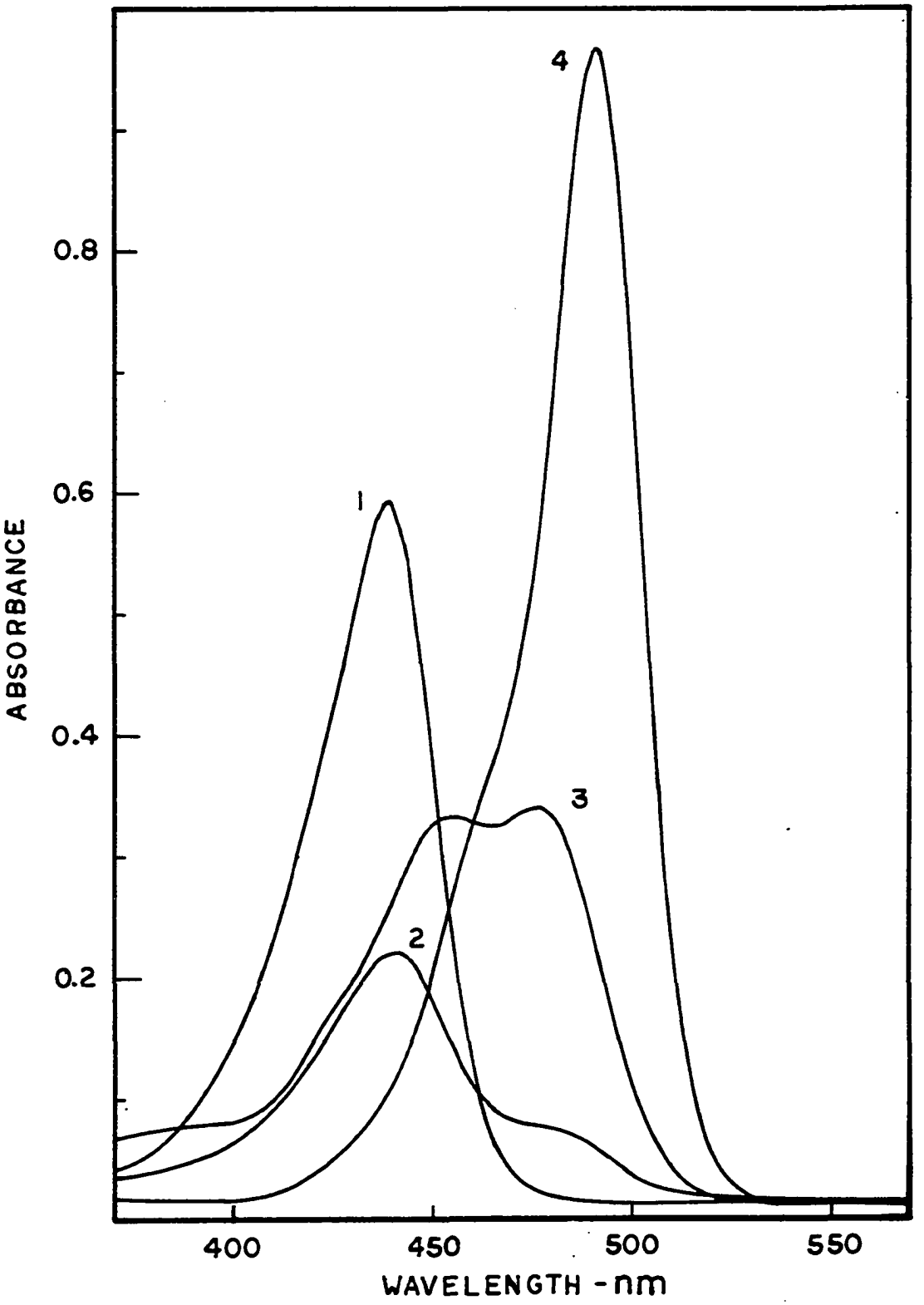
Concentration: 1.0×10^{-5} M

Curve 1: pH = 1.03

Curve 2: pH = 3.12

Curve 3: pH = 5.22

Curve 4: pH = 8.11


4
0.8
0.6
1
ABSORBANCE
0.4
3
2
0.2
400
450
500
550
WAVELENGTH - nm

The interesting situation is that at pH 5.22, at which pH, the monoanion, HFl^-, is present but two absorption bands appear, of about equal intensity. The position of the two maxima indicate that the monoanion is a mixture of the two forms, the yellow, zwitter ion-carboxylate structure and the quinone-carboxylic acid structure. This is in agreement with Markuszewski (ref. 3, p. 99) who shows actually six structures for the monoanion, two resonance forms for the zwitter ion structure and four forms for the quinone structure, the latter being based on resonance forms and distribution of the negative charge.

4. 4-, 5-, and 6-Sulfofluorescein

The absorption spectra of three sulfofluorescein are presented in Figures 18, 19 and 20; the absorption bands are also tabulated for convenience, Table 4.

The spectra, and the changes in the spectra with pH, of the three sulfofluoresceins are very similar, and very similar also to the corresponding spectra of fluorescein. These similarities are interpreted to mean that the prototropic forms of the sulfofluoresceins are similar to those of fluorescein, with, of course, the modifications introduced by the presence of the sulfonic acid group.

As will be seen by examination of Table 4, the parallelism is exact with one exception, 4-sulfofluorescein at pH 3, a difference which is a matter of intensity of absorbance

Figure 18. Absorption spectrum of 4-sulfofluorescein in aqueous solution.

Concentration: 1.0×10^{-5} M

Curve 1: pH = 1.01

Curve 2: pH = 3.12

Curve 3: pH = 5.20

Curve 4: pH = 8.11

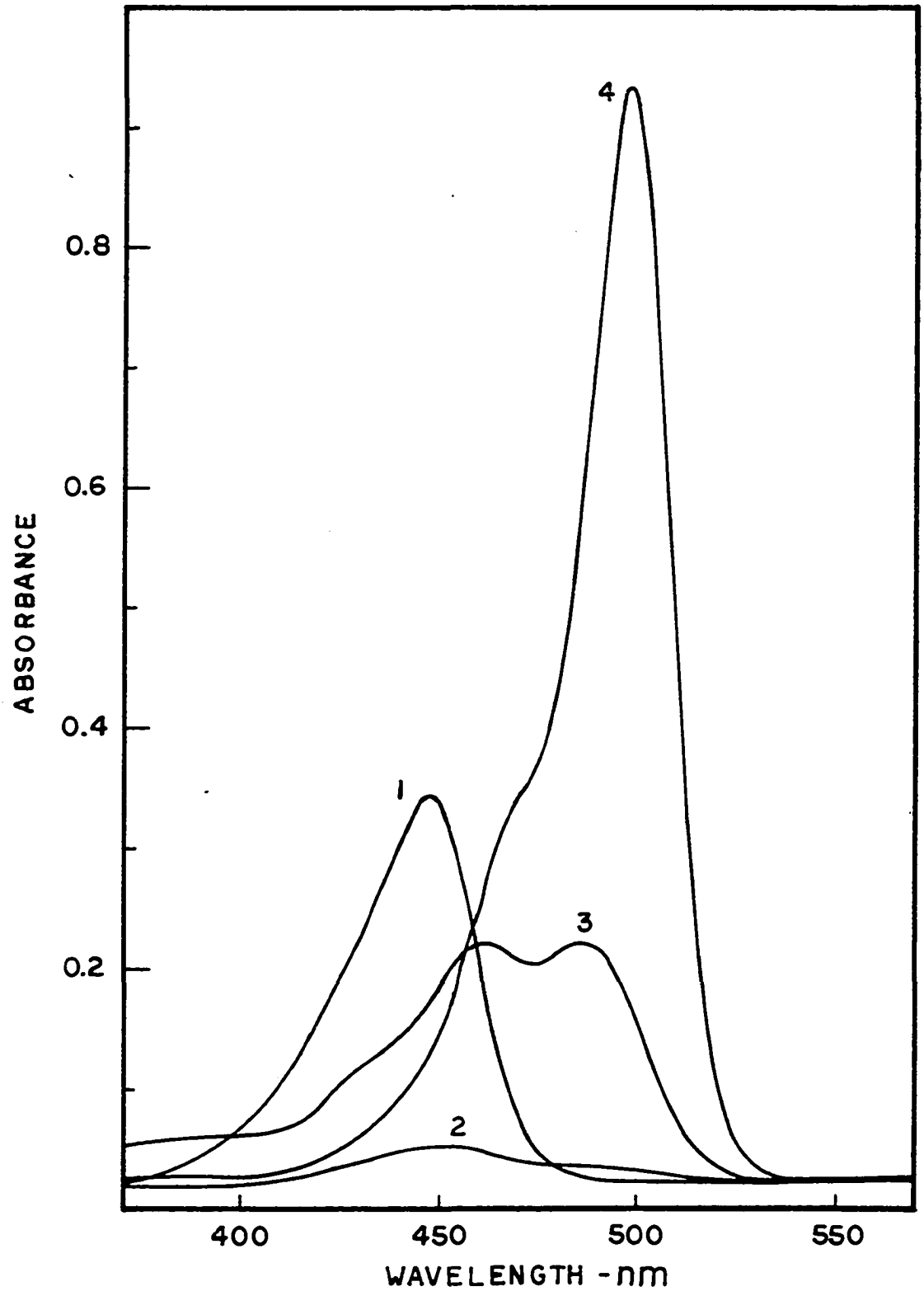

4
0.8
0.6
ABSORBANCE
0.4
1
3
0.2
2
400
450
500
550
WAVELENGTH - nm

Figure 19. Absorption spectrum of 5-sulfofluorescein in aqueous solution.

Concentration: 1.0×10^{-5} M

Curve 1: pH = 1.02

Curve 2: pH = 3.14

Curve 3: pH = 5.22

Curve 4: pH = 8.12

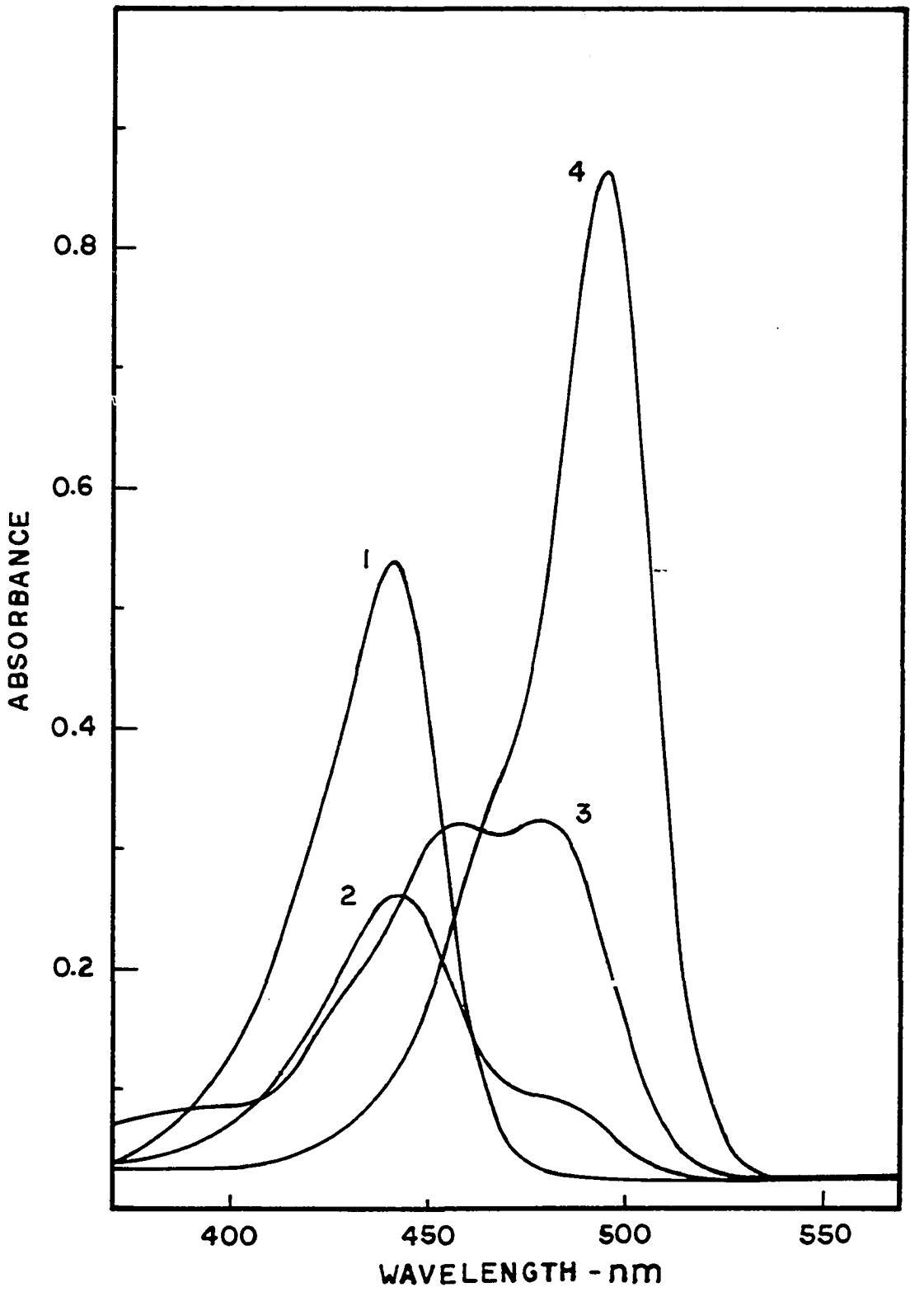

ABSORBANCE
0.8
0.6
0.4
0.2
1
2
3
4
400
450
500
550
WAVELENGTH - nm

Figure 20. Absorption spectrum of 6-sulfofluorescein in aqueous solution.

Concentration: 1.0×10^{-5} M

Curve 1: pH = 1.04

Curve 2: pH = 3.13

Curve 3: pH = 5.24

Curve 4: pH = 8.14

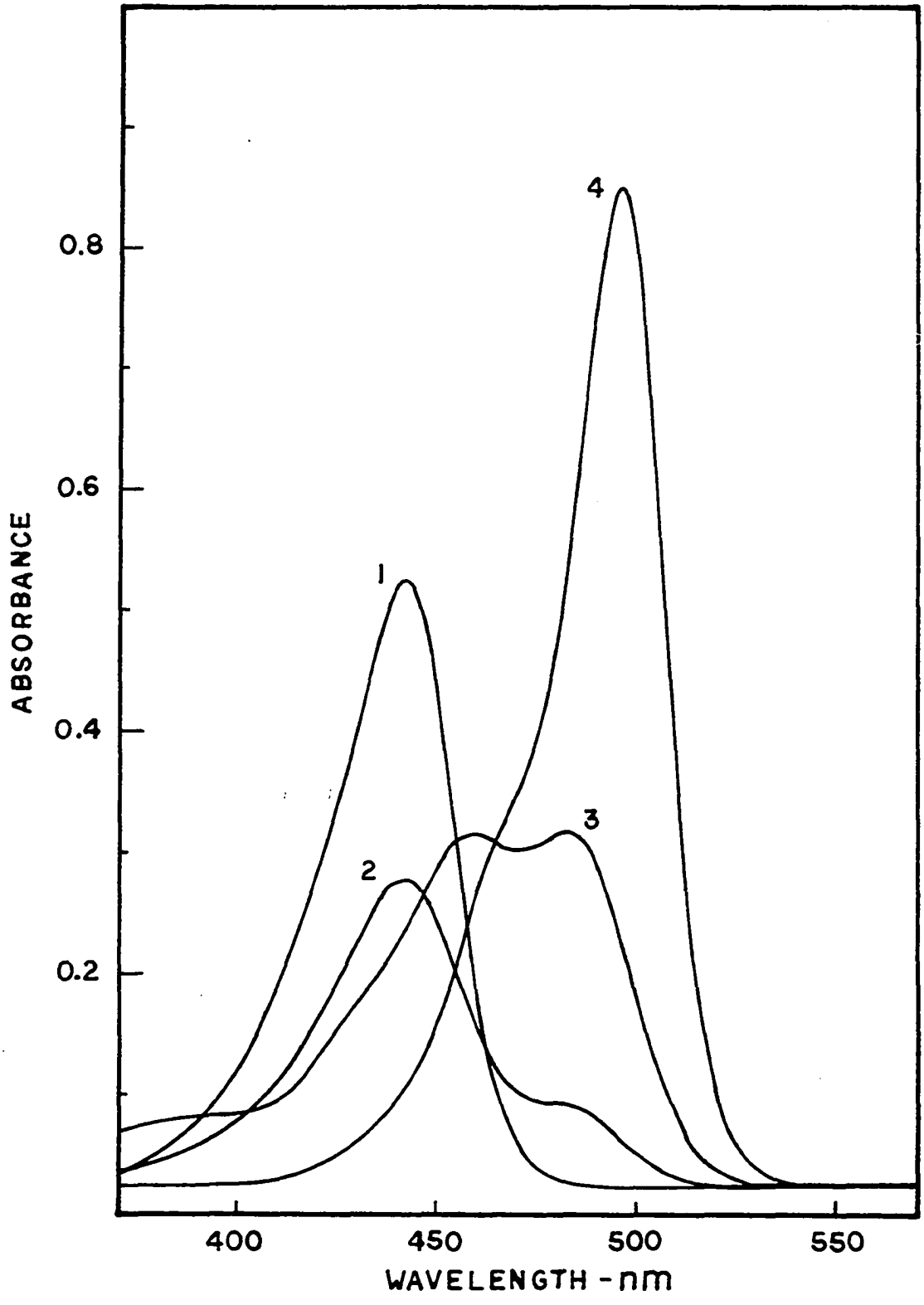

ABSORBANCE
0.8
0.6
0.4
0.2
1
2
3
4
400
450
500
550
WAVELENGTH - nm

Table 4. Absorption bands of fluorescein and three sulfofluoresceins in aqueous solution

Compound	pH	Absorption Band-nm	Predominant Form
		Maximum	
Fluorescein	1.03	438	H_3Fl^+, monocation
4-Sulfofluorescein	1.01	446	H_3Sfl, neutral molecule H_2Sfl^-, monoanion
5-Sulfofluorescein	1.02	442	H_3Sfl, neutral molecule
6-Sulfofluorescein	1.04	443	H_3Sfl, neutral molecule
		Maximum, shoulder	
Fluorescein	3.12	440, 475	H_2Fl, neutral molecule
4-Sulfofluorescein	3.12	Colorless	H_2Sfl^-, monoanion
5-Sulfofluorescein	3.14	443, 480	H_2Sfl^-, monoanion
6-Sulfofluorescein	3.15	443, 480	H_2Sfl^-, monoanion
		Maximum, maximum	
Fluorescein	5.12	455, 476	HFl^-, monoanion
4-Sulfofluorescein	5.10	462, 487	H_2Sfl^-, monoanion $HSfl^{2-}$, dianion
5-Sulfofluorescein	5.12	458, 480	$HSfl^{2-}$, dianion
6-Sulfofluorescein	5.12	459, 483	$HSfl^{2-}$, dianion
		Maximum	
Fluorescein	8.12	491	Fl^{2-}, dianion
4-Sulfofluorescein	8.10	498	Sfl^{3-}, trianion
5-Sulfofluorescein	8.12	495	Sfl^{3-}, trianion
6-Sulfofluorescein	8.14	496	Sfl^{3-}, trianion

only. Because of the presence of the sulfonate group, the prototropic forms of the three sulfofluoresceins carry one more negative charge than the corresponding prototropic form of fluorescein.

As with fluorescein, as discussed in Section 3, immediately above, two explanations are possible for the great decrease in absorbance without change in the wavelength of the maximum on passing from the neutral species, H_3Sfl, of the sulfofluoresceins, to the monoanion, H_2Sfl^-. That the monoanion is almost completely colorless in the case of 4-sulfofluorescein makes the second explanation most plausible. Indeed, the structure of the neutral and monoanion species of sulfofluorescein and of the neutral species of fluorescein will be a central theme in the following parts of this dissertation.

C. Absorbance at Specific Wavelengths and as a Function of pH and the Dissociation Constants of Fluorescein and the Sulfofluoresceins

1. Introduction

Provided that a wavelength can be found at which only one prototropic form (ionic species) of an acid can be found, the variation of absorbance with pH can be used to determine the dissociation constant of the acid. Experimentally, the

determination is carried out using buffers varying in pH in small intervals and prepared so as to have the same ionic strength. The buffers are prepared to cover the value $pH = pK_a$ and to extend on either side of this value such that the absorbance no longer changes with pH so that the absorbance of the two species, acid and salt, can be obtained.

The evaluation of the acid dissociation constants from absorbance data is based on the mathematical equation defining the latter. For a monobasic acid HA

$$HA \rightleftharpoons H^+ + A^- \qquad K_a = \frac{[H^+][A^-]}{[HA]} \tag{1}$$

Rearrangement of equation (1) gives

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \tag{2}$$

From equation (2) it is evident that when $[HA] = [A^-]$, the log term becomes zero and $pH = pK_a$. Thus, a plot of pH <u>vs.</u> the log term can be used to evaluate the acid dissociation constant, and if the change in the concentration of the acid or its salt is reflected by a change in absorbance at a particular wavelength then equation (2) can be expressed in terms of absorbance:

$$pH = pK_a + \log \left(\frac{A_a - A_m}{A_m - A_b}\right) \tag{3}$$

in which A_a is the absorbance of the acid, determined at a point at which this absorbance no longer varies with pH, and A_b is the absorbance of the salt at a point at which this

absorbance is no longer chainging with pH. The third term, A_m, is the total absorbance of the acid and its salt at any point which falls within the pH region under study.

Theoretically, pH = pK_a, when the log term in equation (3) is equal to zero; this corresponds to the point of inflection in the plot of absorbance versus pH. Ideally, the point of inflection should coincide with the mid-point. A difference between the two may arise when application is made to a polybasic acid owing to overlap in the neutralization of the successive protons. Another error may occur if the wavelength of maximum absorbance shifts greatly as the value of pH changes.

2. Apparatus and procedure

The absorbance of yellow fluorescein in aqueous solution as a function of pH was measured using the Hach DR-2 Spectrophotometer, supplied with a flow-through cell.

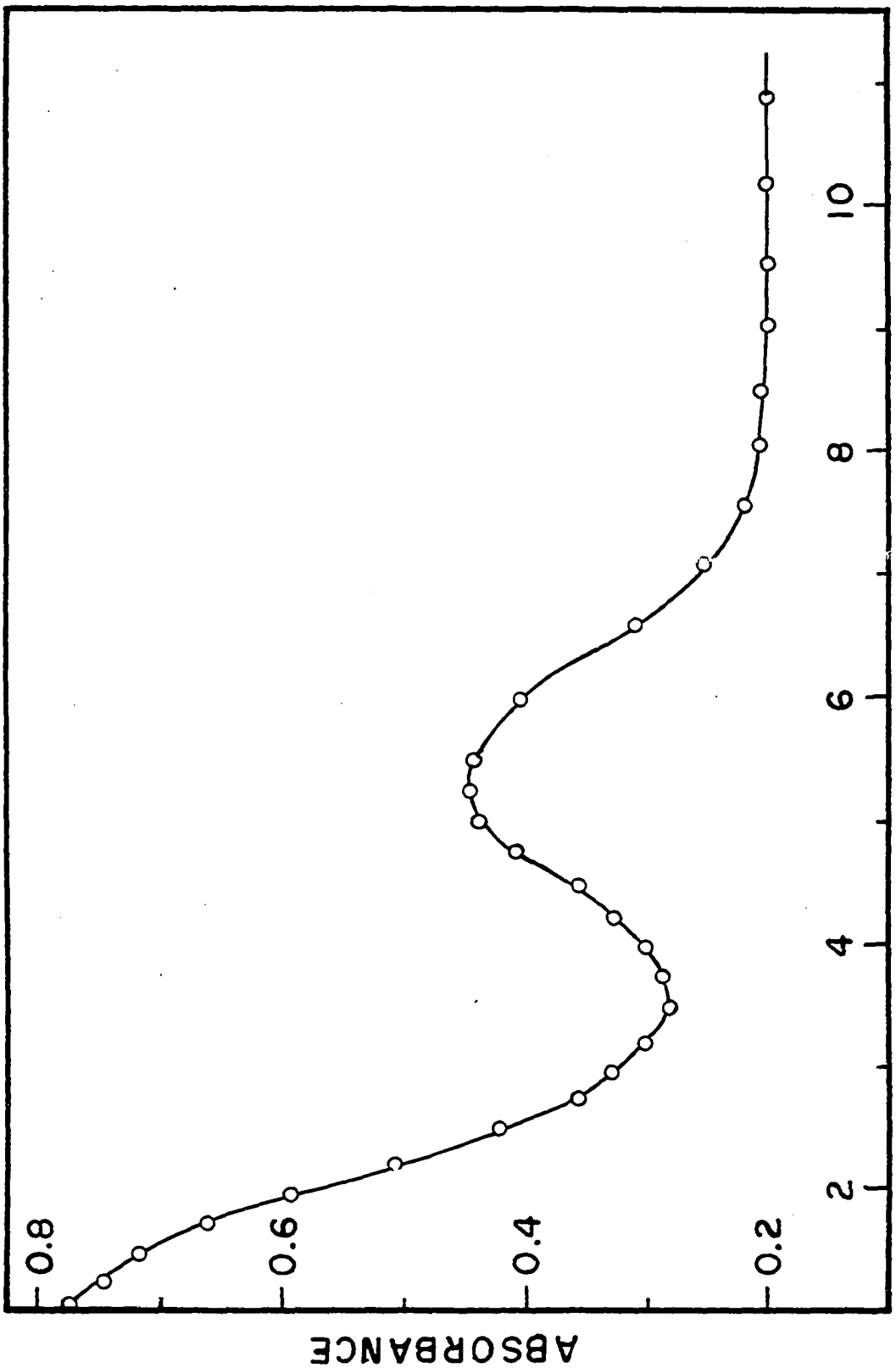
The buffers used were prepared at intervals of about 0.25-0.5 pH over the region pH 1-11 as described in Chapter V, Section b, using 0.1 M solutions of hydrochloric acid, potassium hydrogen phthalate, potassium chloride, potassium hydroxide, and boric acid in a 0.1 M potassium chloride solution. The pH of the buffer solution was determined before and after the addition of fluorescein, using a Hach 8594 pH meter set on the expanded scale.

The concentrations of the yellow fluorescein solutions used for obtaining the absorbance measurements were 8.0×10^{-6} M prepared as described in Chapter V, Section b. Each solution was used to obtain two absorbance readings, one at a wavelength of 440 nm and another at 490 nm.

3. Fluorescein

The absorbance curve of yellow fluorescein as a function of pH, at 440 nm, is presented in Figure 21. The absorbance is maximum at pH 1, the region at which the monocation (H_3Fl^+) predominates, then the absorbance decreases to reach a minimum at about pH 3.4, the region at which the neutral molecule, H_2Fl, predominates. As the neutral molecule is further neutralized to form the monoanion, HFl^-, the absorbance increases to reach another maximum at about pH 5.3, the region in which the monoanion predominates. The absorbance decreases, as the fraction of the monoanion in solution decreases, to reach a minimum and essentially constant value at about pH 8.0, the region at which the dianion, Fl^{2-}, is the predominant form. Thus, the mid-point of the absorption curve which lies between pH 1 and pH 3.4 occurs at pH 2.2 and corresponds to $pK_{H_3Fl^+}$. The mid-point which lies between pH 3.4 and pH 5.3 occurs at pH 4.5 and corresponds to pK_{H_2Fl}. Again, the mid-point which lies between pH 5.3 and pH 8.0 occurs at pH 6.6, corresponding to pK_{HFl^-}. These values are in good agreement with those

Figure 21. Absorbance of fluorescein, 8.0×10^{-6} M, as a function of pH at a wavelength of 440 nm.


ABSORBANCE
0.8
0.6
0.4
0.2
2
4
6
8
10
pH

(2.2, 4.4, and 6.7) determined by Lindqvist (23) obtained by absorbance measurement at ionic strength of 0.01.

The absorbance curve of fluorescein as a function of pH, at a wavelength of 490 nm, is presented in Figure 22. The absorbance is very low at pH less than 2, and rises slowly between pH 2 and 3. The absorbance rises between pH 3.5 and 5.3 at which the monoanion is the predominant form. Above pH 5.3, the region at which the dianion begins to increase, an inflection in the absorption is observed, and the curve rises rapidly and reaches a maximum at about pH 8.0. Beyond pH 8.5, the absorbance is essentially constant. The mid-point of the absorbance curve occurs at pH 6.2 and corresponds to pK_{HFl^-}, a value in good agreement with that (6.28) obtained by Markuszewski (3) using fluorescence measurements at ionic strength of 0.1 and excitation at 489 nm.

The plots of pH *vs.* the log term in equation (3) is presented in Figures 23 and 24 for the determination of the values of pK_a of fluorescein, where the absorbance is measured at 440 nm. The plot in Figure 24 is for the determination of pH_{HFl^-} only, the absorbance being measured at 490 nm. The slopes and the intercepts of the straight lines were computed by the least-squares method and are presented in Tables 5 and 6. The values of $pK_{H_3Fl^+}$ and pK_{H_2Fl} determined by the intercepts in Figures 23a and 23b

Figure 22. Absorption of fluorescein, 8.0×10^{-6} M, as a function of pH at a wavelength of 490 nm.

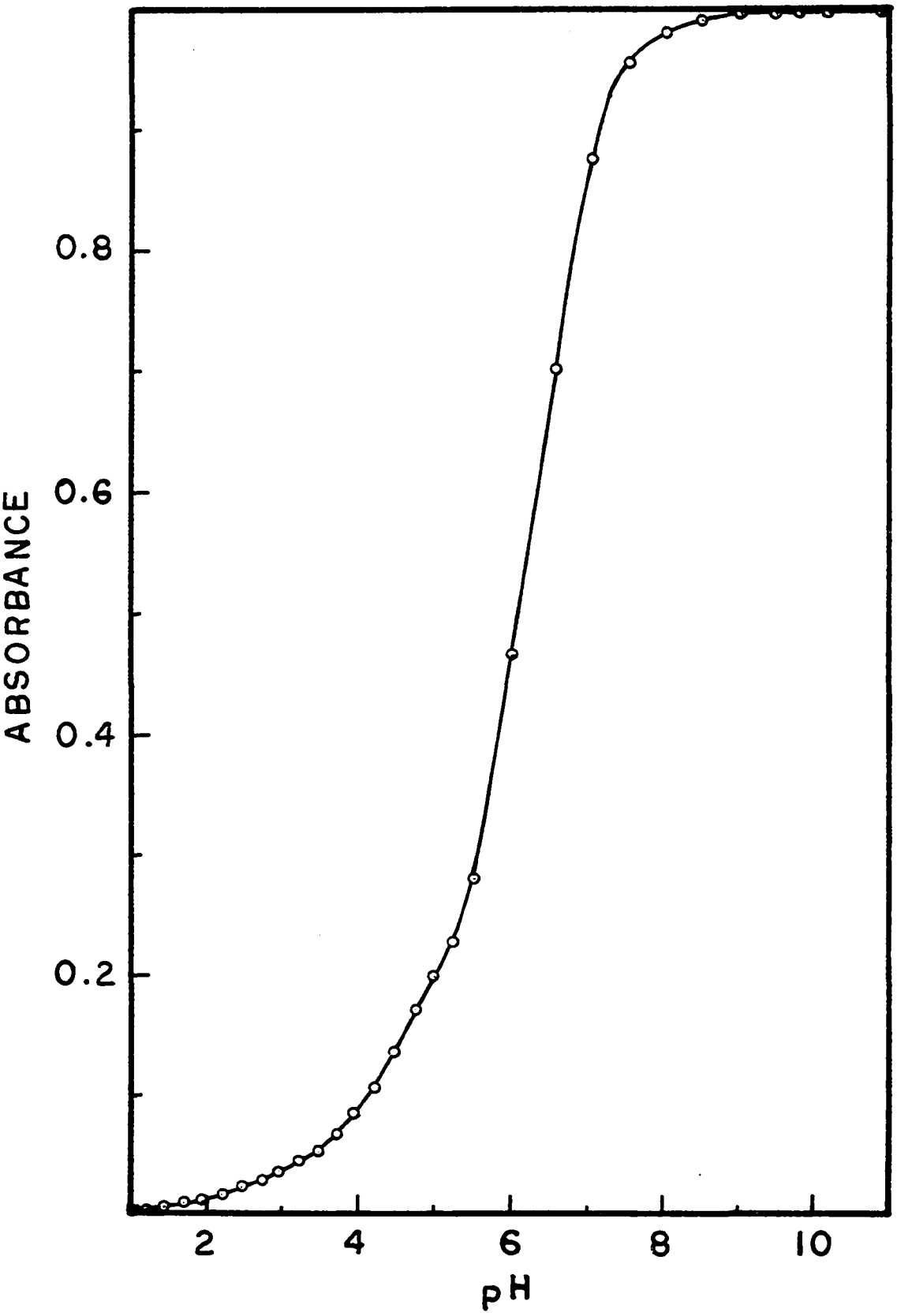

ABSORBANCE
0.8
0.6
0.4
0.2
2
4
6
8
10
pH

Figure 23. Mathematical treatment of the absorbance of fluorescein at 440 nm.

$$\log \left(\frac{A_1 - A_m}{A_m - A_2}\right) \text{ vs. pH}$$

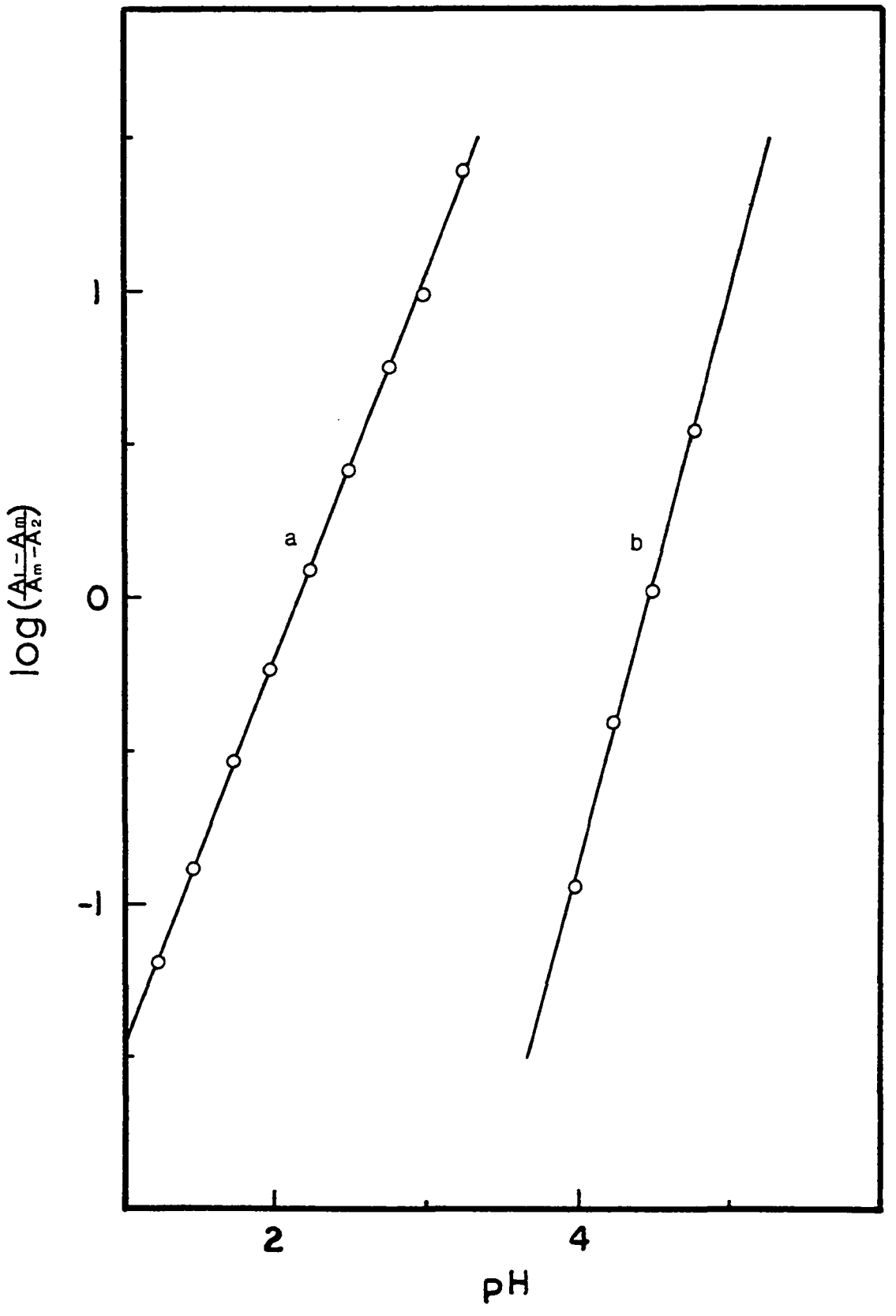

1
0
-1
log ((A_L-A_m)/(A_m-A_2))
a
b
2
4
pH

Table 5. Absorbance of fluorescein as a function of pH at 440 nm

pH	A_m	$\log \left(\frac{A_1 - A_m}{A_m - A_2}\right)$	
1.22	0.745	-1.19	$\mu = 0.1$
1.45	0.719	-0.89	$A_1 = A_{H_3Fl^+} = 0.775$
1.72	0.664	-0.534	$A_2 = A_{H_2Fl} = 0.284$
1.96	0.595	-0.237	slope = 0.79
2.22	0.507	0.080	intercept (log term = 0) = 2.15
2.48	0.421	0.412	
2.74	0.358	0.751	
2.96	0.330	0.985	
3.22	0.303	1.40	
3.97	0.300	-0.948	$A_1 = A_{H_2Fl} = 0.284$
4.23	0.328	-0.413	$A_2 = A_{HFl^-} = 0.442$
4.48	0.365	0.022	slope = 0.53
4.76	0.407	0.546	intercept (log term = 0) = 4.46

are 2.15 and 4.46, respectively. The value of pK_{HFl^-} determined by the intercept in Figure 24 is 6.15.

By examination of Figure 17, it becomes clear that the molar absorptivity of the monoanion, HFl^-, is much less than the molar absorptivity of the dianion, Fl^{2-}, of fluorescein at 490 nm. And the absorbance of the monoanion becomes very minimal beyond pH 6.0, at which point the fraction of Fl^{2-} starts to increase very rapidly. Thus, the log term, $\log \left(\frac{A_m - A_{H_2Fl^-}}{A_{Fl^{2-}} - A_m} \right)$ in equation (3) could be reduced to $\log \left(\frac{A_m}{A_{Fl^{2-}} - A_m} \right)$. The validity of this approximation is demonstrated by the slope of line (b), Figure 24, a value of 1.02 and very close to the theoretical value 1.0. This is in contrast to the slope of line (a), a value of 2.40. The experimental points, slope and intercept are presented in Table 6. The value of pK_{HFl^-} determined by the intercept of line (b), Figure 24, is 6.15.

4. 4-Sulfofluorescein

Buffers of ionic strength 0.1 covering the pH range 1 to 11 at intervals of 0.25 to 0.50 pH were prepared as described in Part B above. Buffers of ionic strength 1.0 covering the pH range 0 to 4 at intervals of 0.25 to 0.50 pH were prepared by mixing 1.0 M solutions of hydrochloric acid, potassium chloride, and potassium hydroxide. pH was

Figure 24. Mathematical treatment of the absorbance of fluorescein at 490 nm.

$$\log \left(\frac{A_m}{A_2 - A_m}\right) \text{ vs. pH}$$

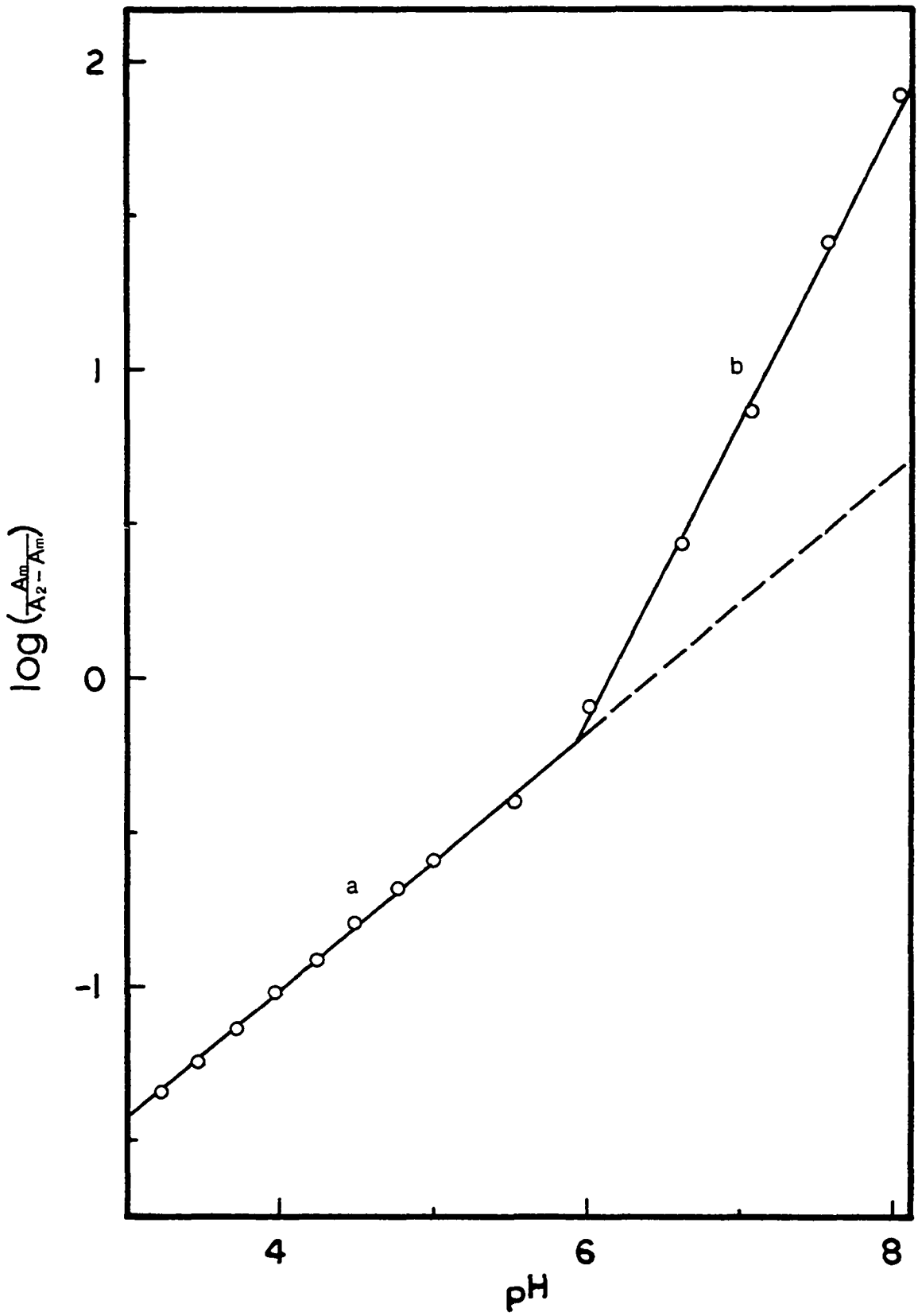

2
1
0
-1
log ($\frac{A_m}{A_2 - A_m}$)
a
b
4
6
8
pH

Table 6. Absorbance of fluorescein as a function of pH at 490 nm

pH	A_m	$\log \left(\frac{A_m}{A_2 - A_m}\right)$	
3.22	0.043	-1.35	$\mu = 0.1$
3.46	0.053	-1.25	$A_2 = A_{Fl2-} = 0.996$
3.72	0.067	-1.14	slope = 2.40
3.97	0.086	-1.03	
4.23	0.108	-0.915	
4.48	0.137	-0.797	
4.76	0.171	-0.683	
5.00	0.201	-0.597	
5.52	0.282	-0.403	
6.01	0.449	-0.086	
6.62	0.724	0.425	slope = 1.02
7.07	0.876	0.863	
7.56	0.959	1.41	intercept (log term = 0) = 6.15
8.04	0.983	1.88	

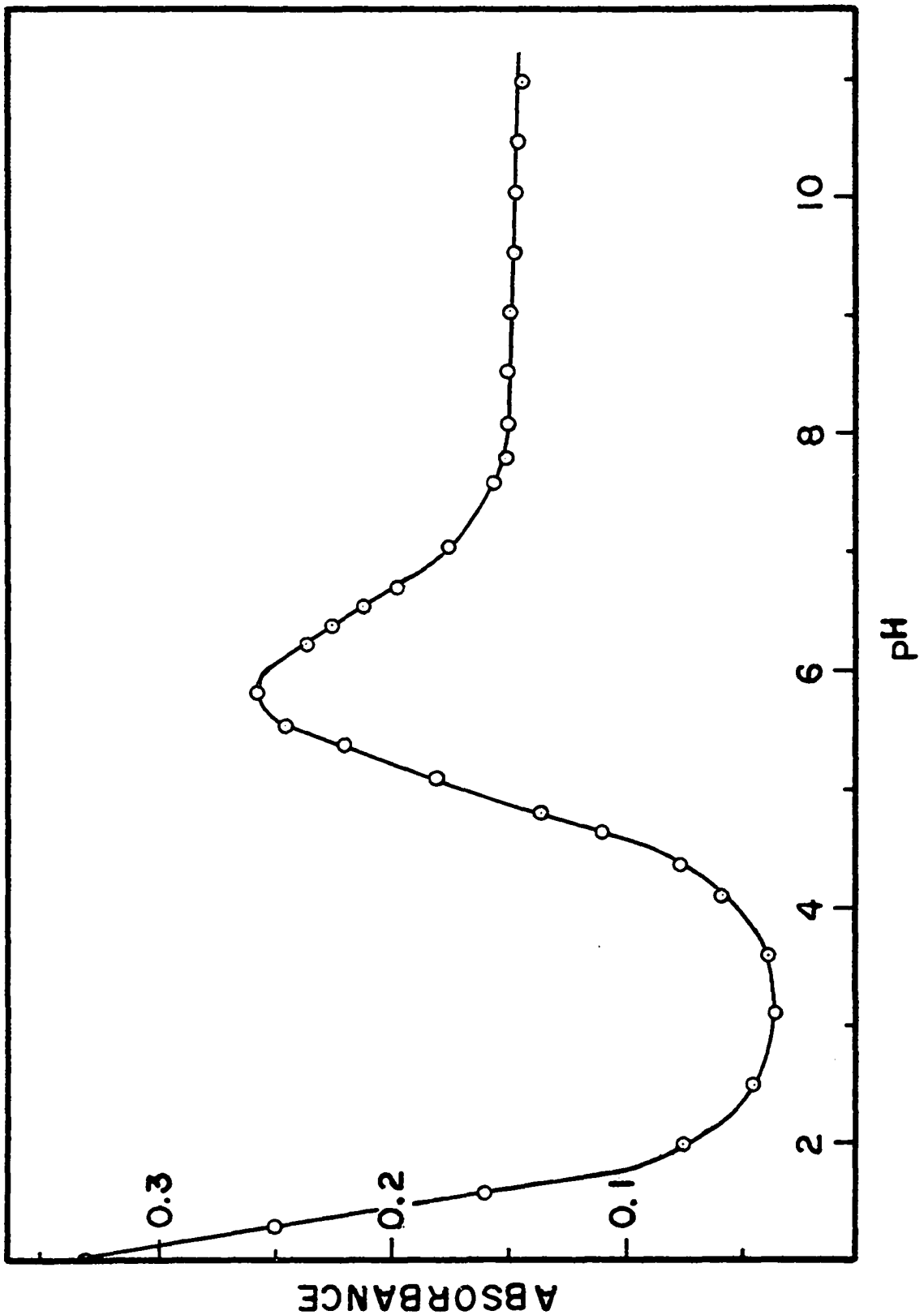
measured with the Hach Model 8594 pH Meter set on the expanded scale; the meter was calibrated against two buffer solutions prepared according to specifications of the National Bureau of Standards.

The absorbance of 4-sulfofluorescein, 5.0×10^{-6} M, as a function of pH, at 446 nm, is presented in Figure 25. The absorbance has a relatively high value at pH 1, the region in which 4-sulfofluorescein exists as a mixture of the neutral molecule, H_3Sfl, and the monoanion, H_2Sfl^-. With increasing pH, the absorbance decreases, reaching a minimum at pH 3.2; at this point the monoanion is the predominant species and the absorbance is less than 0.02. This decrease in absorbance is interpreted as evidence that the monoanion, H_2Sfl^-, is present as the colorless lactone.

Above pH 3.2, the absorbance increases, reaching a maximum in the region of pH 5.8. At this point the dianion, $HSfl^{2-}$, is the predominant form. The mid-point occurs at pH 4.9, at which point the pH is equal to the negative logarithm of the dissociation constant involved, $pK_{H_2Sfl^-}$. As the pH rises further, the absorbance drops gradually reaching a constant value about pH 8, at which point the trianion, Sfl^{3-}, is the predominant form. The mid-point occurs at pH 6.7, corresponding to $pK_{HSfl^{2-}}$.

Because the absorbance did not level off at pH 1, Figure 25, the absorbance measurements were extended to pH 0

Figure 25. Absorbance of 4-sulfofluorescein, 5.0×10^{-6} M, as a function of pH at a wavelength of 446 nm.


ABSORBANCE
0.3
0.2
0.1
2
4
6
8
10
pH

by using buffer solutions of ionic strength 1.0. The measurements were made in the region pH 0 to 4, Figure 26. At pH 0 the neutral molecule, H_3Sfl, is the predominant species and at pH 3.2, the absorbance reaches a minimum, the monoanion, H_2Sfl^-, being the predominant form. The mid-point occurs at pH 1.2, corresponding to pK_{H_3Sfl}. This is in very close agreement with the value 1.23 found by solubility measurements as a function of pH at ionic strength 0.1, Chapter VII.

The absorbance of 4-sulfofluorescein, 5.0×10^{-6} M, as a function of pH, at 498 nm, is presented in Figure 27. The absorbance is very low at pH less than 3, increases slowly between pH 4 and 5, rises rapidly between pH 6 and 7, and reaches a maximum and essentially constant value at pH 8 and above. The trianion is the predominant form in this region. The mid-point of the absorbance curve as a function of pH occurs at pH 6.2, corresponding to $pK_{HSfl^{2-}}$, a value in very close agreement with that determined by fluorescence measurement, 6.24, reported in Chapter VI.

The dissociation constants were obtained using the graphical method, discussed in the previous section. A plot of pH vs. $\log \left[\frac{A_1 - A_m}{A_m - A_2}\right]$ (equation 3). The values for pK_{H_3Sfl}, $pK_{H_2Sfl^-}$, and $pK_{HSfl^{2-}}$, determined from the intercepts, are presented in Figures 28, 29a and 29b, respectively. The experimental points, intercepts, and slopes

Figure 26. Absorbance of 4-sulfofluorescein, 2.5×10^{-6} M, as a function of pH at a wavelength of 446 nm.

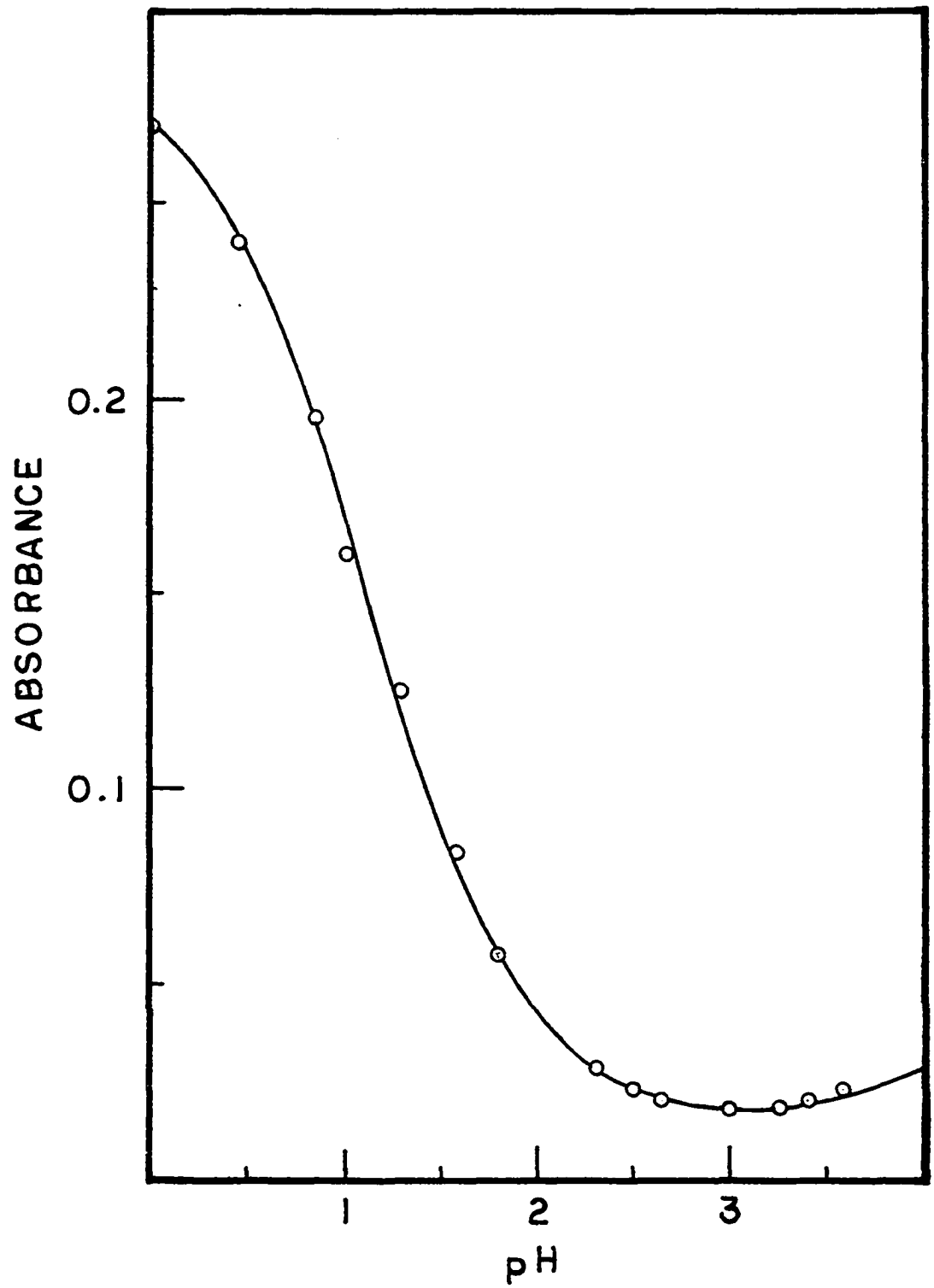

ABSORBANCE
0.2
0.1
1
2
3
pH

Figure 27. Absorbance of 4-sulfofluorescein, 5.0×10^{-6} M, as a function of pH at a wavelength of 498 nm.

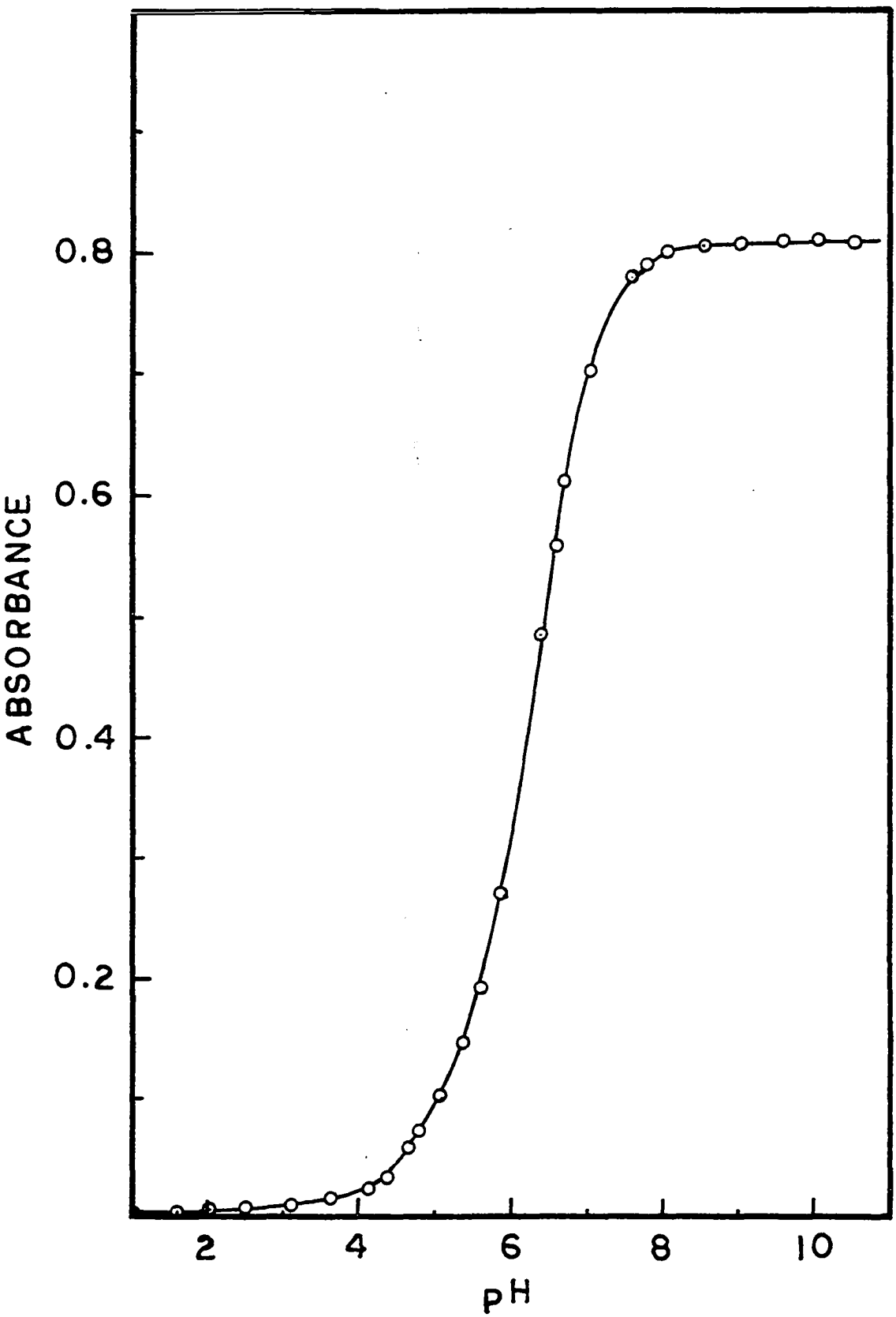

ABSORBANCE
0.8
0.6
0.4
0.2
2
4
6
8
10
pH

Figure 28. Mathematical treatment of the absorbance of 4-sulfofluorescein at 446 nm.

$$\log \left(\frac{A_1 - A_m}{A_m - A_2} \right) \text{ vs. pH}$$

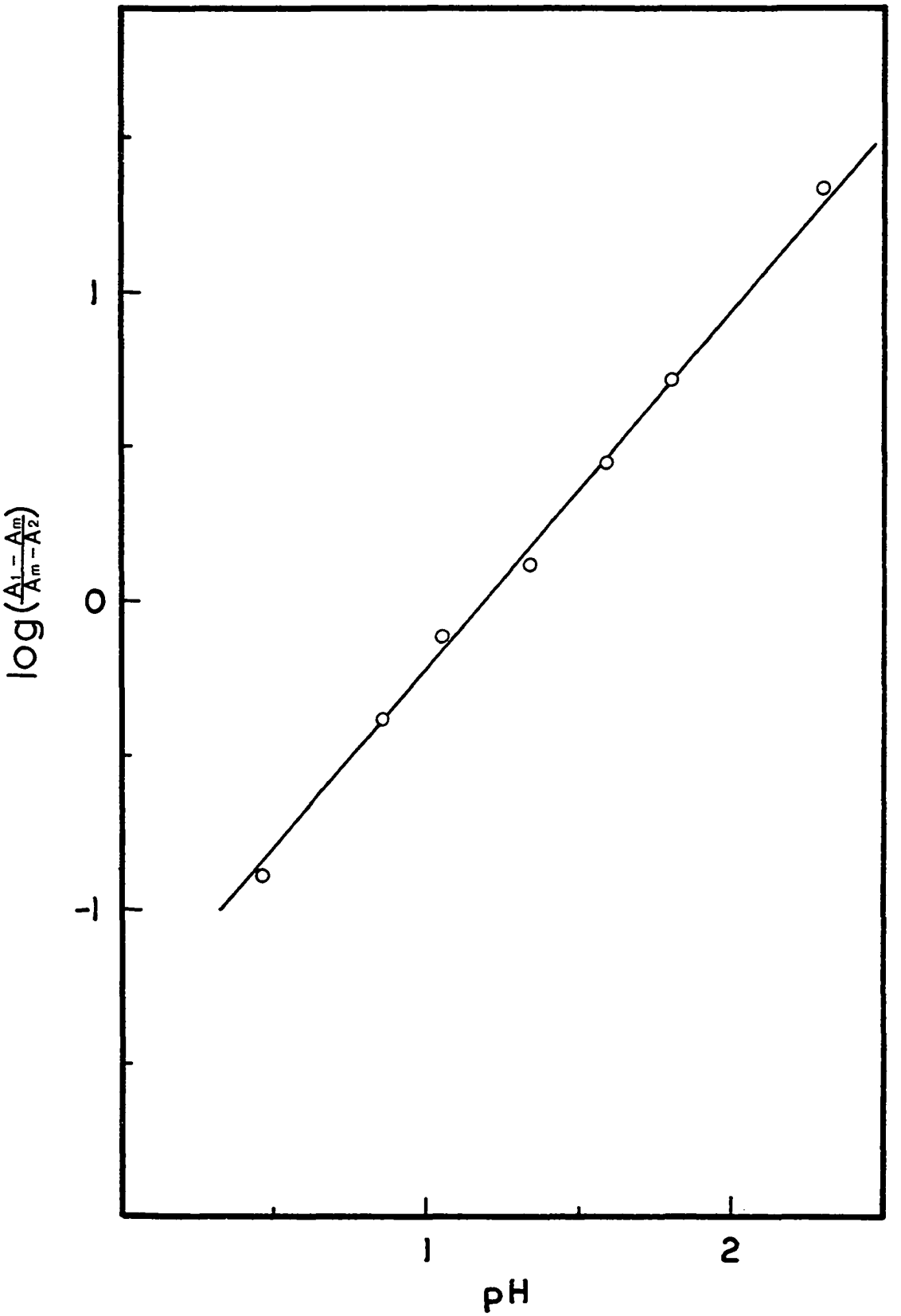
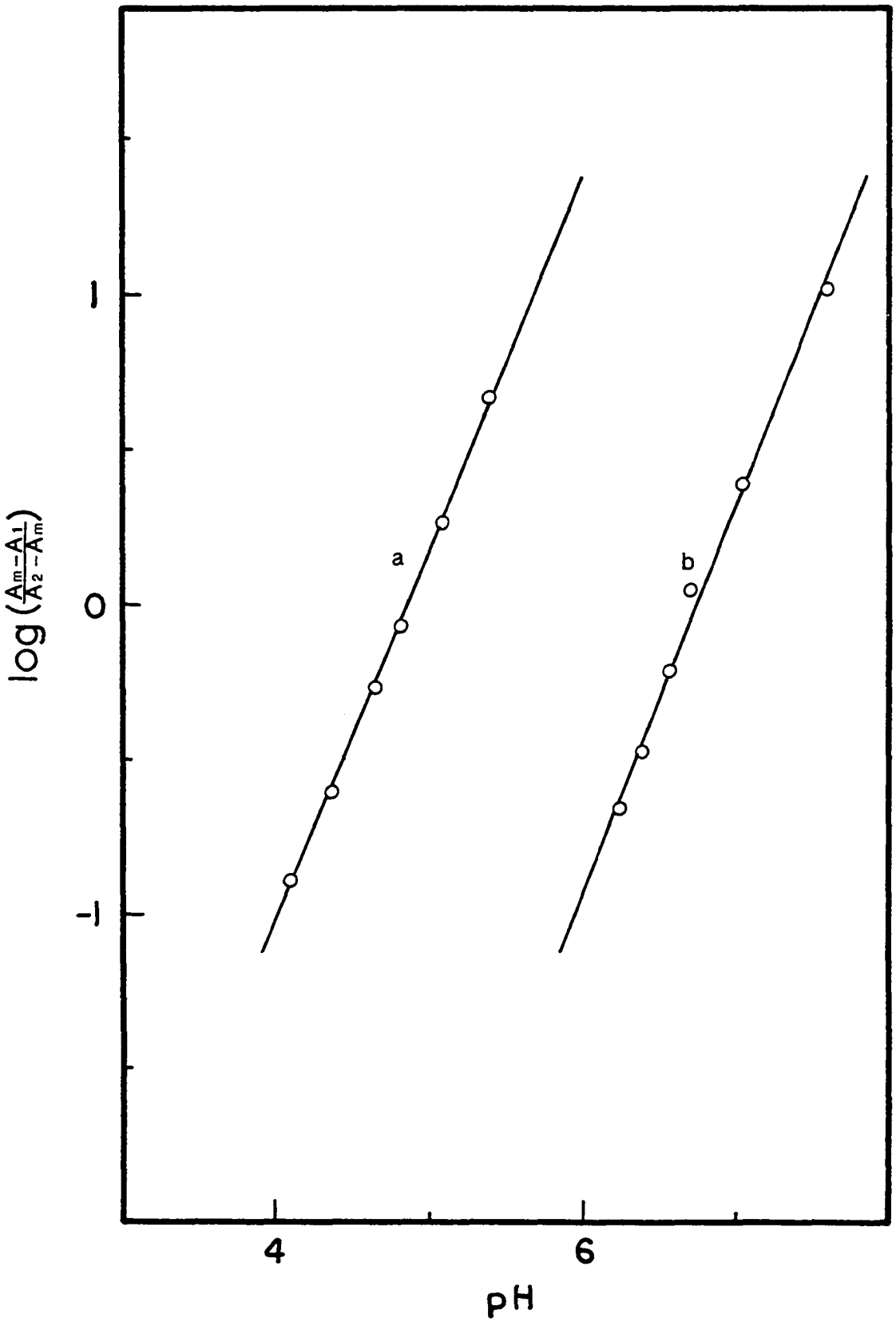

1
0
-1
$\log\left(\frac{A_1 - A_m}{A_m - A_2}\right)$
1
2
pH

Figure 29. Mathematical treatment of the absorbance of 4-sulfofluorescein at 446 nm.

$$\log \left(\frac{A_m - A_1}{A_2 - A_m}\right) \text{ vs. pH}$$


1
0
-1
log ((Am-A1)/(A2-Am))
a
b
4
6
pH

are given in Table 7, the measurement, having been made at wavelength 446 nm. The least-squares method was employed for the calculation of intercept and slope.

Table 7. Absorbance of 4-sulfofluorescein as a function of pH at 446 nm

pH	A_m	$\log \left(\frac{A_1 - A_m}{A_m - A_2}\right)$	
0.45	0.240	-0.884	μ = 1.0
0.85	0.196	-0.387	$A_1 = H_{H_3Sfl} = 0.269$
1.05	0.161	-0.122	$A_2 = A_{H_2Sfl^-} = 0.018$
1.30	0.126	0.122	slope = 0.84
1.58	0.084	0.448	intercept (log term = 0) = 1.18
1.80	0.058	0.722	
2.30	0.029	1.339	
4.10	0.056	-0.895	μ = 0.1
4.36	0.076	-0.602	$A_1 = A_{H_2Sfl^-} = 0.030$
4.64	0.110	-0.273	$A_2 = A_{HSfl^{2-}} = 0.260$
4.81	0.135	0.076	slope = 0.82
5.07	0.178	0.256	intercept (log term = 0) = 4.85
5.38	0.219	0.663	

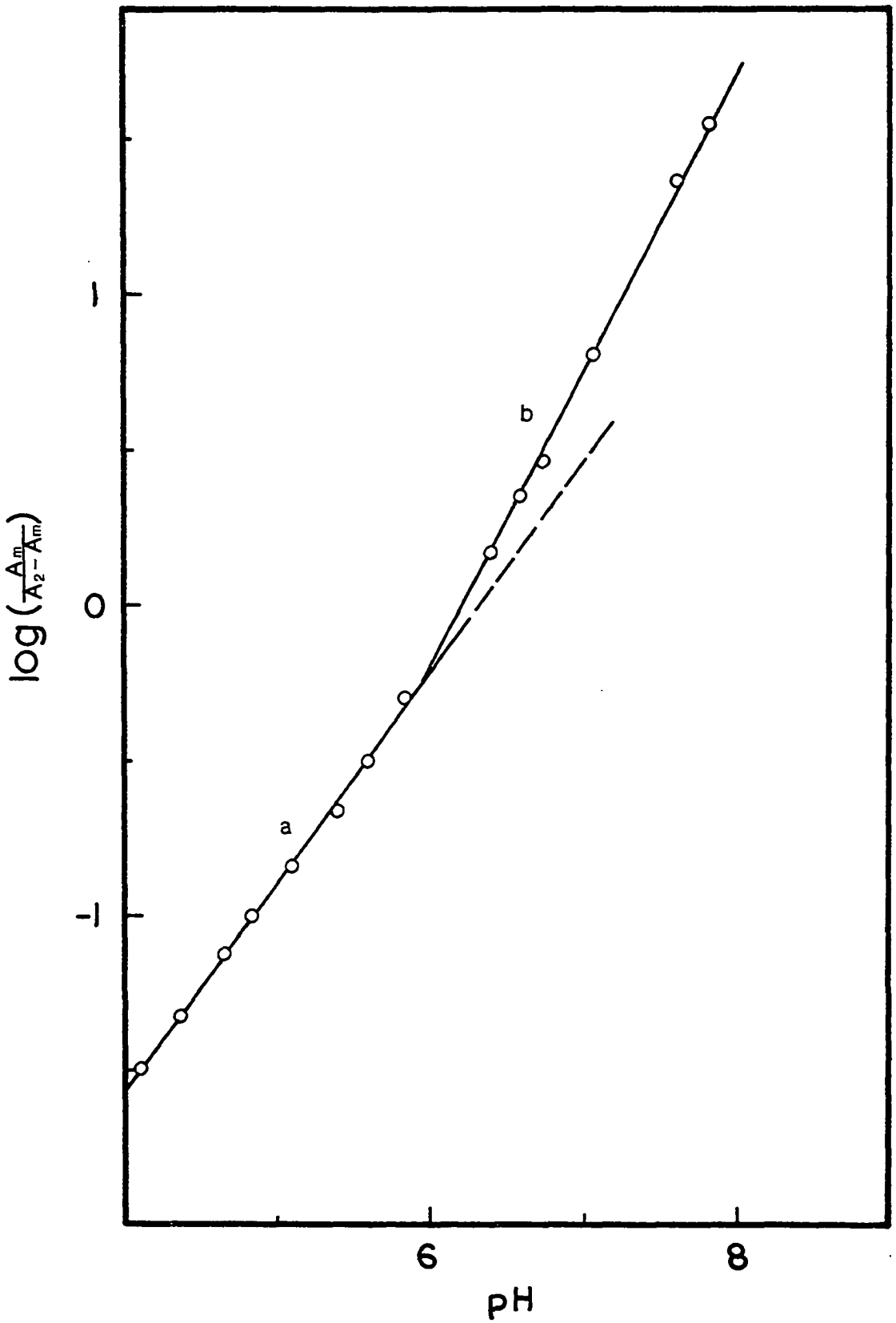
Table 7. (Continued)

pH	A_m	$\log \left(\frac{A_1 - A_m}{A_m - A_2}\right)$	
6.25	0.236	-0.667	$\mu = 0.1$
6.38	0.228	-0.482	$A_1 = A_{HSfl}^{2-} = 0.256$
6.71	0.213	-0.217	$A_2 = A_{Sfl}^{3-} = 0.143$
6.57	0.197	0.039	slope = 0.80
7.05	0.175	0.403	intercept (log term = 0) = 6.75
7.60	0.153	1.013	

The evaluation of pK_{HSfl}^{2-} using the absorbance points determined at 498 nm is presented in Figure 30. The term $\log \left[\frac{A_m}{A_{SF}^{3-} - A_m}\right]$ instead of $\log \left[\frac{A_m - A_{HSF}^{2-}}{A_{SF}^{3-} - A_m}\right]$ is used. This approximation is valid, for the absorbance of the dianion at 498 nm is negligible when compared to the absorbance of the trianion of 4-sulfofluorescein, Figure 18. Again, the approximation is most valid beyond pH 6.0, the point at which the fraction of the trianion starts to increase very rapidly. The value of pK_{HSfl}^{2-} determined by the intercept of line (b), Figure 30, is 6.21, which is in excellent

Figure 30. Mathematical treatment of the absorbance of 4-sulfofluorescein at 498 nm.

$$\log \left(\frac{A_m}{A_2 - A_m}\right) \text{ vs. pH}$$


1
0
-1
log ($\frac{A_m}{A_2 - A_m}$)
a
b
6
8
pH

agreement with the value (6.24) determined by fluorescence measurements reported in Chapter VI. The intercept and slope determined by the least-squares method, and the absorbance points measured at 498 nm, are presented in Table 8.

Table 8. Absorbance of 4-sulfofluorescein as a function of pH at 498 nm

pH	A_m	$\log \left(\frac{A_m}{A_2 - A_m}\right)$	
4.10	0.026	-1.48	μ = 0.1
4.36	0.037	-1.32	$A_2 = A_{Sfl}3-$ = 0.810
4.64	0.057	-1.12	slope = 1.49
4.81	0.073	-1.00	
5.07	0.103	-0.837	
5.38	0.146	-0.658	
5.58	0.194	-0.502	
5.83	0.271	-0.299	
6.38	0.483	0.169	
6.57	0.559	0.348	slope = 1.03
6.71	0.611	0.487	intercept (log term = 0) = 6.21
7.05	0.701	0.808	
7.60	0.777	1.37	
7.81	0.788	1.55	

5. 5- and 6-Sulfofluorescein

The absorption curves of 5- and 6-sulfofluorescein, 5.0×10^{-6} M, at 443 nm, are presented in Figures 31 and 32. The two curves are almost identical. The absorbance starts maximum at pH 1, the point at which the neutral molecule, H_3Sfl, predominates. As the pH increases, the absorbance decreases gradually reaching a minimum at about pH 3.5, at which point the monoanion, H_2Sfl^-, is the major ionic form. The decrease in absorbance as the neutral molecule is neutralized to form the monoanion is less than the cases of fluorescein and 4-sulfofluorescein, Figures 21 and 25, respectively. Again, the absorbances of the monoanions of the β-isomers are much larger than those of fluorescein and 4-sulfofluorescein. Here it becomes evident that the formation of the lactone in the case of the β-isomers, in the pH region over which the monoanion predominates, is less favorable when compared to that of fluorescein and especially to that of 4-sulfofluorescein.

The mid-points on the absorption curves, Figures 31 and 32, for 5- and 6-sulfofluorescein occur at pH 2.2 and 6.4 which correspond to pK_{H_3Sfl} and $pK_{HSfl^{2-}}$, respectively. The evaluation of $pK_{H_2Sfl^-}$ for both of the isomers from the mid-points is impractical, since the increase in absorbance between pH 3.5 and pH 5.4 is very small.

Again, the absorption curves of 5- and 6-sulfofluorescein, 5.0×10^{-6} M, at 496 nm, are presented in Figures 33

Figure 31. Absorbance of 5-sulfofluorescein, 5.0×10^{-6} M, as a function of pH at a wavelength of 443 nm.

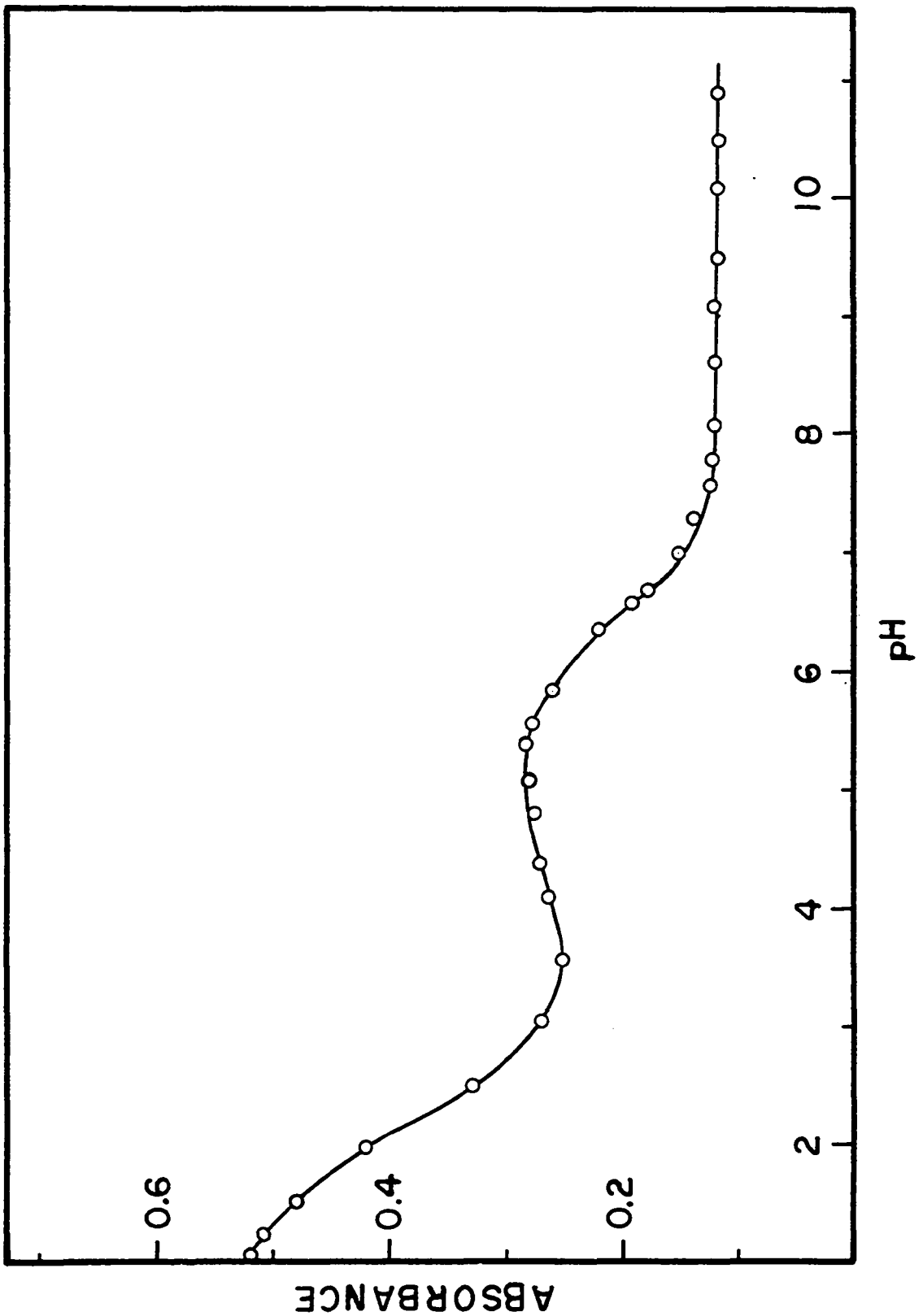
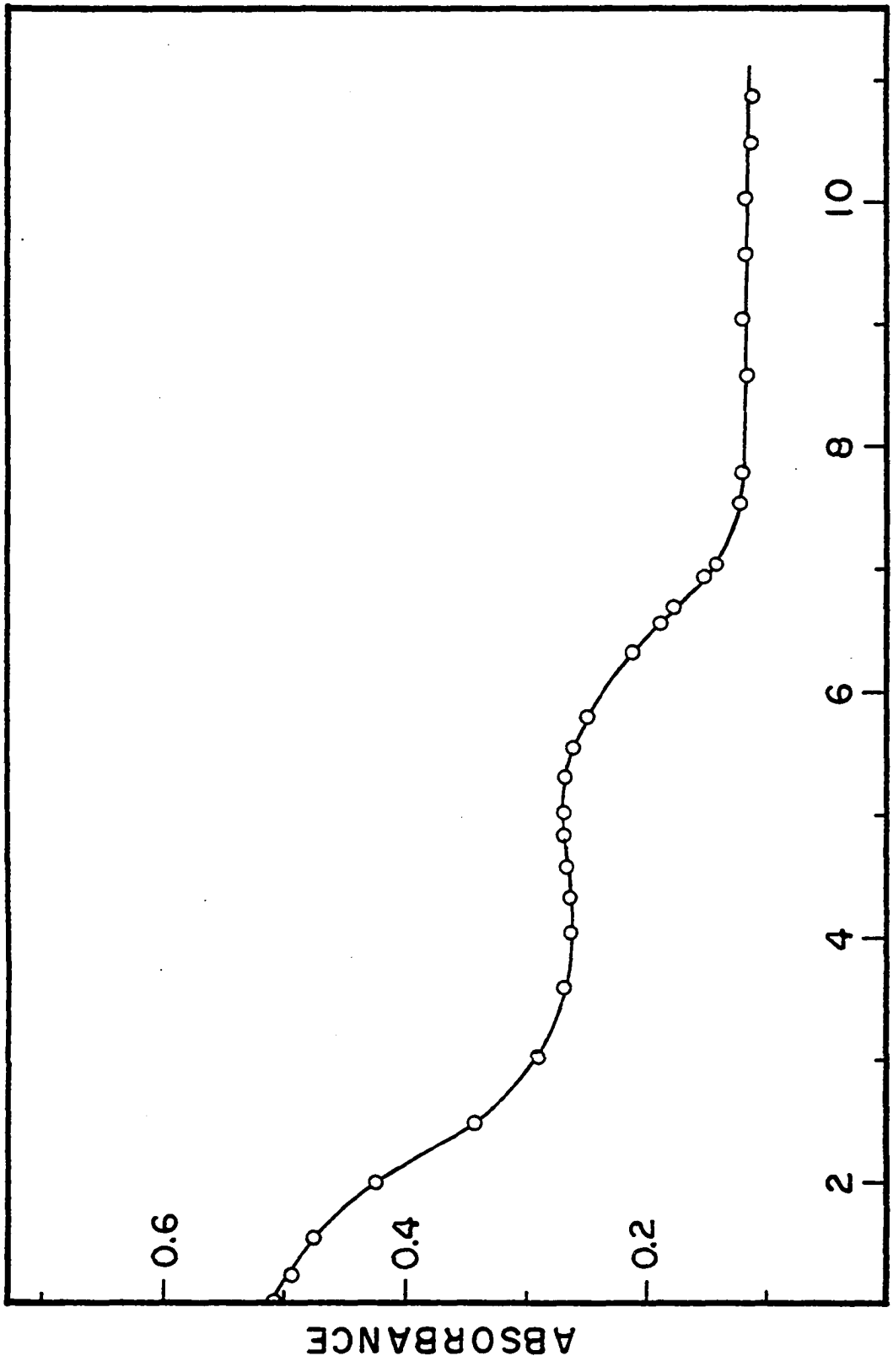

ABSORBANCE
0.6
0.4
0.2
2
4
6
8
10
pH

Figure 32. Absorbance of 6-sulfofluorescein, 5.0×10^{-6} M, as a function of pH, at a wavelength of 443 nm.


ABSORBANCE
0.6
0.4
0.2
2
4
6
8
10
pH

and 34. The two curves are almost identical. The absorbance is minimal at pH less than 2, increases gradually beyond pH 2 and up to pH 5.5, at which a point of inflection is observed. Finally, it rises most rapidly between pH 6 and 7, and reaches a maximum and essentially constant value at pH 8 and above.

Above pH 5.5, the rapid increase in absorbance is due to the formation of the trianion, Sfl^{3-}, thus as explained above, in the graphical evaluation of $pK_{HSfl}{}^{2-}$ of the β-isomers, the straight lines (b) in Figures 36 and 38 will be used. The mid-points of the absorption curves for 5- and 6-sulfofluorescein occur at pH 6.3 and correspond to $pK_{HSfl}{}^{2-}$.

The graphical method was employed to obtain the acid dissociation constants of 5-sulfofluorescein, a plot of pH *vs.* $\log \left(\frac{A_1 - A_m}{A_m - A_2}\right)$ as defined in equation (3). The value of pK_{H_3Sfl}, determined by the intercept in Figure 35, at a wavelength value of 443 nm, is 2.19. This value agrees well with the value of pK_{H_3Sfl} obtained by solubility measurement (2.13) as a function of pH. The value of $pK_{HSfl}{}^{2-}$ determined at 496 nm by the intercept of line b, Figure 36, is 6.13. This value is fairly close to the value of $pK_{HSfl}{}^{2-}$ (6.22) determined by fluorescence measurement in Chapter VI.

Figure 33. Absorbance of 5-sulfofluorescein, 5.0×10^{-6} M, as a function of pH at a wavelength of 496 nm.

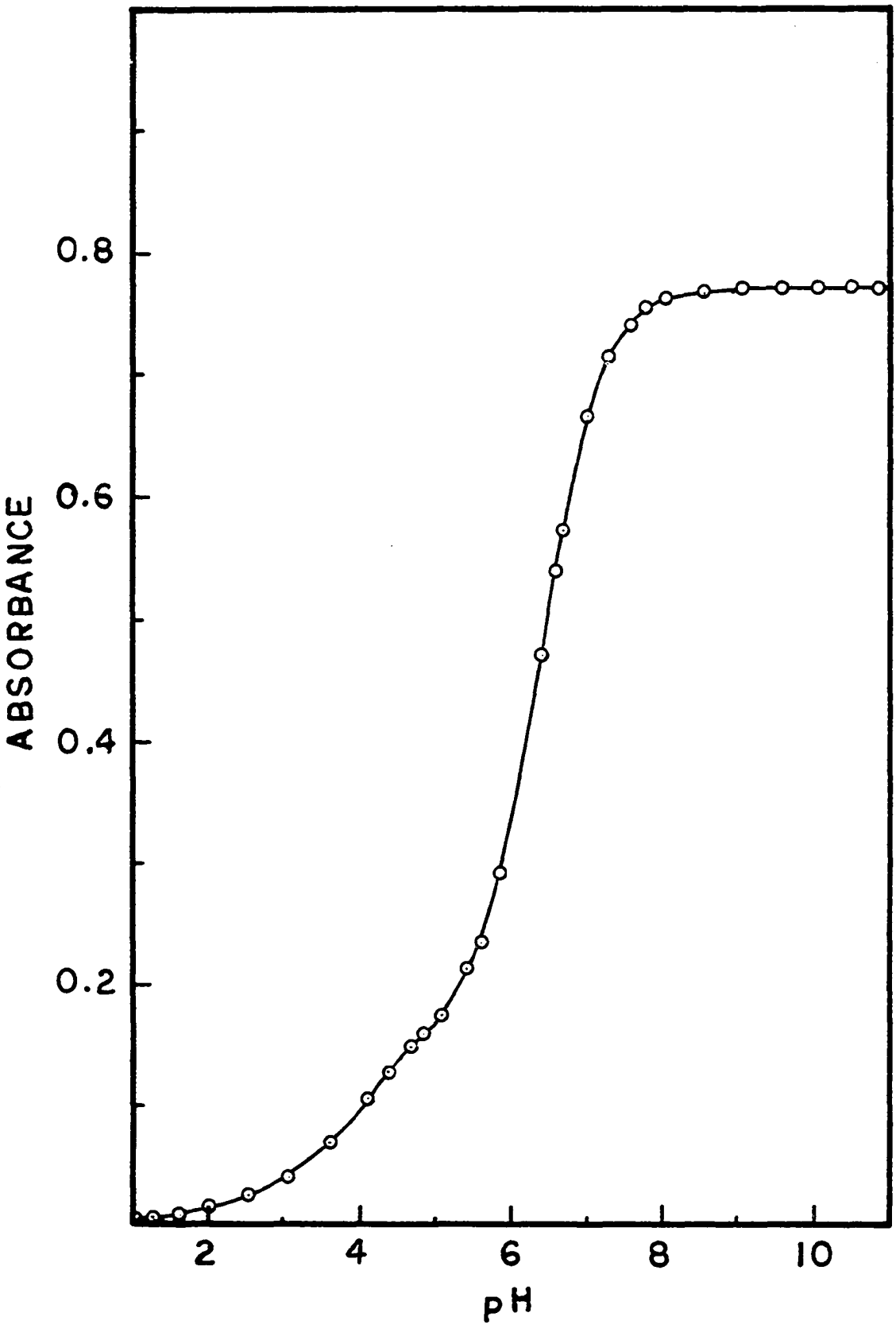

ABSORBANCE
0.8
0.6
0.4
0.2
2
4
6
8
10
pH

Figure 34. Absorption of 6-sulfofluorescein, 5.0×10^{-6} M, as a function of pH at a wavelength of 496 nm.

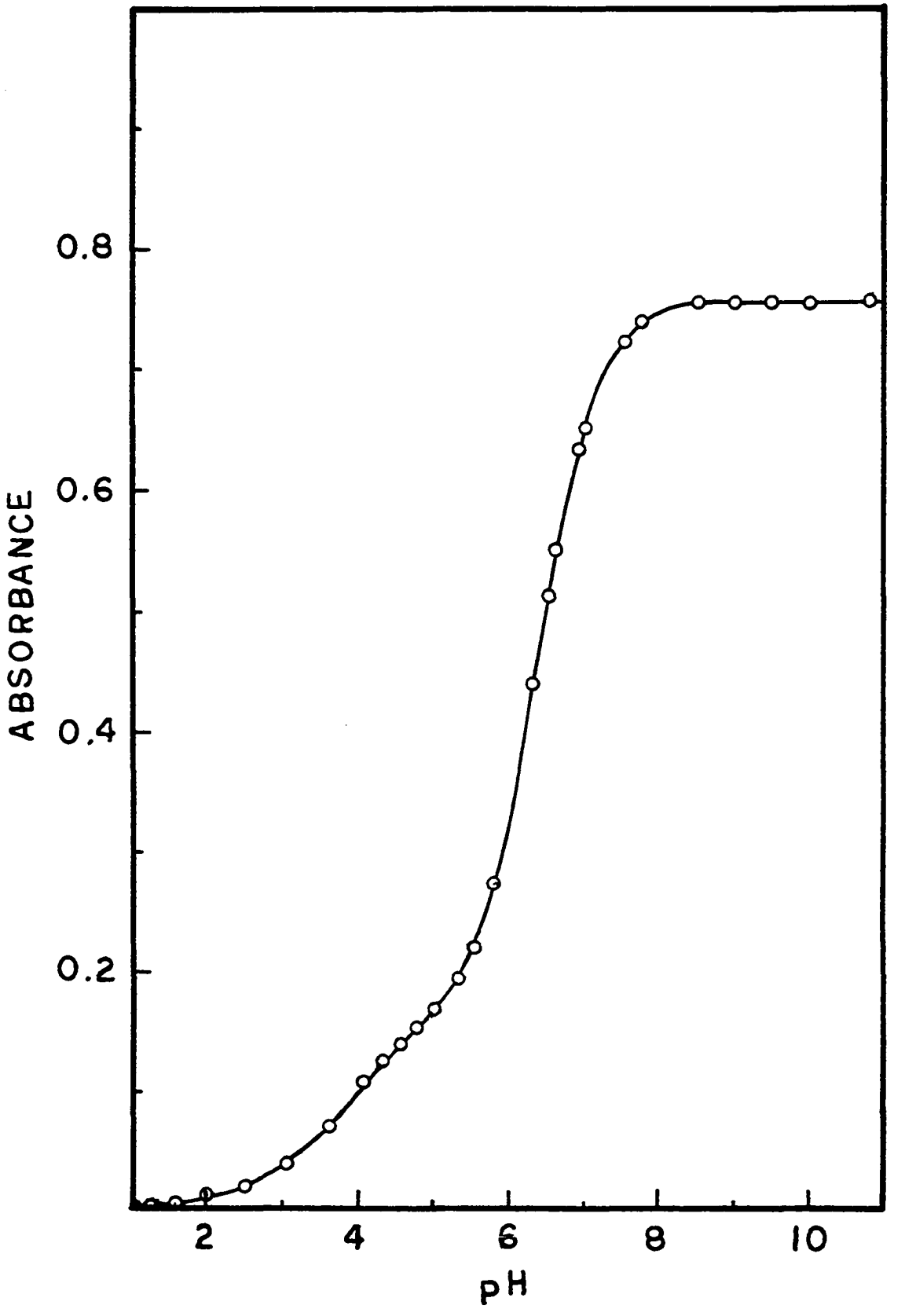

ABSORBANCE
0.8
0.6
0.4
0.2
2
4
6
8
10
pH

Figure 35. Mathematical treatment of the absorbance of 5-sulfofluorescein at 443 nm.

$$\log \left(\frac{A_1 - A_m}{A_m - A_2}\right) \text{ vs. pH}$$

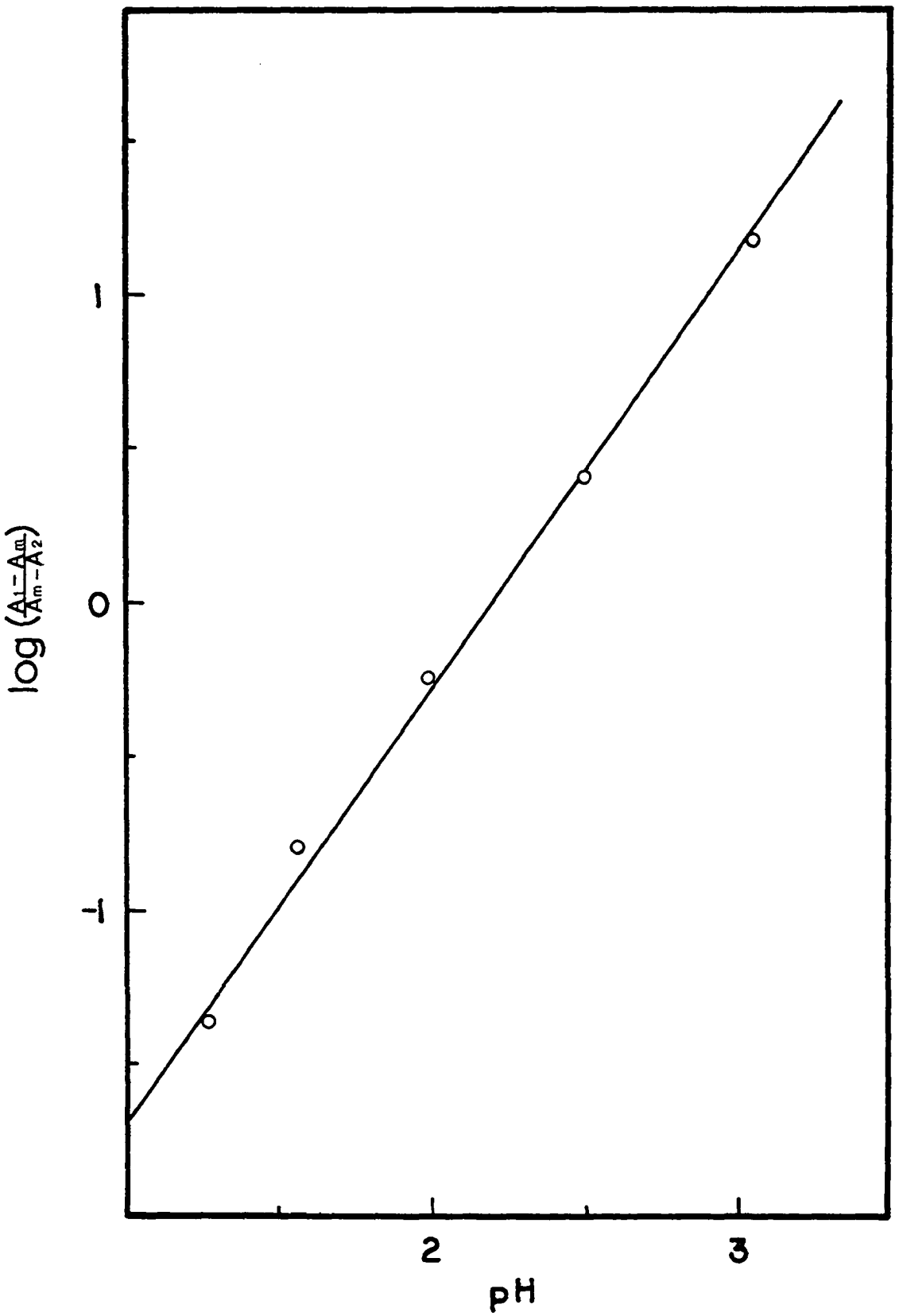
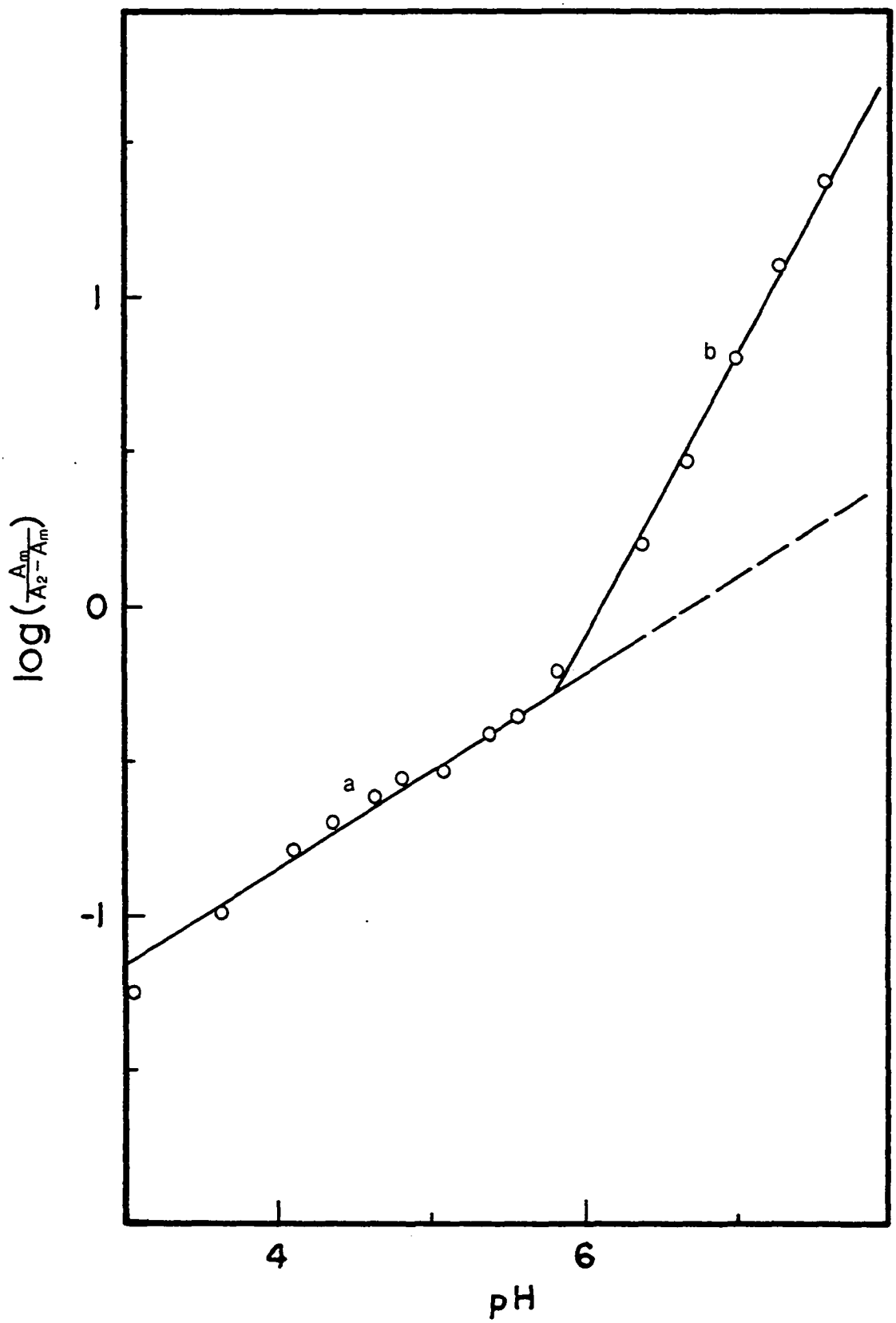

1
0
-1
log $(\frac{A_1-A_m}{A_m-A_2})$
2
3
pH

Figure 36. Mathematical treatment of the absorbance of 5-sulfofluorescein at 496 nm.

$$\log \left(\frac{A_m}{A_2 - A_m}\right) \text{ vs. pH}$$


1
0
-1
log ($\frac{A_m}{A_2 - A_m}$)
a
b
4
6
pH

The intercepts and slopes which were calculated using the least-squares method and the experimental points at 443 and 496 nm are presented in Tables 9 and 10, respectively.

Table 9. Absorbance of 5-sulfofluorescein as a function of pH at 443 nm

pH	A_m	$\log \left(\frac{A_m - A_1}{A_2 - A_m}\right)$	$\mu = 0.1$
1.26	0.509	-1.372	$A_1 = A_{H_3Sfl} = 0.520$
1.57	0.483	-0.799	$A_2 = A_{H_2Sfl^-} = 0.250$
1.99	0.423	-0.251	slope = 0.72
2.49	0.327	0.499	intercept (log term = 0) = 2.19
3.05	0.267	1.173	

Table 10. Absorbance of 5-sulfofluorescein as a function of pH at 496 nm

pH	A_m	$\log \left(\frac{A_m}{A_2 - A_m}\right)$
3.05	0.041	-1.250
3.62	0.072	-0.987
4.10	0.108	-0.787

Table 10. (Continued)

pH	A_m	$\log \left(\frac{A_m}{A_2 - A_m}\right)$	
4.37	0.128	-0.700	μ = 0.1
4.64	0.149	-0.620	$A_2 = A_{Sfl^{3-}} = 0.770$
4.82	0.158	-0.558	slope = 3.19
5.08	0.176	-0.528	
5.38	0.214	-0.415	
5.57	0.235	-0.357	
5.83	0.290	-0.219	slope = 1.09
6.39	0.471	0.197	intercept (log term = 0) = 6.13
6.68	0.572	0.461	
7.00	0.664	0.797	
7.30	0.712	1.089	
7.59	0.738	1.363	

Again, in the evaluation of the acid dissociation constants of 6-sulfofluorescein, the value of pK_{H_3Sfl} (2.20) is determined by the intercept of the plot pH *vs.* $\log\left(\frac{A_1 - A_m}{A_m - A_2}\right)$

as defined in equation (3), Figure 37. The value (2.20) determined by absorbance measurement at 443 nm, is in good agreement with the value (2.14) determined by solubility measurements as a function of pH. The value of pK_{HSfl}^{2-} (6.16) determined at 496 nm by the intercept of line b, Figure 38, is in agreement with that determined by fluorescence measurements (6.25) reported in Chapter VI.

The intercepts and slopes which were calculated by the least-squares method, and the experimental points at 443 and 496 nm are presented in Tables 11 and 12, respectively.

Table 11. Absorbance of 6-sulfofluorescein as a function of pH at 443 nm

pH	A_m	$\log \left(\frac{A_m - A_1}{A_2 - A_m}\right)$	
1.26	0.496	-1.180	$\mu = 0.1$
1.57	0.478	-0.806	$A_1 = A_{H_3Sfl} = 0.511$
2.00	0.426	-0.272	$A_2 = A_{H_2Sfl^-} = 0.267$
2.50	0.343	0.344	slope = 0.90
3.05	0.291	0.962	intercept (log term = 0) = 2.20

Table 12. Absorbance of 6-sulfofluorescein as a function of pH at 496 nm

pH	A_m	$\log \left(\frac{A_m}{A_2 - A_m}\right)$	
4.08	0.107	-0.786	$\mu = 0.1$
4.36	0.128	-0.694	$A_2 = A_{Sfl3-} = 0.760$
4.63	0.146	-0.624	slope = 3.93
4.81	0.155	-0.591	
5.06	0.169	-0.544	
5.37	0.196	-0.459	
5.56	0.220	-0.390	
5.82	0.273	-0.251	slope = 1.14
6.34	0.440	-0.138	intercept (log term = 0) = 6.16
6.56	0.513	0.317	
6.68	0.550	0.418	
6.98	0.635	0.706	
7.58	0.721	1.27	

Figure 37. Mathematical treatment of the absorbance of 6-sulfofluorescein at 443 nm.

$$\log \left(\frac{A_1 - A_m}{A_m - A_2}\right) \text{ vs. pH}$$

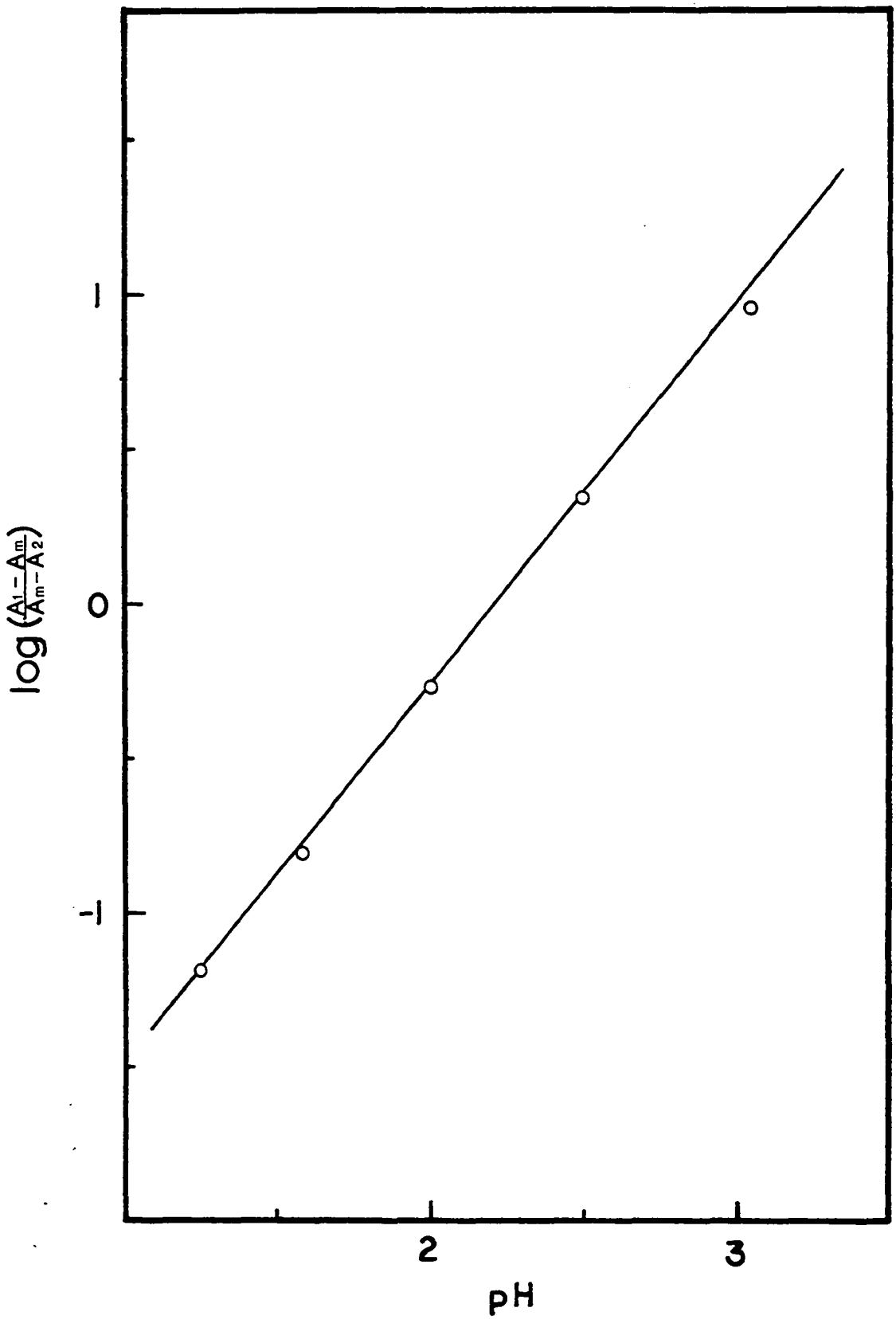
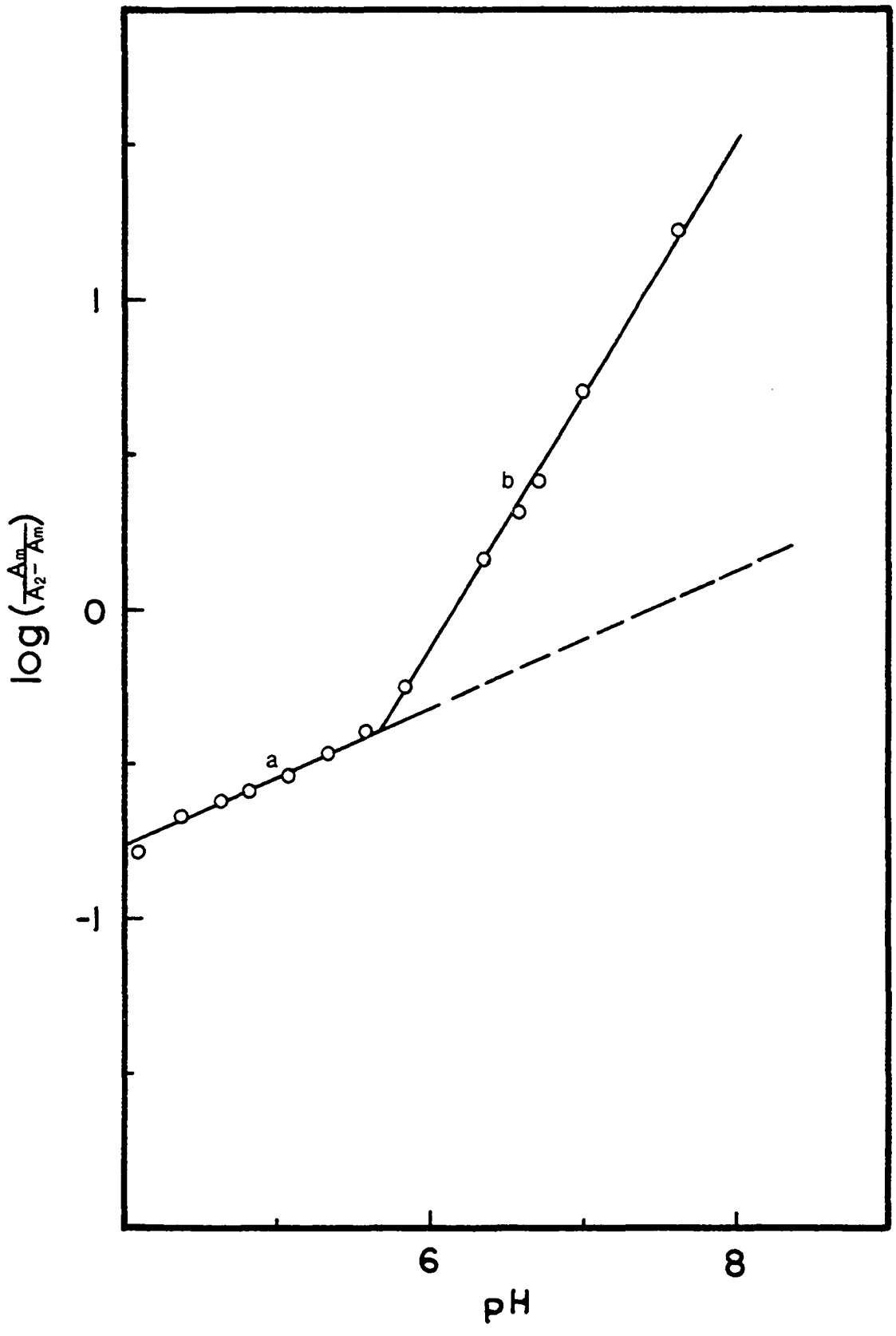

1
0
-1
log $(\frac{A_1 - A_m}{A_m - A_2})$
2
3
pH

Figure 38. Mathematical treatment of the absorbance of 6-sulfofluorescein at 496 nm.

$$\log \left(\frac{A_m}{A_2 - A_m}\right) \text{ vs. pH}$$


log ($\frac{A_m}{A_2 - A_m}$)
1
0
-1
a
b
6
8
pH

D. Results and Discussion

The absorption spectra of fluorescein and of 4-, 5-, and 6-sulfofluorescein in aqueous solution at various values of pH are presented in Figures 17, 18, 19, and 20, respectively. The absorption bands are also tabulated, Table 4. These absorption curves are very similar and this is interpreted to mean that prototropic forms of the sulfofluoresceins are similar to those of fluorescein. Because of the presence of the sulfonate group, the prototropic forms of the three sulfofluoresceins carry one more negative charge than the corresponding prototropic form of fluorescein.

I have proposed the following: fluorescein at pH ≈ 1.0 exists as the monocation, H_3Fl^+, and assumes the zwitter ion form, with a maximum absorption band at 498 nm. In the basic region, pH $\geq$ 8.0, fluorescein exists as the dianion, Fl^{2-}, and assumes the quinone form, with a maximum absorption band at 491 nm. At about pH 5.0 the monoanion of fluorescein, HFl^-, predominates with two maxima, at 455 and 476 nm, which are attributed to the zwitter ion and the quinone forms, respectively. Finally, at about pH 3.0 the neutral molecule of fluorescein, H_2Fl, predominates with two absorption bands, a maximum at 440 nm attributed to the zwitter ion and a shoulder centered at 475 attributed to the quinone form.

The absorption curves of 5- and 6-sulfofluorescein in aqueous solution at various values of pH are presented in Figures 19 and 20, and the absorption bands are tabulated in Table 4. On the basis of the acid dissociation constants evaluated in the present work and the distribution curves of the different species of 5- and 6-sulfofluorescein as a function of pH, the following conclusions are drawn.

At about pH 1.0 the neutral molecule, H_3Sfl, of 5- and 6-sulfofluorescein predominates and has an absorption band corresponding to that of the monocation, H_3Fl^+, of fluorescein. At pH ≈ 3.0 the monoanion, H_2Sfl^-, predominates and has absorption bands corresponding to those of the neutral molecule, H_2Fl, of fluorescein. At pH ≈ 5.0 the dianion, $HSfl^{2-}$, predominates and has absorption bands corresponding to those of the monoanion, HFl^-, of fluorescein. Finally, at pH ≈ 8.0 the trianion, Sfl^{3-}, predominates and has an absorption band corresponding to that of the dianion, Fl^{2-}, of fluorescein.

Again, the absorption curves of 4-sulfofluorescein in aqueous solution at varying values of pH are presented in Figure 18, and the absorption bands are tabulated in Table 4. On the basis of the acid dissociation constants evaluated in this work, and the distribution curves of the different forms of 4-sulfofluorescein as a function of pH, the following conclusions have been drawn.

At pH ≈ 1.0 the neutral, H_3Sfl, and the monoanion, H_2Sfl^-, of 4-sulfofluorescein and one absorption band is observed at 446 nm, corresponds to that of the monocation, H_3Fl^+, of fluorescein, which exists as the zwitter ion. The low absorption band of 4-sulfofluorescein at pH 1.01 could be explained by pointing out that at about pH 3.0, the point at which the monoanion, H_2Sfl^-, predominates the solution is practically colorless. Thus, the monoanion of 4-sulfofluorescein exists predominantly in the lactone form. And in turn at pH 1.01 only the neutral molecule, H_3Sfl, absorbs. At pH ≈ 5.0 the monoanion, H_2Sfl^-, and the dianion, $HSfl^{2-}$, predominate, and because the monoanion is colorless, the absorption bands at 462 and 487 nm are of the dianion of 4-sulfofluorescein corresponding to those of the monoanion of fluorescein, which assumes the zwitter ion and the quinone forms. At pH ≈ 8.0 the trianion, Sfl^{3-}, of 4-sulfofluorescein predominates and has an absorption band at 498 nm corresponding to that of the dianion of fluorescein, which exist predominantly as the quinone.

Markuszewski had proposed that the monocation and the neutral molecule of fluorescein in aqueous solution assume exclusively the zwitter ion form; the monoanion assumes the zwitter ion and the quinone forms; the dianion exists predominantly as the quinone form. He excluded the presence of the lactone form in water. My proposal modifies that of

Markuszewski's in two aspects. First, the lactone form does exist in aqueous solution, especially at about pH 3.0, at which point the neutral molecule of fluorescein predominates. Second, in the pH region 1.0 to 8.0 the three forms, namely the zwitter ion, the lactone and the quinone exist in an equilibrium, and this equilibrium is dependent on the pH of the solution. These modifications are based on the absorbance study presented above and other lines of evidence to be offered later in this dissertation.

In summary, my proposals that the prototropic forms of fluorescein in aqueous solution are as follows: the monocation is present as the zwitter ion form; the neutral molecule exists mainly as the lactone and to a certain extent as the zwitter ion and a small fraction as the quinone form; the monoanion exists as the zwitter ion but a considerable fraction as the quinone; the dianion exists predominantly as the quinone.

The same equilibria are applicable to the prototropic forms of 5- and 6-sulfofluorescein. The neutral molecule exists predominantly as the zwitter ion; the monoanion is present as the lactone, the zwitter ion and a small fraction as the quinone form; the dianion exists as the zwitter ion and a considerable fraction as the quinone; the trianion exists predominantly as the quinone.

With respect to 4-sulfofluorescein, the presence of the lactone is realized the most, and the prototropic forms of

this isomer are as follows: the neutral molecule exists predominantly as the zwitter ion; the colorless monoanion exists predominantly as the lactone; the dianion is present as the zwitter ion and as the quinone form; the trianion exists predominantly as the quinone.

Examination of the absorption curves and the absorption bands tabulated in Table 5 reveals that the presence of the sulfonate group has two effects on the absorbance of fluorescein. First, a bathochromic shift ranging from 5 to 10 nm; second, the formation of the colorless lactone becomes most favored in the case of 4-sulfofluorescein, Structure VII, with a sulfonate group in the ortho position with respect to the carboxylic acid group. The readiness of the monoanion, H_2Sfl^-, of this isomer to form the lactone is attributed to the high negative charge density caused by the adjacent sulfonate and carboxylate groups, causing the carboxylate group to close and form the lactone ring.

The readiness of a particular isomer to form the lactone in solution seems to have a tremendous effect on its acid-base properties. The first acid dissociation constant for fluorescein, $pK_{H_3Fl^+} = 2.15$; for 4-sulfofluorescein $pK_{H_3Sfl} = 1.18$, for 5-sulfofluorescein $pK_{H_3Sfl} = 2.19$, and for 6-sulfofluorescein = 2.20. The agreement between fluorescein and the β-isomers of sulfofluorescein is very good, and these pK values are in the range expected for a

carboxylic acid in the ortho position to an electowithdrawing group. By analogy, salicilic acid, with a hydroxyl group in the ortho position with respect to the carboxylic acid group, has a pK value of 2.89, phthalic acid with a carboxyl group in the ortho position has a pK value of 2.80.

The first acid dissociation constant for 4-sulfofluorescein stands different and approximately 10 times larger than that of fluorescein and of the β-isomers. This difference is explained by examination of the equilibria involved in the dissociation of the acid and the formation of the lactone;

$$H_3Sfl \text{ (zwitter ion)} \rightleftharpoons H_2Sfl^- \text{ (zwitter ion)} + H^+$$
$$\Downarrow\Uparrow$$
$$H_2Sfl^- \text{ (lactone)}$$

By the application of the fundamental equilibria principles, as the formation of the lactone, H_2Sfl^-, is more favored, the dissociation of the acid, H_3Sfl, will proceed further to the right, and the net effect is a larger acid dissociation constant. The absorbance curves of 4-sulfofluorescein, Figures 18 and 25, as indicated above confirm that this isomer favors the formation of the colorless lactone much more than fluorescein and the β-isomers of sulfofluorescein.

The argument that $pK_{H_3Sfl} = 1.18$ should be attributed to the dissociation of the sulfonic acid rather than the carboxylic acid could be refuted by the following: the fact

that the neutral molecule of 4-sulfofluorescein is present in solution as the zwitter ion, the acid dissociation constant of the sulfonic acid would be very large, $pK \leq 0$, especially in the presence of electrowithdrawing groups on the phthalate ring, such as the carboxylic and the pyrylium-type ring. Second, since the carboxylic acids are weaker than the sulfonic acid, the proton is more likely to associate with the carboxylic group rather than the sulfonate group.

Further effects, due to the formation of the lactone, on the chemistry in solution of fluorescein and of the three sulfofluorescein, will be encountered and discussed later in this dissertation.

VI. FLUORESCENCE OF THE SULFOFLUORESCEINS

A. Apparatus and Procedure

The excitation and emission spectra of 4-, 5-, and 6-sulfofluorescein at pH 9.4 were obtained on an Aminco-Bowman Spectrophotofluorometer equipped with a Hewlett-Packard X-Y Recorder. The fluorescence excitation and emission spectra of 4-, 5-, and 6-sulfofluorescein are shown in Figures 39, 42 and 45, respectively. The maxima found in these spectra are essentially all at the same wavelengths and these wavelengths were used for setting the monochromators in the more detailed studies of the measurements of the fluorescence as functions of pH, that is, the excitation monochromator was set at about 490 nm and the emission monochromator at 520 nm.

Buffers for the region pH 1 to 11 were prepared at intervals of 0.25 to 0.50 units, as in the section on absorbance measurements, Chapter V, using 0.1 M solutions of hydrochloric acid, potassium hydrogen phthalate, boric acid in 0.1 M potassium chloride, potassium hydroxide, and potassium chloride; the ionic strength was maintained at $\mu = 0.1$. The pH of the buffer solutions was measured before and after the addition of the sulfofluorescein, using a Hach Model 8594 pH meter, set on the expanded scale, and a high-alkalinity Beckman glass electrode and a saturated calomel reference electrode.

A stock solution, 5.0×10^{-4} M, of the compound under investigation, was prepared by dissolving the appropriate amount of the compound in about 30 ml of 0.1 M potassium hydroxide and diluting with deionized water to one liter. A 1.00-ml aliquot of the stock solution was placed in a 100-ml volumetric flask and diluted to the mark with the appropriate buffer. The final concentration of the solution was brought to 5.0×10^{-6} M. The intensity of the fluorescence of each of these buffered solutions was then measured on an Aminco-Bowman Spectrofluorometer. The data, relative fluorescence of 4-, 5- and 6-sulfofluorescein as a function of pH, are presented in Figures 40, 43 and 46, respectively, and the mathematical treatment of the data is given in Figures 41, 44 and 47, respectively.

B. Introduction

The intensity and the changes in intensity with pH of the relative fluorescence of fluorescein, and of each of the three isomers of sulfofluorescein studied in the present work are very similar. The fluorescence of each compound is very low at pH below 3, increases slowly between pH 3 and 5, rises rapidly between pH 6 and 7, reaches a maximum at pH about 8.0, and remains constant at all pH greater than 8.0.

The third acid dissociation constant can be obtained from the data shown in Figures 40, 43 and 46; the point of

inflection corresponds to the negative logarithm of the dissociation constant, pK_3. The value can be estimated by inspection but is better obtained graphically. The graphical evaluation is based on a formula relating relative fluorescence and acid dissociation constant similar to that used to obtain dissociation constants from absorbence data, Chapter V.

The fundamental chemical and mathematical equations are

$$HSfl^{2-} = Sfl^{3-} + H^+ \qquad K_{HSfl^{2-}} = \frac{[Sfl^{3-}][H^+]}{[HSfl^{2-}]} \tag{4}$$

Rearrangement of equation (4) gives

$$pH = pK_{HSfl^{2-}} + \log \frac{[Sfl^{3-}]}{[HSfl^{2-}]} \tag{5}$$

Examination of the absorption spectra of the various isomeric sulfofluoresceins, Figures 18, 19 and 20, reveals that the absorption of all species at 495 nm is small except for the trianion, Sfl^{3-}. Because the intensity of fluorescence is directly proportional to the intensity of light absorbed, it can be assumed that the fluorescence is a measure of the concentration of the trianion. Thus, equation (5) becomes

$$pH = pK_{HSfl^{2-}} + \log \left(\frac{F_m}{F_t - F_m}\right) \tag{6}$$

in which F_t is the fluorescence of the trianion Sfl^{3-}, that is, the fluorescence at pH greater than 8.0 at which this

fluorescence reaches a constant value and no longer increases with pH, and F_m is the fluorescence of any solution along the curve. A plot of pH versus the log term in equation (6) is then used to obtain the acid dissociation constant, that is, pH = $pK_{HSfl^{2-}}$ at the point of intercept at which the log term becomes zero.

Equation (6) is most valid, that is, the fluorescence of the dianion, $HSfl^{2-}$, becomes negligible, at pH greater than 5.5, at which region the concentration of the trianion rises rapidly to become the predominant species, while the concentration of dianion decreases rapidly. The fluorescence of the dianion at pH less than 5.5 cannot be neglected, and this fact will become apparent in the following section, upon examining Figures 44 and 47, which represent the mathematical treatment of fluorescence as a function of pH.

The data are best handled by the least-squares method, both the slope and the intercept being obtained. The data and mathematical treatment and results are given in Tables 13, 14 and 15, and Figures 41, 44 and 47, for the respective sulfofluoresceins.

C. Fluorescence of 4-Sulfofluorescein as a Function of pH

The fluorescence excitation and emission spectra of 4-sulfofluorescein in solution of pH 9.4 are shown in Figure

39. The maxima in these spectra occur at 495 nm and 520 nm, respectively.

The more detailed measurements of fluorescence, at small intervals of pH of over the range 1 to 11, were made with the excitation monochromator set at 495 nm and the emission monochromator set at 520 nm. The data obtained are presented in Table 13 and shown graphically in Figure 40. The plot of the mathematical function developed above is given in Figure 41.

The value obtained for the third dissociation constant is 6.24, a value in good agreement with that, 6.21, determined by measurement of absorbance at 498 nm, Chapter V, Part C, Section 4.

Table 13. Relative fluorescence of 4-sulfofluorescein as a function of pH

pH	F_m	$\log \left(\frac{F_m}{F_t - F_m}\right)$	
4.64	2.5	-1.571	$\mu = 0.1$
5.07	5.8	-1.189	$F_t = F_{Sfl^{3-}} = 95.5$
5.38	10.5	-0.908	slope = 0.99
5.58	16.0	-0.696	intercept = 6.24
5.83	26.5	-0.416	
6.38	55.5	0.142	

Table 13. (Continued)

pH	F_m	$\log \left(\frac{F_m}{F_t - F_m}\right)$
6.57	65.5	0.339
6.71	72.0	0.486
7.05	82.5	0.825

D. Fluorescence of 5-Sulfofluorescein as a Function of pH

The fluorescence excitation and emission spectra of 5-sulfofluorescein in solution of pH 9.4 are shown in Figure 42. The maxima in these spectra occur at 492 nm and at 520 nm, respectively.

The more detailed measurements of the intensity of fluorescence as a function of pH, at small intervals of pH, over the range 1 to 11, were made with the excitation monochromator set at 492 nm and the emission monochromator set at 520 nm. The data are presented in Table 14 and shown graphically in Figure 43. The plot of the mathematical function, developed in Part B, above, is given in Figure 44.

Figure 39. The excitation and emission spectra of 4-sulfofluorescein.

Concentration: 1.0×10^{-5} M; pH = 9.4

a. Excitation spectrum.

Emission monochromator set at 520 nm.

b. Emission spectrum.

Excitation monochromator set at 495 nm.

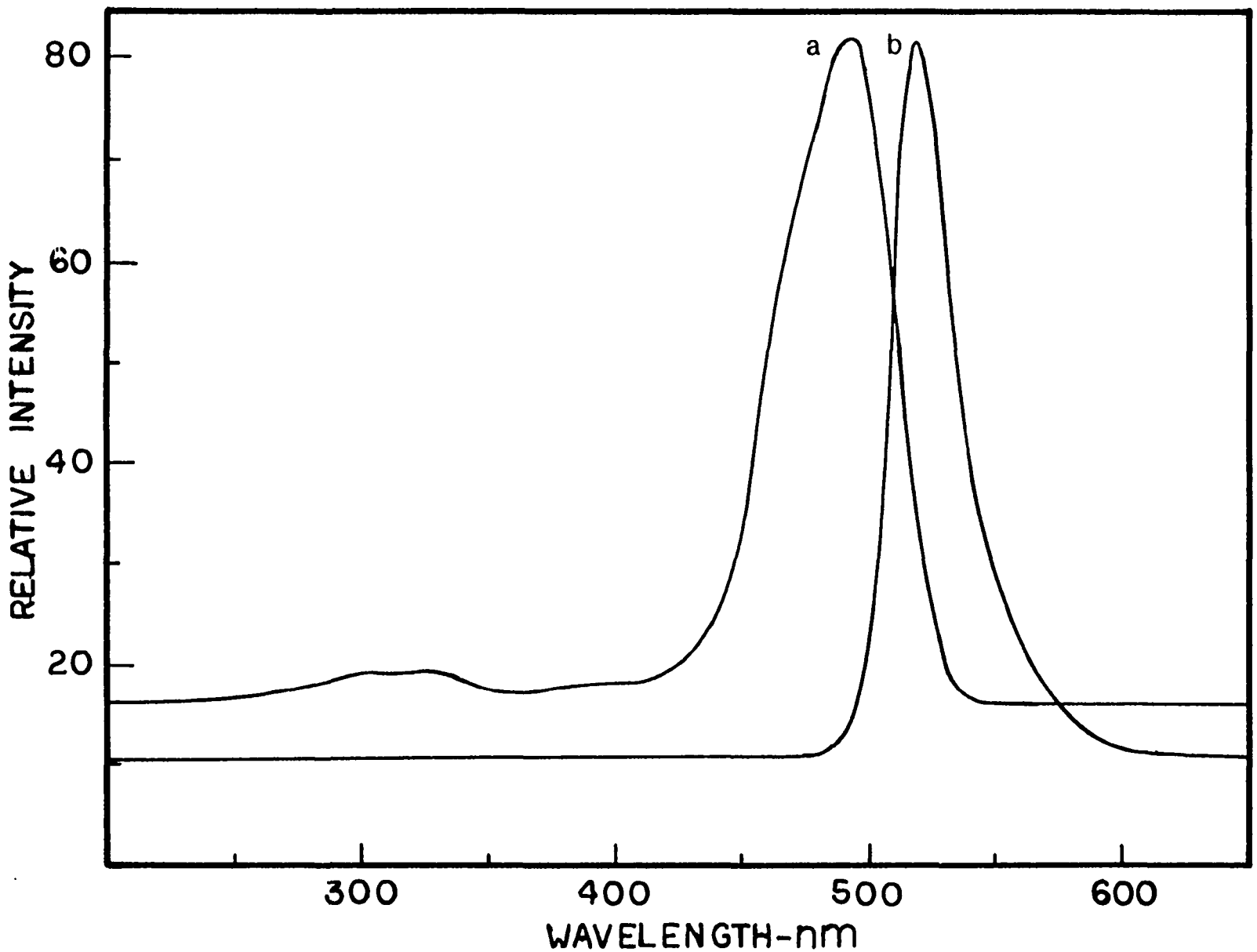

80
60
40
20
RELATIVE INTENSITY
a
b
300
400
500
600
WAVELENGTH-nm

Figure 40. Relative fluorescence of 4-sulfofluorescein as a function of pH.

Concentration: 5.0×10^{-6} M

Excitation monochromator set at 495 nm

Emission monochromator set at 520 nm

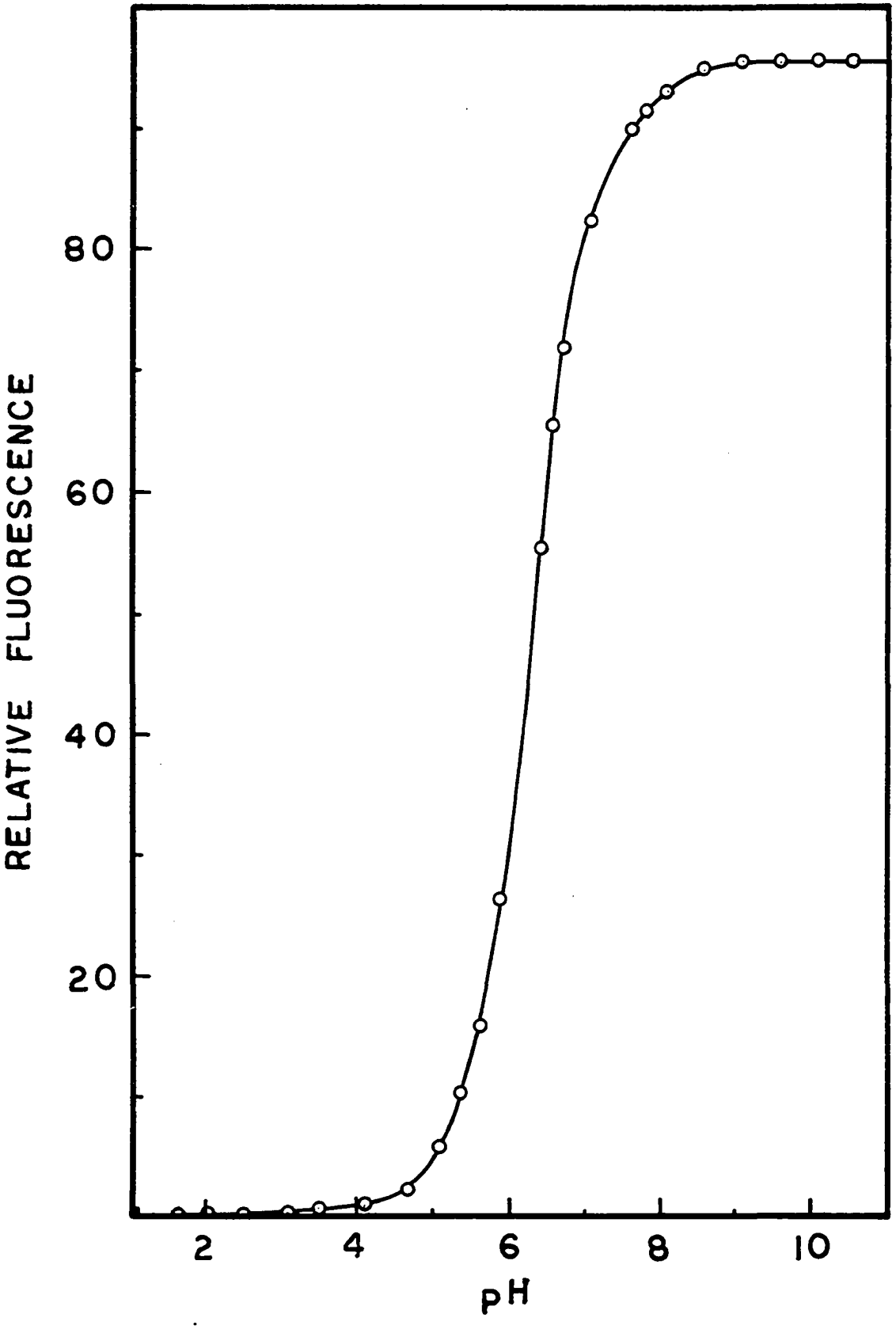

RELATIVE FLUORESCENCE
80
60
40
20
2
4
6
8
10
pH

Figure 41. Mathematical treatment of the fluorescence of 4-sulfofluorescein.

$$\log \left(\frac{F_m}{F_t - F_m}\right) \text{ vs. pH}$$

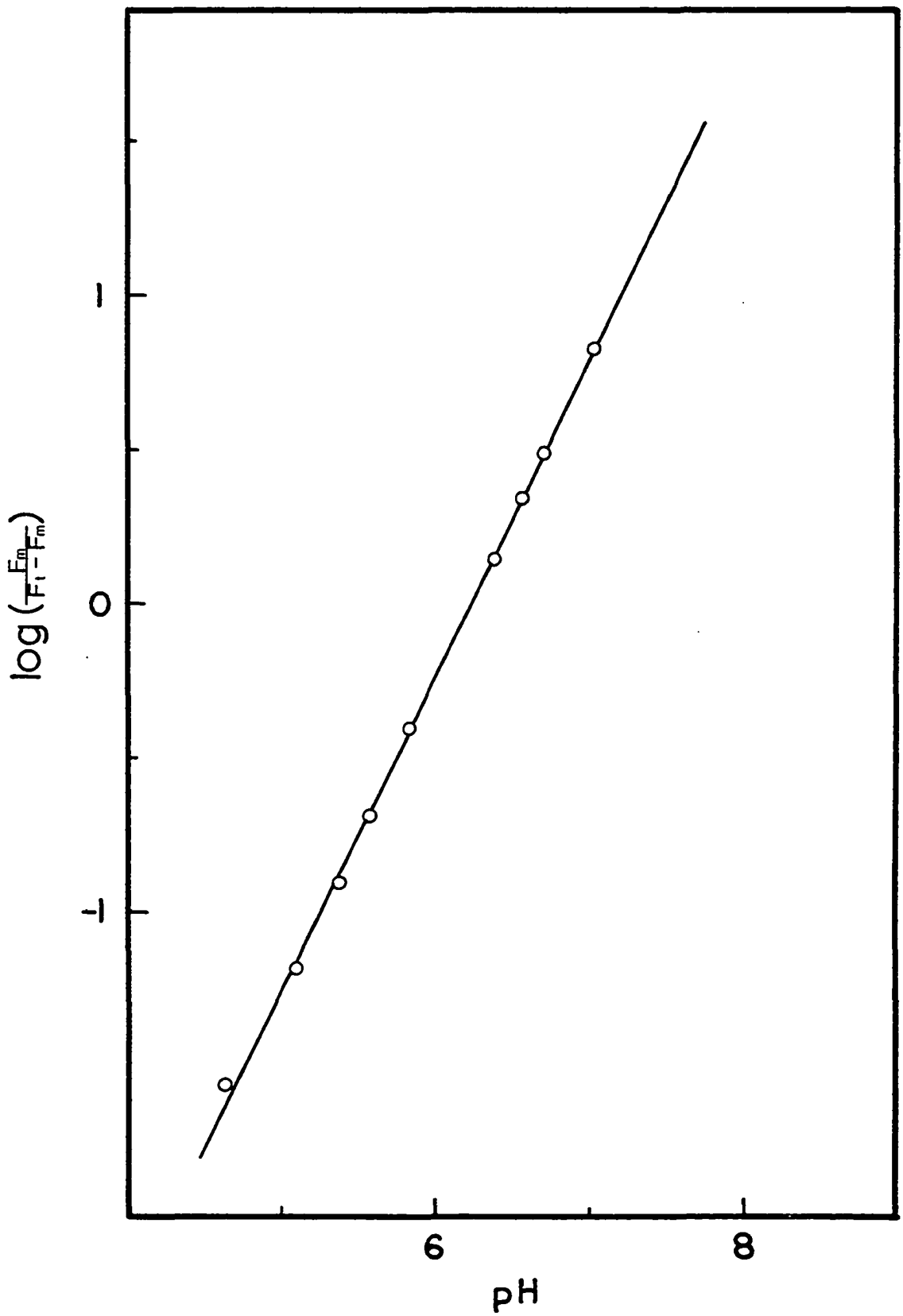

1
0
-1
log ($\frac{F_m}{F_t - F_m}$)
6
8
pH

Figure 42. Excitation and emission spectra of 5-sulfofluorescein.

Concentration: 1.0×10^{-5} M; pH = 9.4

a. Excitation spectrum.

Emission monochromator set at 520 nm.

b. Emission spectrum.

Excitation monochromator set at 492 nm.

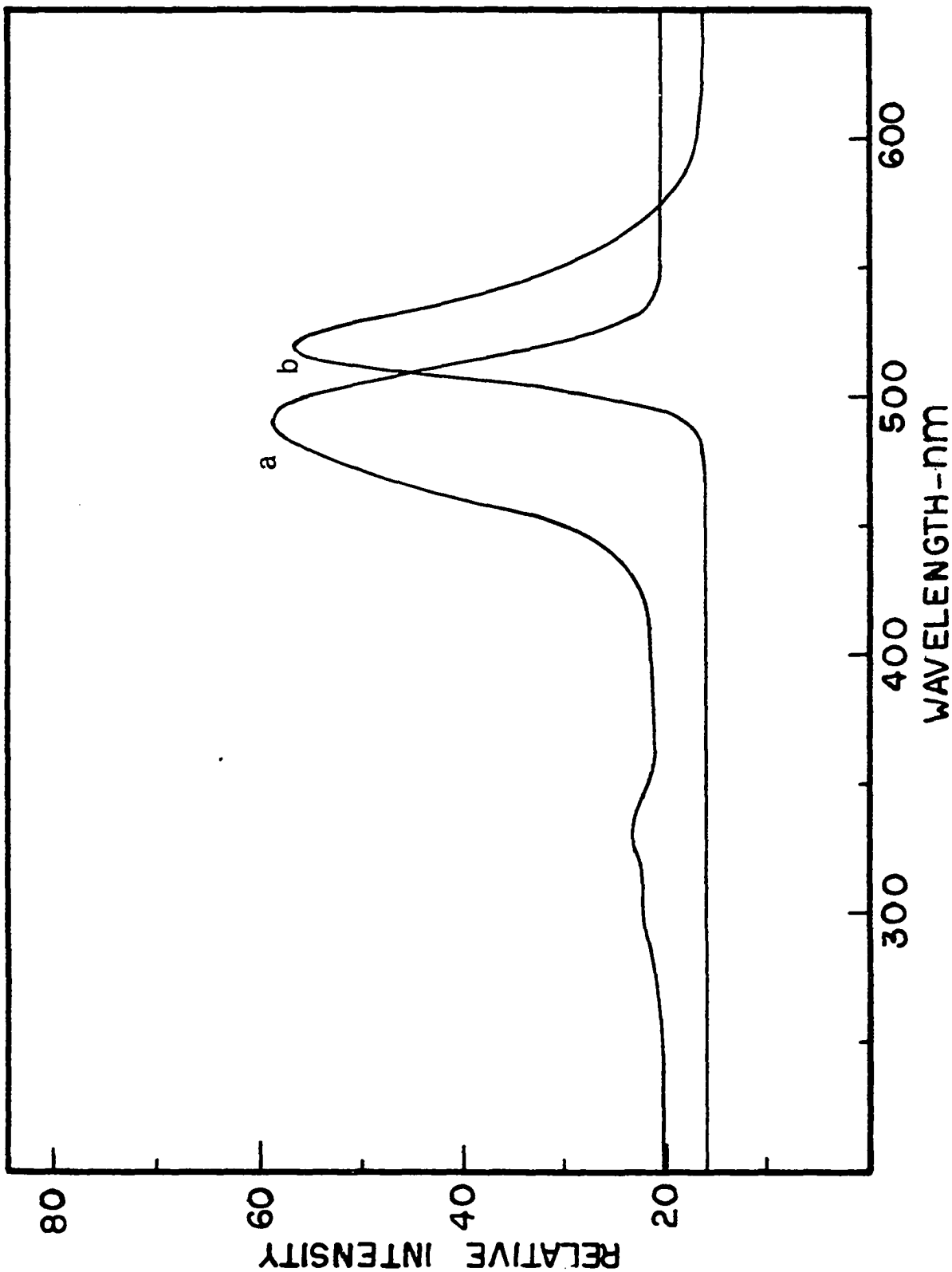

RELATIVE INTENSITY
80
60
40
20
300
400
500
600
WAVELENGTH-nm
a
b

Table 14. Relative fluorescence of 5-sulfofluorescein as a function of pH

pH	F_m	$\log \left(\frac{F_m}{F_t - F_m}\right)$	
3.05	1.0	-1.952	$\mu = 0.1$
3.62	2.0	-1.646	$F_t = F_{Sfl^{3-}} = 90.5$
4.10	3.3	-1.422	slope = 2.09
4.37	4.3	-1.302	
4.64	5.5	-1.189	
4.82	6.5	-1.111	
5.08	9.0	-0.957	
5.38	13.0	-0.775	
5.57	17.5	-0.620	
5.83	26.0	-0.395	
6.39	52.0	0.131	slope = 1.04
6.68	65.0	0.406	intercept (log term = 0) = 6.22
7.00	77.0	0.756	point of intersection = 5.35
7.30	83.0	1.044	
7.59	86.5	1.335	

Figure 43. Relative fluorescence of 5-sulfofluorescein as a function of pH.

Concentration: 5.0×10^{-6} M

Excitation monochromator set at 492 nm

Emission monochromator set at 520 nm

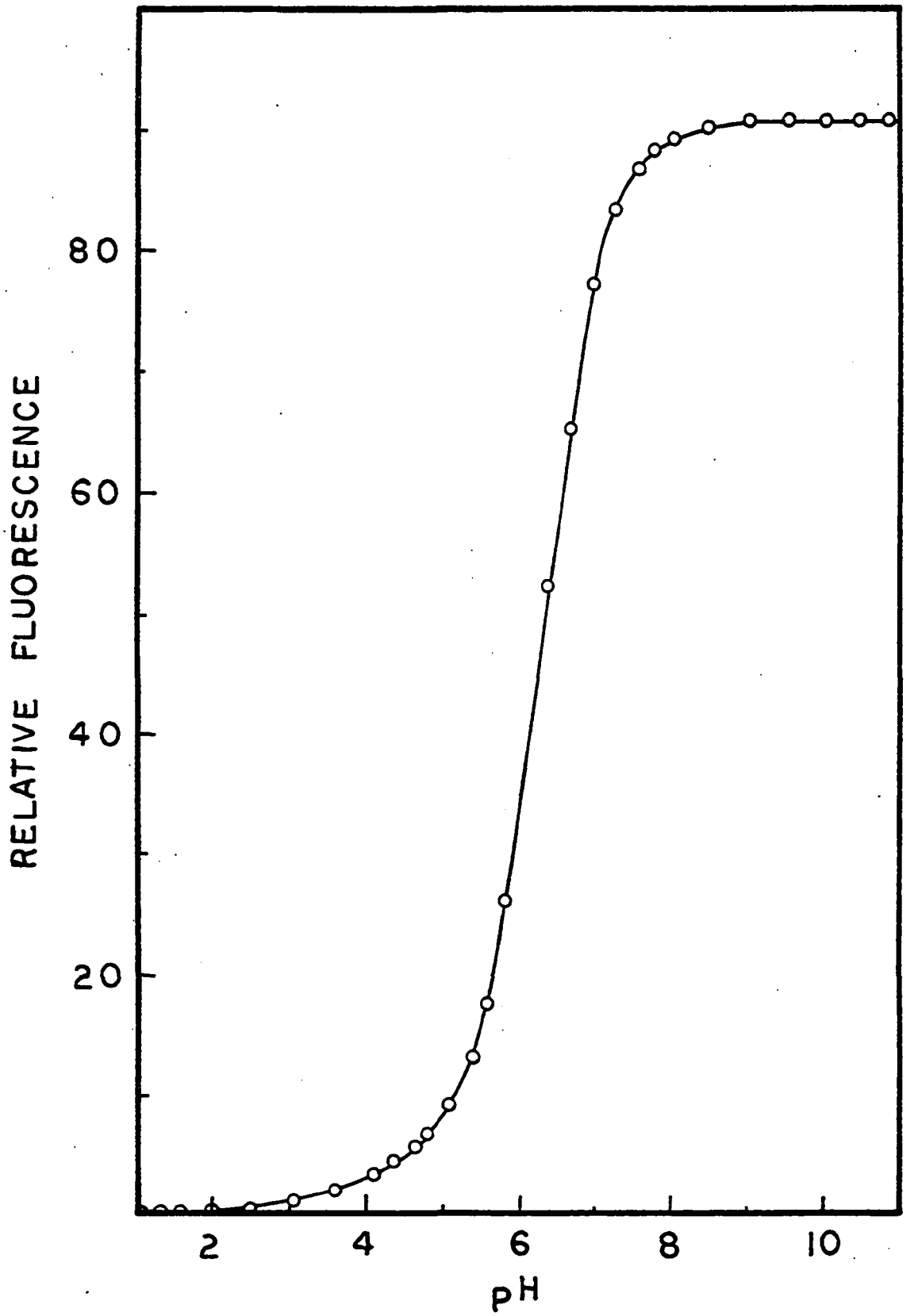
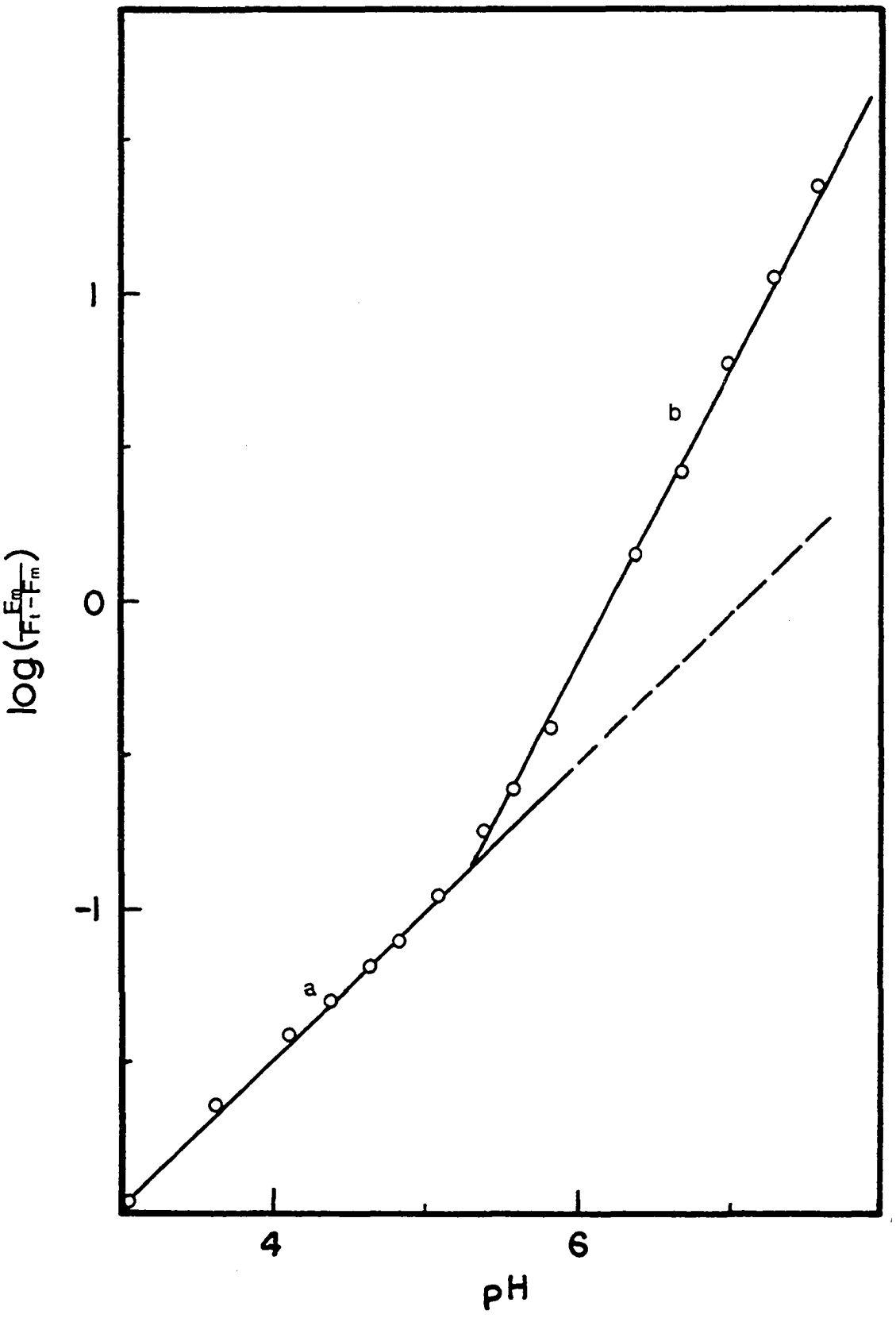

RELATIVE FLUORESCENCE
80
60
40
20
2
4
6
8
10
pH

Figure 44. Mathematical treatment of the fluorescence of 5-sulfofluorescein as a function of pH.

$$\log \left(\frac{F_m}{F_t - F_m}\right) \text{ vs. pH}$$


1
0
-1
log (Ft - Fm / Em)
a
b
4
6
pH

In Figure 44 there are two lines intersecting at pH = 5.35; line (a) represents mathematically the change in fluorescence of the dianion, $HSfl^{2-}$, as a function of pH, while line (b) represents that of the trianion, Sfl^{3-}. Ideally, if the fluorescence of the dianion is negligible over the entire pH range, a plot of pH vs. log $(\frac{F_m}{F_t - F_m})$ in Figure 44 is expected to give one straight line and a slope of 1.0, obviously this is not the case here, for the slope of line (a) is 2.09 while that of line (b) is 1.04. The intercept of line (b) is used to obtain $pK_{HSfl^{2-}} = 6.22$, for the reasons stated above.

At the point of intersection, at pH 5.35, the concentration of H_2Sfl^{2-} is maximum and the relation holds:

$$pH = \frac{pK_{H_2Sfl^-} + pK_{HSfl^{2-}}}{2} \qquad (7)$$

Using the values $pK_{HSfl^{2-}} = 6.22$ and pH = 5.35, equation (7) yields $pK_{H_2Sfl^-} = 4.48$.

E. Fluorescence of 6-Sulfofluorescein as a Function of pH

The fluorescence excitation and emission spectra of 6-sulfofluorescein in solution of pH 9.4 are shown in Figure 45. The maxima in these spectra occur at 492 nm and 520 nm, respectively.

Figure 45. Excitation and emission spectra of 6-sulfofluorescein.

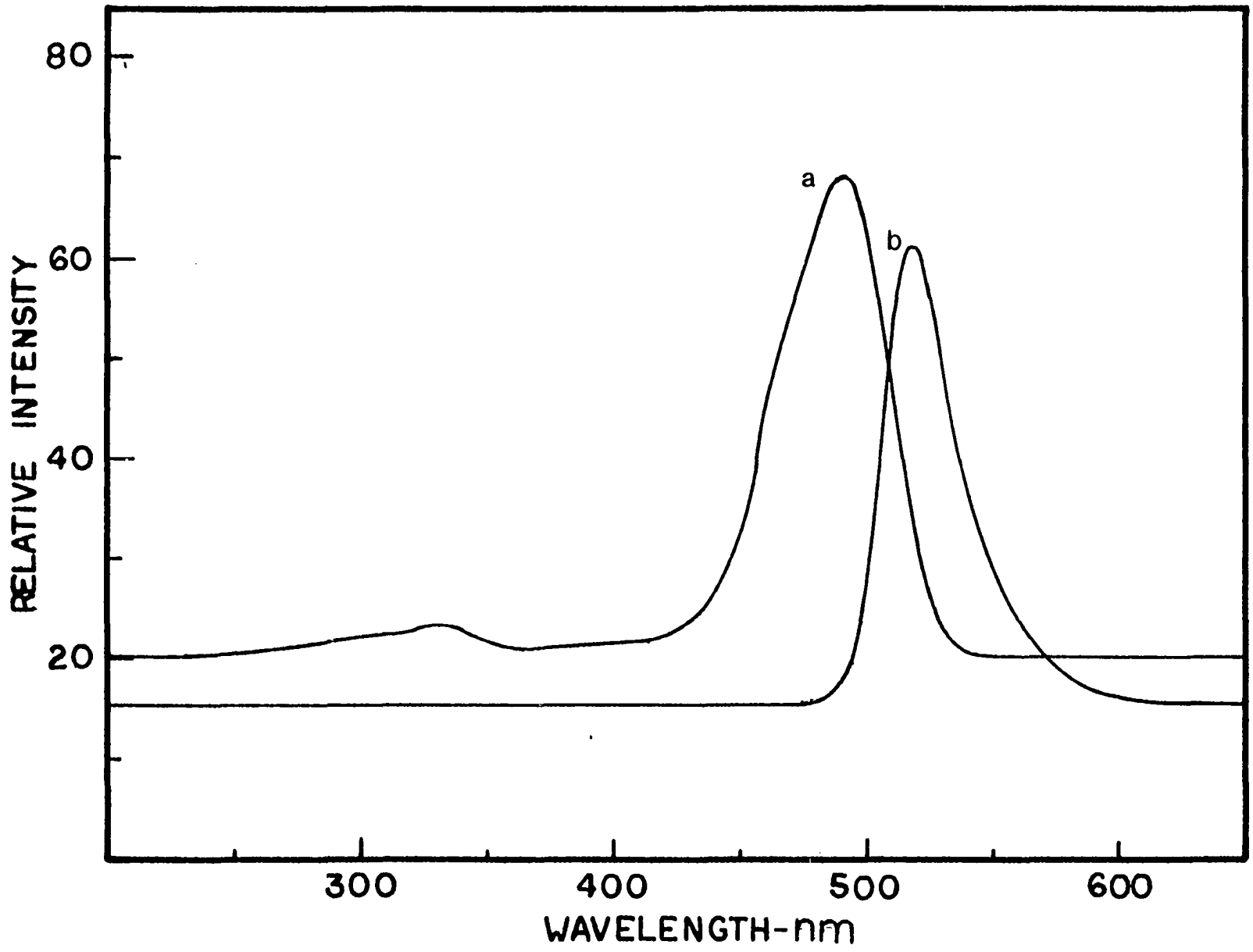
Concentration: 1.0×10^{-5} M; pH = 9.4

a. Excitation spectrum.

Emission monochromator set at 520 nm.

b. Emission spectrum.

Excitation monochromator set at 492 nm.


80
60
40
20
RELATIVE INTENSITY
a
b
300
400
500
600
WAVELENGTH-nm

The more detailed measurements of intensity of fluorescence as a function of pH, at small intervals over the range 1 to 11, were made with the excitation monochromator set at 492 nm and the emission monochromator set at 520 nm. The data are presented in Table 15 and shown graphically in Figure 46. The plot of the mathematical function developed in Part B, above, is shown in Figure 47.

Table 15. Relative fluorescence of 6-sulfofluorescein as a function of pH

pH	F_m	$\log \left(\frac{F_m}{F_t - F_m}\right)$	
3.05	1.0	-1.944	$\mu = 0.1$
3.61	2.0	-1.638	$F_t = F_{Sfl^{3-}} = 89.0$
4.08	3.0	-1.457	slope = 2.19
4.36	4.0	-1.327	
4.63	5.0	-1.225	
5.06	8.0	-1.005	
5.37	12.0	-0.807	
5.56	16.0	-0.659	slope = 1.02
5.82	24.5	-0.420	intercept (log term = 0) = 6.25
6.38	49.5	0.088	point of intersection = 5.35
6.56	58.0	0.272	

Table 15. (Continued)

pH	F_m	$\log \left(\frac{F_m}{F_t - F_m}\right)$
6.68	63.5	0.396
6.98	74.5	0.711
7.58	85.0	1.327
7.80	86.5	1.539

Again in Figure 47 there are two lines intersecting at pH 5.35; line (a) with a slope of 2.19 and line (b) with a slope of 1.02. The presence of two lines rather than one is due to the fluorescence of the dianion, $HSfl^{2-}$, at a pH less than 5.35 as explained above, Part D. The dissociation constants are obtained as in the case of 5-sulfofluorescein; the intercept of line (b) is used to obtain $pK_{HSfl^-} = 6.25$, and using the values $pK_{HSfl^-} = 6.25$ and the point of intersection pH = 5.35, equation (7) yields $pK_{H_2Sfl^-} = 4.45$.

F. Conclusion

The fluorescence excitation and emission spectra of 4-, 5- and 6-sulfofluorescein are almost identical; they are almost identical also to the spectra of fluorescein as

Figure 46. Relative fluorescence of 6-sulfofluorescein as a function of pH.

Concentration: 5.0×10^{-6} M

Excitation monochromator set at 492 nm

Emission monochromator set at 520 nm

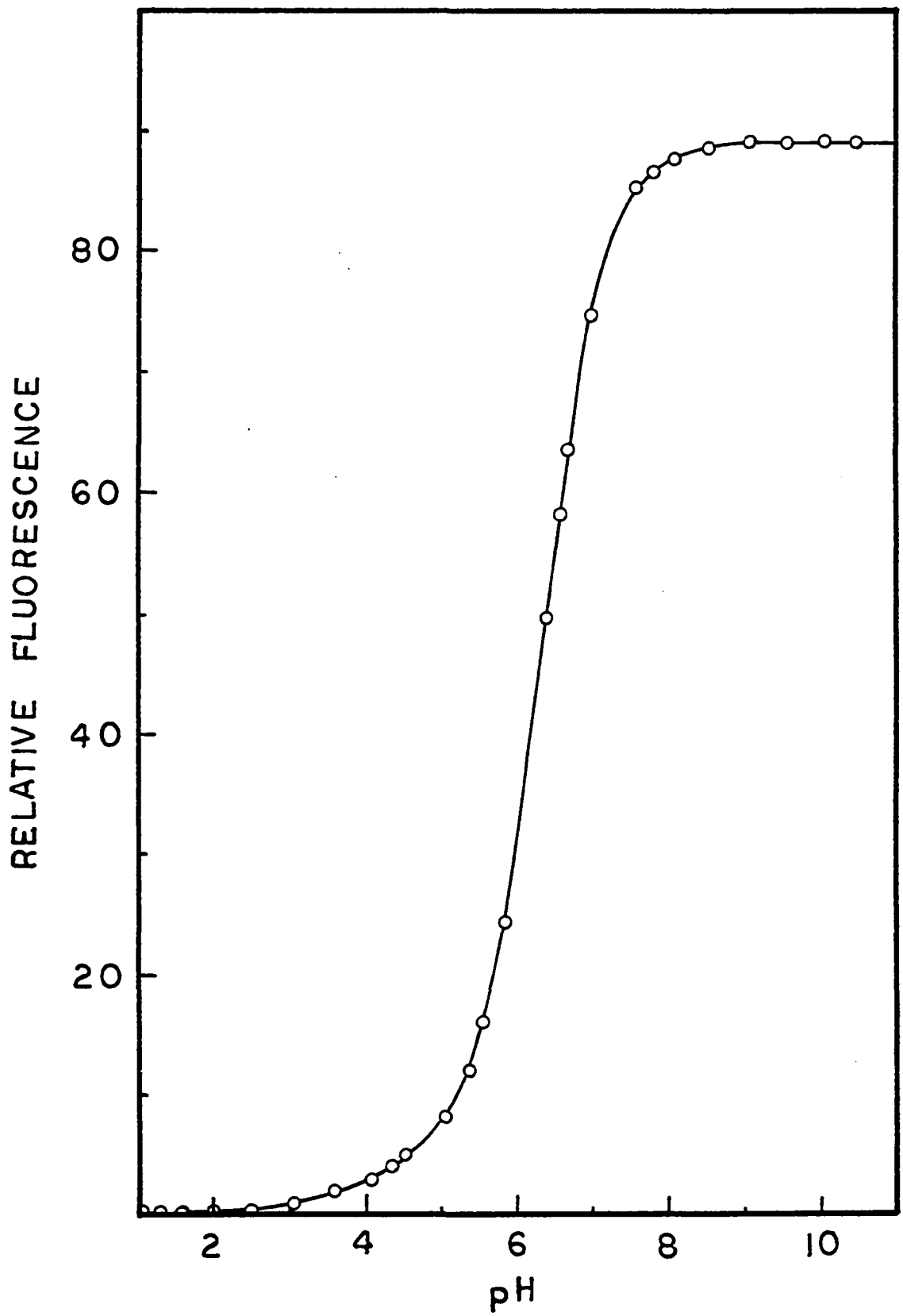
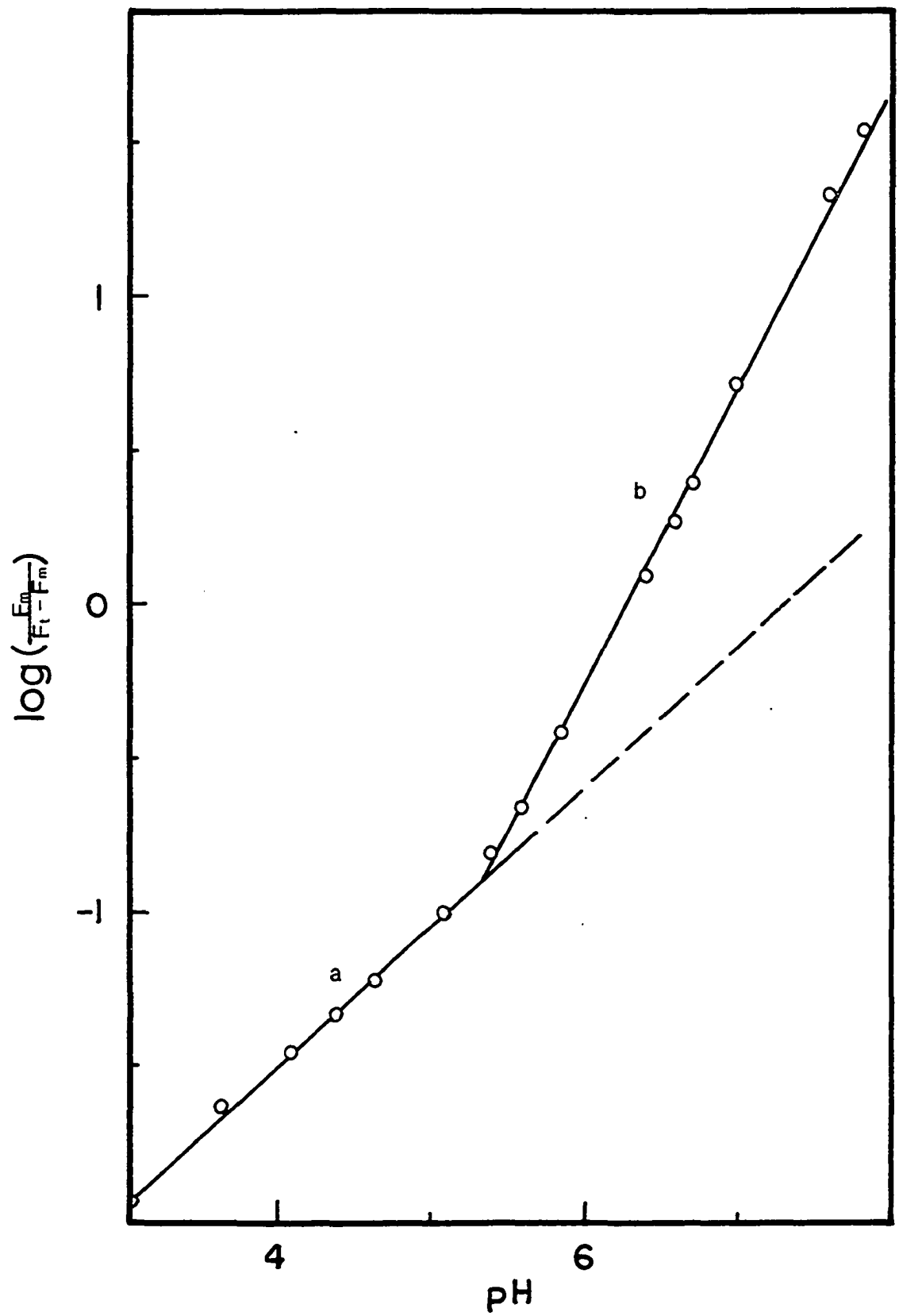

RELATIVE FLUORESCENCE
80
60
40
20
2
4
6
8
10
pH

Figure 47. Mathematical treatment of the fluorescence of 6-sulfofluorescein as a function of pH.

$$\log \left(\frac{F_m}{F_t - F_m}\right) \text{ vs. pH}$$


log ($\frac{F_m}{F_t - F_m}$)
1
0
-1
a
b
4
6
pH

reported by Markuszewski (3). The maximum in the excitation spectra of all four compounds falls between 490 and 495 nm and these maxima are close to the maxima found in Chapter V, above.

The intensity and the changes in intensity with pH of all four compounds, also are almost identical. The fluorescence of each compound is very low at pH below 3, increases slowly between pH 3 and 5, rises rapidly between pH 6 and 7, reaches a maximum at pH about 8.0, and remains constant at all pH greater than 8.0.

Using the data of relative fluorescence as a function of pH, it is possible to calculate the third acid dissociation constant of the three sulfofluoresceins; the values found are: 6.24, 6.22 and 6.25 of 4-, 5-, and 6-sulfofluorescein, respectively. These values are almost identical to the third dissociation constant of fluorescein, 6.28, determined by Markuszewski (3) by measurements of fluorescence.

Again, using the value of pH at the point of intersection in Figures 44 and 47 and substituting the value of the third dissociation constant of the respective compound in equation (7), the second dissociation constant of 5- and 6-sulfofluorescein are calculated to be 4.48 and 4.45. These values are almost identical to the corresponding dissociation constant of fluorescein, 4.42, determined by Markuszewski in the same manner.

In obtaining the values of the third dissociation constant of the three sulfofluoresceins an assumption was made, that the fluorescence of the dianion, $HSfl^{2-}$, is minimal in contrast to that of the trianion, Sfl^{3-}, when the excitation monochromator is set at 490 to 495 nm. This assumption holds true for the isomer 4-sulfofluorescein for the entire pH range, at which the measurements of fluorescence were made. This fact is confirmed by obtaining one line, Figure 41, with a slope of 0.99, very close to the theoretical slope of 1.0. In the case of 4- and 5-sulfofluoresceins, Figures 44 and 47, and in the case of fluorescein (ref. 3, p. 164) two linear curves are obtained; the slope of the line at pH less than 5.4 of each of the three compounds is about 2, while the slopes of the lines at pH above 5.4 are: 1.04, 1.02 and 1.07, respectively. These last values are close to the theoretical value of 1.0.

The presence of two linear curves in Figures 44 and 47 of the mathematical treatment of fluorescence of 5- and 6-sulfofluorescein as a function of pH, is an indication that the fluorescence of the dianion, $HSfl^{2-}$, becomes negligible above pH 5.4 only, at which region the relative concentration of dianion decreases rapidly while that of the trianion, Sfl^{3-}, increases. The same argument applies to the corresponding prototropic forms of fluorescein the monoanion, HFl^{-}, and the dianion, Fl^{2-}. This is in contrast

to the assumption made by Markuszewski that the monoanion of fluorescein is nonfluorescent.

VII. SOLUBILITY OF THE SULFOFLUORESCEINS

A. Experimental Work

1. Preparation of buffers

Buffers were prepared in the pH region from 1 to 6 at intervals of 0.25 - 0.5 pH units using 0.1 M solutions of hydrochloric acid, potassium hydrogen phthalate, boric acid in 0.1 M potassium chloride, potassium hydroxide, and potassium chloride; the ionic strength was maintained at $\mu = 0.1$. The pH of each buffer solution was measured before and after saturation with the sulfofluorescein, using a Hach Model 8594 pH meter. The pH meter was set on the expanded scale and a high-alkalinity Beckman glass electrode and a saturated calomel electrode were used. The pH meter was calibrated against standard buffer solutions prepared according to the specifications of the National Bureau of Standards.

2. Solubility measurements

Approximately 25 ml of each buffer solution was placed in a 50-ml volumetric flask. The flask was covered and the top was wrapped with a parafilm. Into each bottle a sufficient amount of the sulfofluorescein was added to insure saturation of the solution. The flasks were then shaken on a Burrell mechanical shaker for at least 36 hours at room temperature 23±1°. Each solution was then filtered through

a porous bottom crucible, and the final pH was measured. An aliquot of 1.00 ml of each solution was diluted to 100.0 ml with 0.1 M potassium hydroxide and the absorbance of the solution was measured, at the wavelength of maximum absorption for the particular isomer, using a Hach DR-2 Spectrophotometer, supplied with a flow-through cell and a digital readout.

3. Preparation of calibration curves

About 120.0 mg of the appropriate isomer, purified by the use of a strong cation exchange resin, was placed into a 250.0-ml volumetric flask and each diluted to the mark with 0.1 M potassium hydroxide, then 10.0-ml aliquot of the solution was diluted to exactly 100.0 ml with 0.1 M potassium hydroxide. Then aliquots of 1, 2, 3, 4, 5, and 6 ml of the last solution were taken and diluted again to 100.0 ml with 0.1 M potassium hydroxide for absorbence measurements, using a Hach DR-2 Spectrophotometer.

Calibration curves were plotted for each of the isomers of sulfofluorescein. Using these calibration curves and the absorbance measurements of the various saturated solutions for each of the sulfofluoresceins, the solubility was calculated.

B. Evaluation of the Intrinsic Solubility and the Acid Dissociation Constants

Following the method of Krebs and Speakman (24), solubility data can be used to evaluate the acid dissociation constant of the first replaceable hydrogen atom of the neutral molecule of sulfofluorescein, H_3Sfl. This constant is defined by the reaction and the mathematical equation:

$$H_3Sfl \rightleftharpoons H^+ + H_2Sfl^- \qquad K_{H_3Sfl} = \frac{[H^+][H_2Sfl^-]}{[H_3Sfl]} \tag{8}$$

The assumption is made that the solubility of the unionized acid, H_3Sfl, the so-called intrinsic solubility, S_i, is constant and independent of pH range under study. That is,

$$S_i = [H_3Sfl] \tag{9}$$

The solubility of the acid, S_o, in a solution of a given pH, is the sum of the concentration of the unionized acid, and that of the ionized species.

$$S_o = [H_3Sfl] + [H_2Sfl^-] \tag{10}$$

Combination of equations (8), (9), and (10) yields:

$$S_o = S_i + \frac{K_{H_3Sfl} \cdot S_i}{[H^+]} \tag{11}$$

and

$$\frac{S_o}{S_i} - 1 = \frac{K_{H_3Sfl}}{[H^+]} \tag{12}$$

and

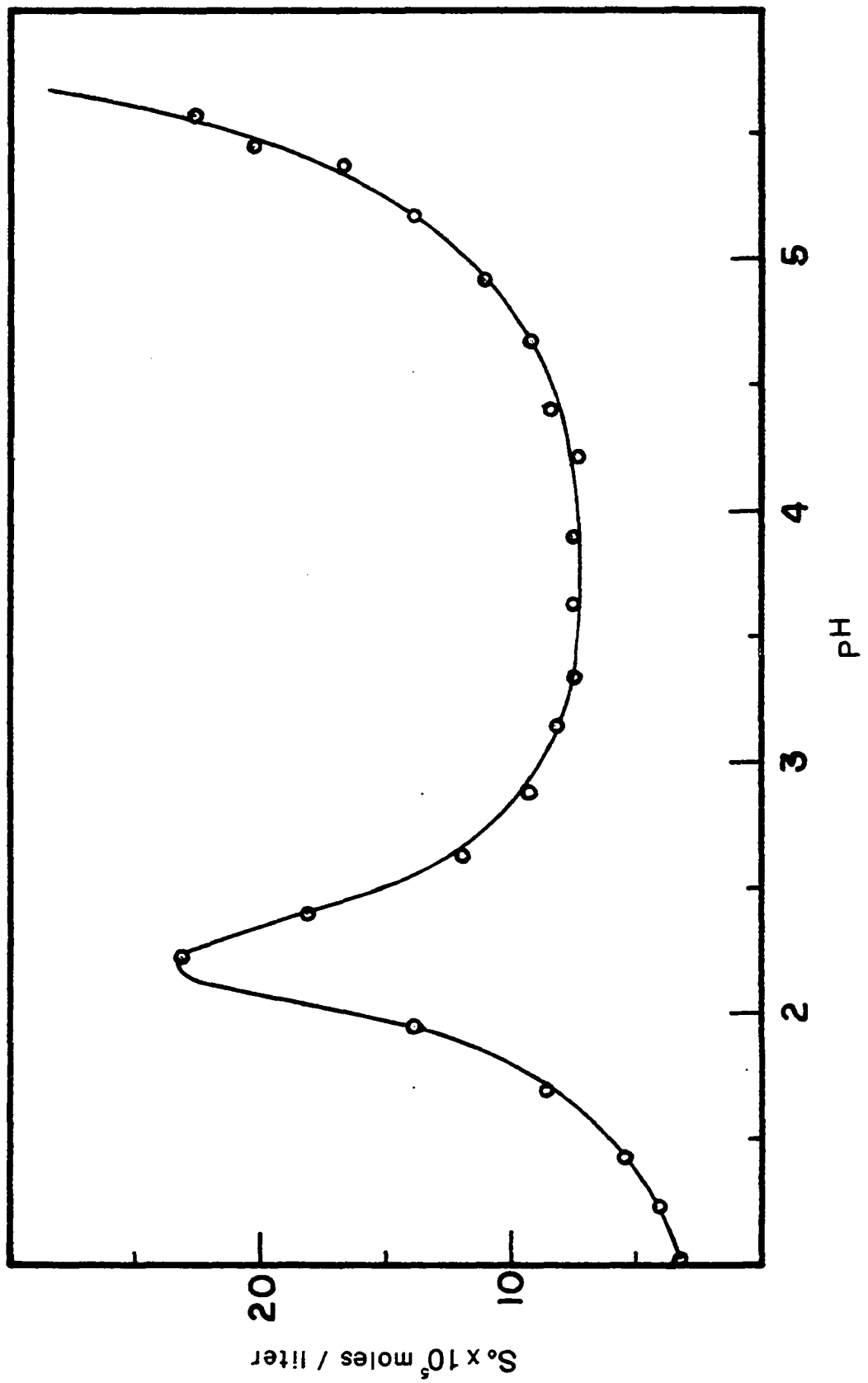
$$\log \left(\frac{S_o}{S_i} - 1\right) = pH - pK_{H_3Sfl} \tag{13}$$

Two treatments of the data obtained from the solubility measurements are made. First, a plot of S_o *vs.* $\frac{1}{[H^+]}$, equation (11), gives a straight line, the intercept being the intrinsic solubility, and the slope being $K_{H_3Sfl}S_i$. Second, using the value of S_i, a plot of $\log \left(\frac{S_o}{S_i} - 1\right)$ *vs.* pH, equation (13), gives a straight line of unit slope, the intercept of which yields a value of pK_{H_3Sfl}.

C. Solubility of 4-Sulfofluorescein as a Function of pH

The solubility of 4-sulfofluorescein as a function of pH is presented in Figure 48. This isomer exhibits an unusual behavior. First, the solubility increases to reach a maximum at pH about 2.2; next, it decreases as rapidly to reach a minimum at pH about 3.3; next, it remains essentially constant to about pH 4.3; finally, it rises again with increasing pH. This unusual behavior is explained by recalling the equilibria which were given in Chapter V, to

Figure 48. Solubility of 4-sulfofluorescein as a function of pH.


$S_o \times 10^5$ moles / liter
20
10
2
3
4
5
pH

explain the value found for the first dissociation constant, $pK_{H_3Sfl} = 1.18$, which is unusual for a carboxylic acid. In addition to the dissociation reaction, another reaction, the interconversion to the lactone structure, is involved,

$$\text{(zwitter ion) } H_3Sfl \rightleftharpoons \text{(zwitter ion) } H_2Sfl^- + H^+$$
$$\Updownarrow$$
$$\text{(lactone) } H_2Sfl^-$$

The formation of the colorless lactone was established in the course of the study dealing with absorbance as a function of pH; see Figure 26. The absorbance was practically zero in the region of pH 3.2, a region in which the monoanion is the predominant species. The solubility is explained on the same basis. As the pH increases, the solubility of the acid increases owing to the formation of the monoanion in the zwitter ion form. The monoanion is the predominant species at pH about 2.2. Above this pH, a shift to the lactone structure occurs and the solubility drops. The reason being that the lactone, Structure XVc, is less ionic in character than the zwitter ion, Structure XVb. The solubility rises again above pH 4 owing to the formation of the dianion and the trianion.

The evaluation of the intrinsic solubility and the acid dissociation constant of 4-sulfofluorescein, was based on data obtained over the pH range 1 to 2 and equations (11) and (13) as discussed in the previous section. The

experimental and calculated points are given in Table 16, and presented graphically in Figures 49 and 50. The slopes and the intercepts were calculated using the least-squares method.

Table 16. Solubility of 4-sulfofluorescein as a function of pH

pH	$S_o \times 10^5$ moles/liter	$\frac{1}{[H^+] \cdot 10}$	$\log (\frac{S_o}{S_i} - 1)$	
1.03	3.27	1.07	-0.175	$\mu = 0.1$
1.22	3.88	1.66	-0.009	$S_i = 1.96 \times 10^{-5}$ moles/liter
1.44	5.62	2.76	0.271	$pK_{H_3Sfl} = 1.23$
1.71	8.66	5.13	0.534	slope = 1.03
1.96	13.82	9.12	0.782	
2.22	23.13	16.60	1.033	

The intrinsic solubility, S_i, was evaluated by extrapolation of the straight line in Figure 49 to $\frac{1}{[H^+]} = 0$, in which $S_i = 1.96 \times 10^{-5}$ moles/liter, and the slope expressed as $\frac{\Delta S_o}{\Delta(1/[H^+])} = 1.283 \times 10^{-6}$ moles^2/liter^2.

Figure 49. Determination of the intrinsic solubility, S_i, and the first dissociation constant K_{H_3Sfl} of 4-sulfofluorescein by solubility measurements.

$S_o \times 10^5$ moles/liter *vs.* $\frac{1}{[H^+] \cdot 10}$

Intercept $(\frac{1}{[H^+]} = 0) = 1.96$

$S_i = 1.96 \times 10^{-4}$ moles/liter

Slope $= S_i \times K_{H_3Sfl} = 1.28 \times 10^{-6}$ moles^2/liter^2

$pK_{H_3Sfl} = 1.19$

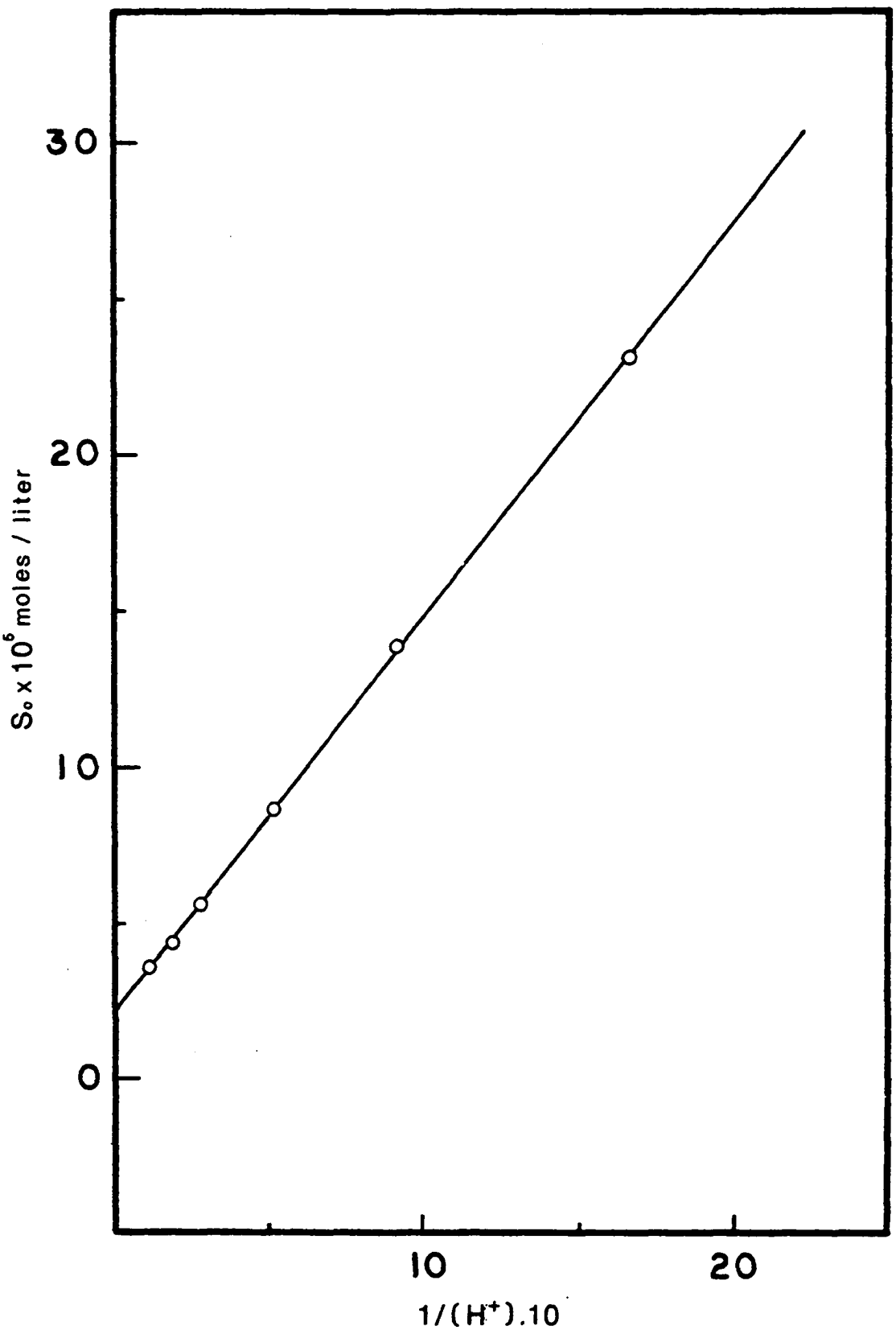

30
20
10
0
$S_o \times 10^6$ moles / liter
10
20
1/(H+).10

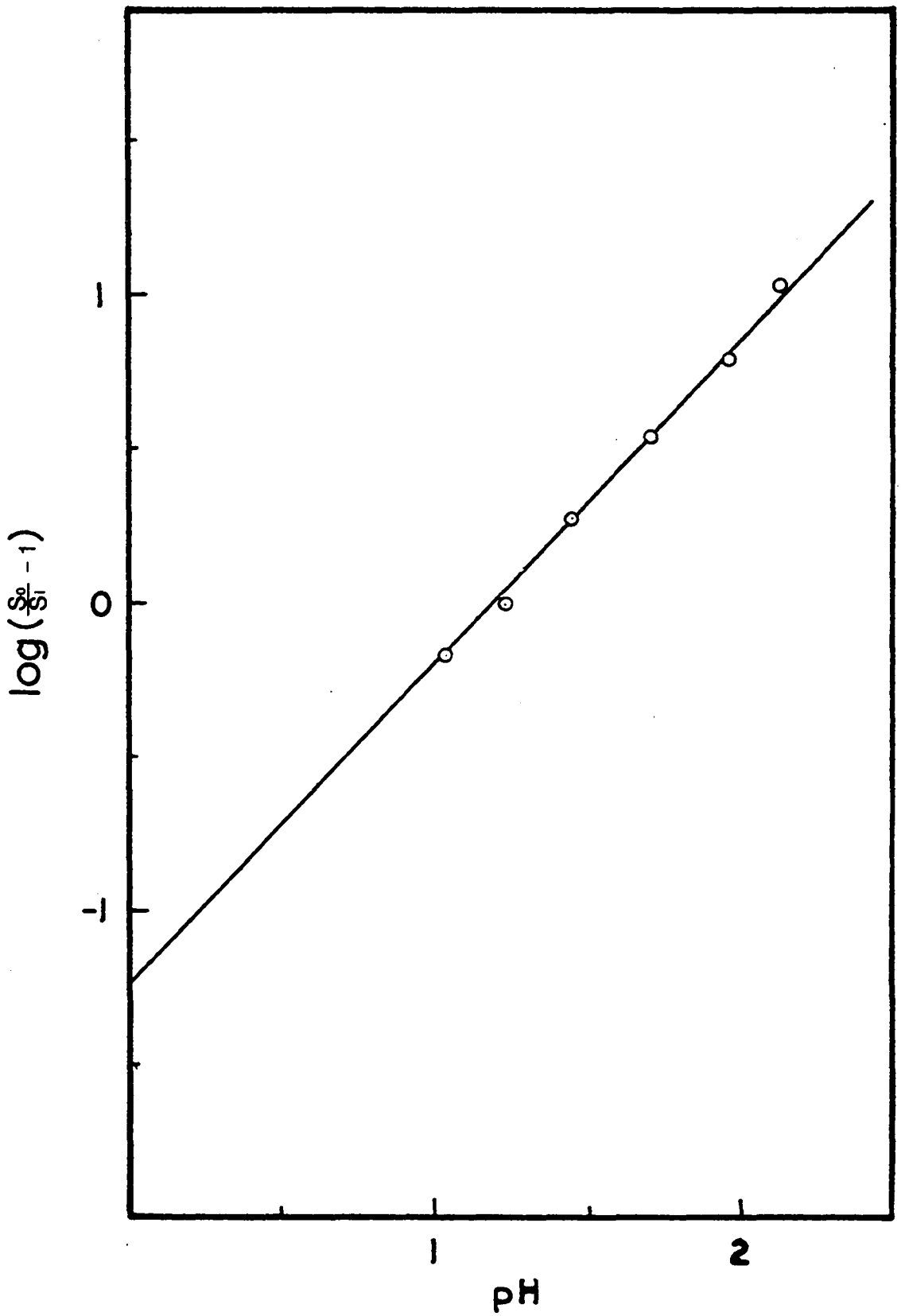
Figure 50. Determination of the first acid dissociation constant of 4-sulfofluorescein from solubility measurements.

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

$S_i = 1.96 \times 10^{-5}$ moles/liter

Intercept (pH = 0) = -1.23

$pK_{H_3Sfl} = 1.23$


1
0
-1
log ($\frac{S_o}{S_i}$ - 1)
1
2
pH

Rearrangement of equation (11) yields:

$$\text{Slope} = K_{H_3Sfl} \times S_i \qquad (14)$$

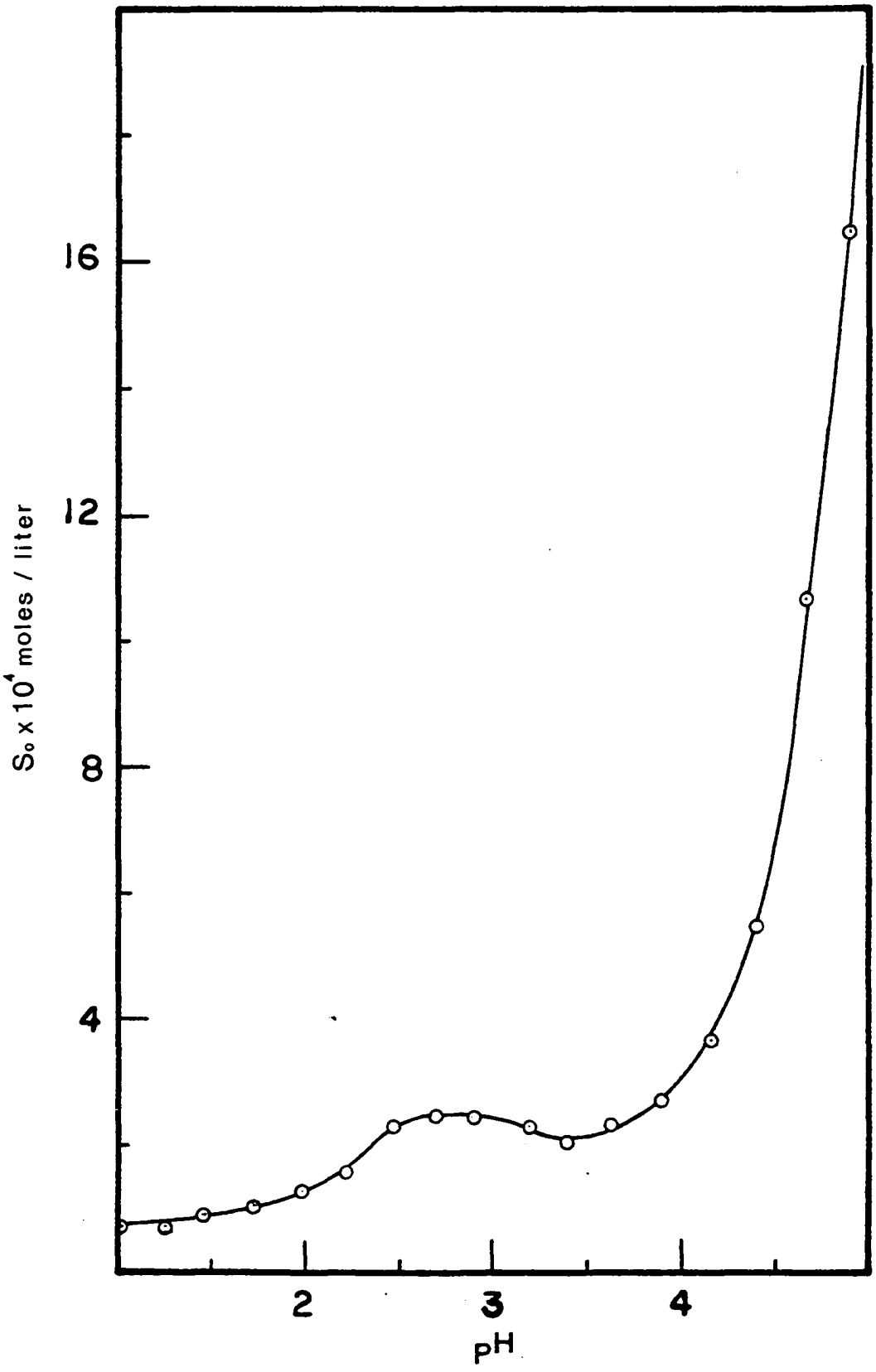
Using the value of 1.96×10^{-5} for S_i, the value pK_{H_3Sfl} was calculated to be 1.19.

The value $pK_{H_3Sfl} = 1.23$ was also determined by extrapolation of the straight line, in Figure 49, to pH = 0, the slope being 1.03. The value of the acid dissociation constant found by these solubility measurements, 1.19 and 1.23, is in a good agreement with that, 1.18, determined by absorbance measurements at ionic strength, $\mu = 1.0$, Chapter V.

D. Solubility of 5-Sulfofluorescein as a Function of pH

The solubility of 5-sulfofluorescein as a function of pH is presented graphically in Figure 51. The solubility increases slowly at higher pH and reaches a maximum at pH about 2.7 then drops gradually to reach a minimum at pH about 3.4 at which point the monoanion predominates. Again, this behavior in the acidic region is attributed to the formation of the lactone. The relative change in solubility between the maximum and the minimum is not as drastic as in the case of 4-sulfofluorescein. This is another confirmation that the formation of the lactone by the monoanion of

Figure 51. Solubility of 5-sulfofluorescein as a function of pH.


16
12
8
4
$S_o \times 10^4$ moles / liter
2
3
4
pH

5-sulfofluorescein is not as favored as in the case of 4-sulfofluorescein, yet should not be excluded.

The solubility rises slowly again between pH 3.5 and 4 owing to formation of some of the dianion, then rises rapidly above pH 4.5 owing to the formation of both of the dianion and the trianion.

The intrinsic solubility, S_i, was evaluated by extrapolation of the straight line, in Figure 52, to $\frac{1}{[H^+]} = 0$, in which $S_i = 7.30 \times 10^{-5}$ moles/liter, and the slope expressed as $\frac{\Delta S_o}{\Delta(1/[H^+])} = 5.43 \times 10^{-7}$ moles^2/liter^2.

Rearrangement of equation (11) yields:

$$\text{Slope} = K_{H_3Sfl} \times S_i \qquad (14)$$

Using the value 7.30×10^{-5} for S_i, the value pK_{H_3Sfl} was calculated to be 2.13.

The value $pK_{H_3Sfl} = 2.10$ was also determined by extrapolation of the straight line, in Figure 53, to pH = 0, the slope being = 0.98. The pK_{H_3Sfl} value of the acid dissociation constant determined by solubility measurements, 2.13 and 2.10, is in good agreement with that, 2.19, determined by absorbance measurements, Chapter V.

The experimental and calculated points needed to evaluate the intrinsic solubility and the value of pK_{H_3Sfl} are given in Table 17, in which the slopes and the intercepts were calculated using the least-squares method.

Figure 52. Determination of the intrinsic solubility, S_i, and the first dissociation constant K_{H_3Sfl} of 5-sulfofluorescein by solubility measurements.

$S_o \times 10^5$ moles/liter vs. $\frac{1}{[H^+] \cdot 10}$

Intercept $(\frac{1}{H^+} = 0) = 7.30$

$S_i = 7.30 \times 10^{-5}$ moles/liter

Slope $= S_i \cdot K_{H_3Sfl} = 5.43 \times 10^{-7}$ moles^2/liter^2

$pK_{H_3Sfl} = 2.13$

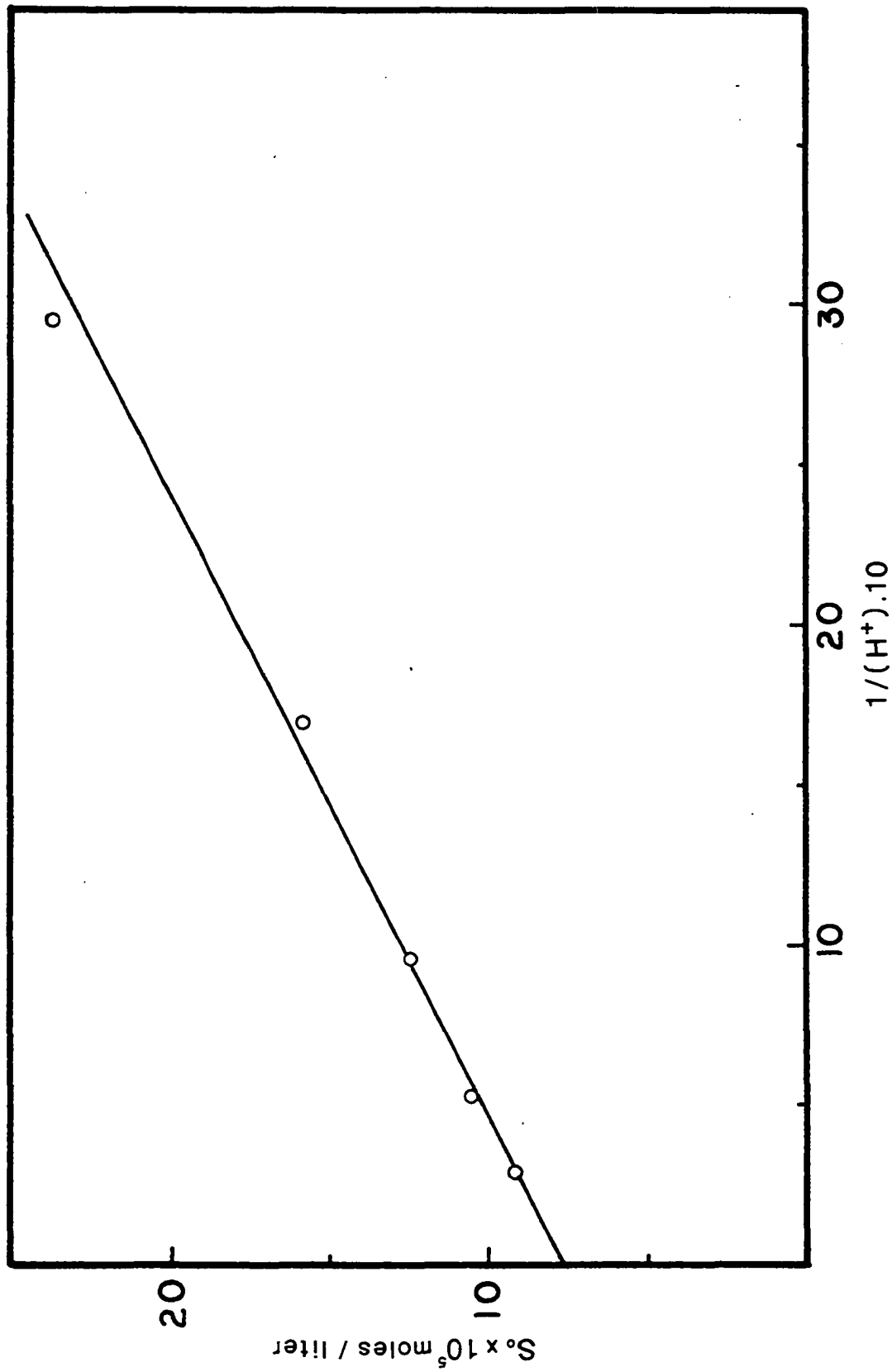

$S_o \times 10^5$ moles / liter
20
10
10
20
30
$1/(H^+).10$

Figure 53. Determination of the first acid dissociation constant of 5-sulfofluorescein from solubility measurements.

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

$S_i = 7.30 \times 10^{-5}$ moles/liter

Intercept (pH = 0) = -2.13

$pK_{H_3Sfl} = 2.13$

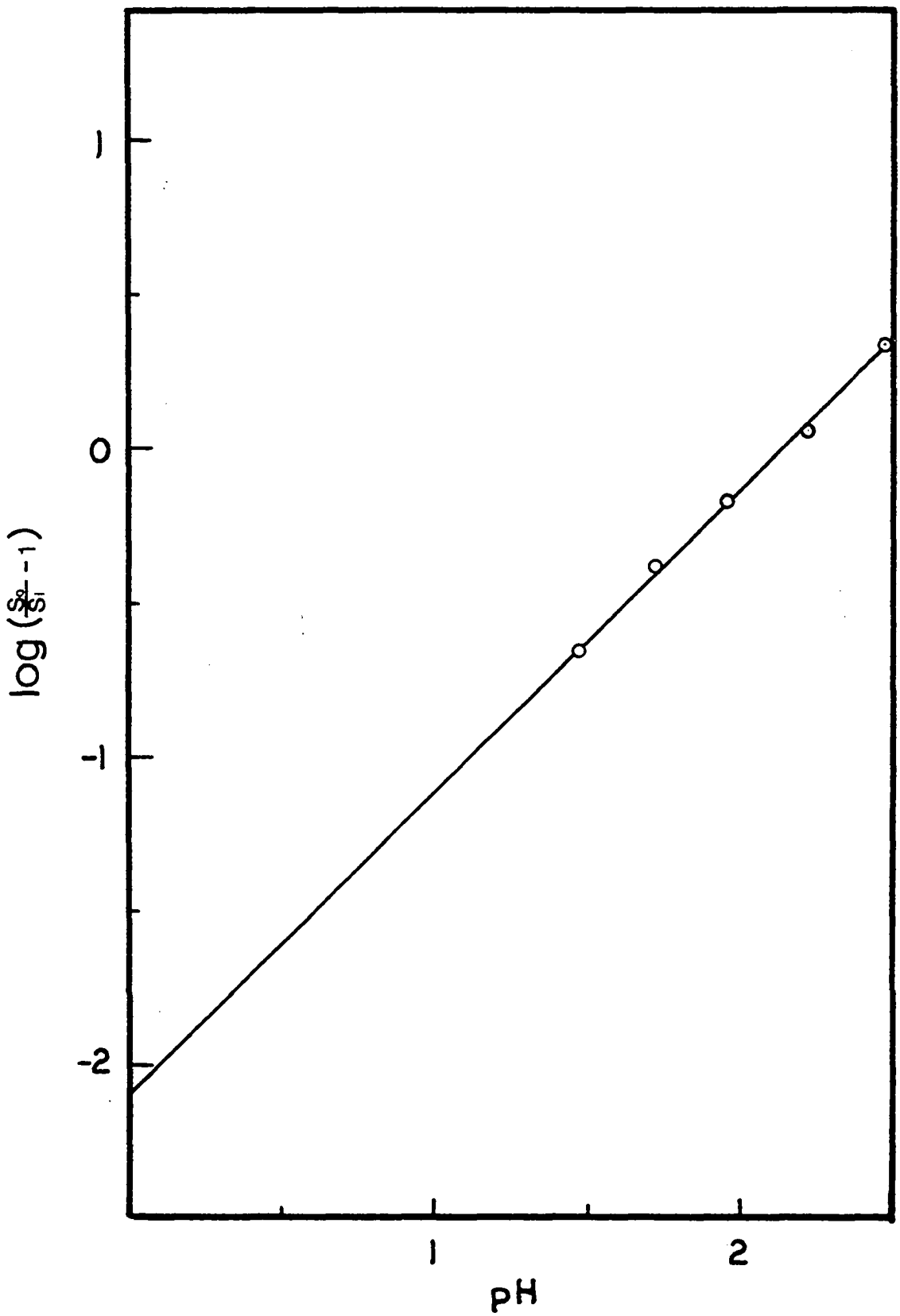

1
0
-1
-2
log ($\frac{S_0}{S_1}$ - 1)
1
2
pH

Table 17. Solubility of 5-sulfofluorescein as a function of pH

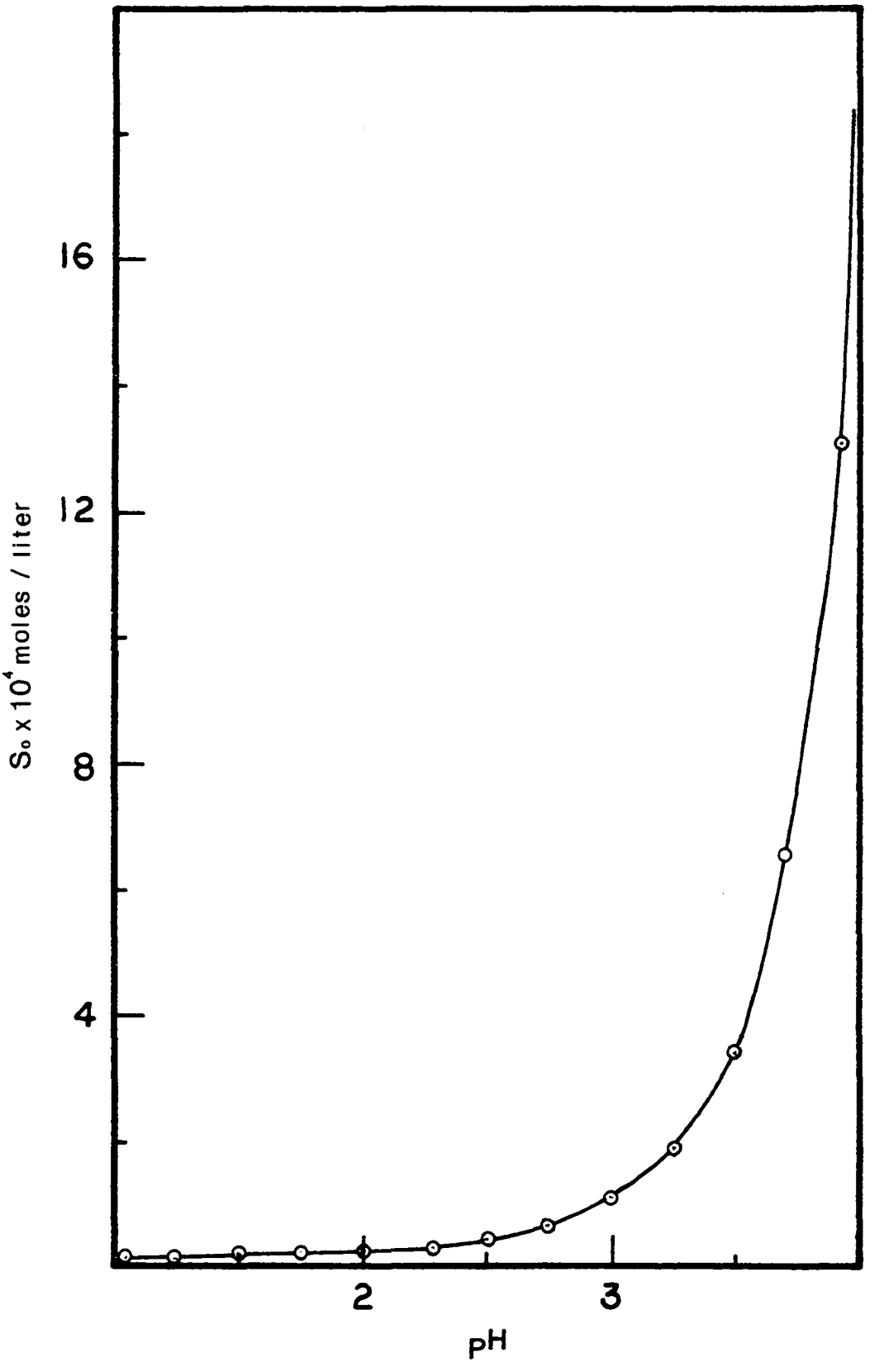
pH	$S_o \times 10^5$ moles/liter	$\frac{1}{[H^+] \cdot 10}$	$\log (\frac{S_o}{S_i} - 1)$	
1.48	9.09	3.02	-0.512	$\mu = 0.1$
1.73	10.5	5.37	-0.292	$S_i = 7.30 \times 10^{-5}$ moles/liter
1.98	12.4	9.55	-0.106	$pK_{H_3Sfl} = 2.10$
2.23	15.8	17.0	0.106	slope = 0.98
2.47	23.7	29.5	0.382	

E. Solubility of 6-Sulfofluorescein as a Function of pH

The solubility of 6-sulfofluorescein as a function of pH, is presented graphically in Figure 54. The solubility is minimal at pH less than 2.5, at which region the neutral molecule predominates, rises slowly between pH 2.5 and 3.0 the region at which the dianion $HSfl^{2-}$ predominates, rises very rapidly beyond pH 3.5 as the formation of the dianion $HSfl^{2-}$ becomes considerable.

Although the solubility of this isomer does not go through a maximum and a minimum between pH 2 and 3.5 as in the case of 4- and 5-sulfofluorescein, yet the solubility is

Figure 54. Solubility of 6-sulfofluorescein as a function of pH.


16
12
8
4
$S_o \times 10^4$ moles / liter
2
3
pH

essentially constant between pH 1 and pH 2.5 and only rises significantly with the formation of the dianion beyond pH 3. Thus, it seems that the equilibrium between the monoanion H_2Sfl^- in the zwitter ion form and the same species in the lactone is keeping the solubility constant until the formation of the dianion $HSfl^{2-}$ becomes considerable.

The intrinsic solubility, S_i, was evaluated by extrapolation of the straight line in Figure 55, to $\frac{1}{[H^+]} = 0$, in which $S_i = 1.32 \times 10^{-5}$ moles/liter, and the slope expressed as $\frac{\Delta S_i}{\Delta(1/[H^+])} = 9.56 \times 10$ moles^2/liter^2. Using the value 1.32×10^{-5} for S_i, the value pK_{H_3Sfl} was calculated to be 2.14.

The value $pK_{H_3Sfl} = 2.12$ was also determined by extrapolation of the straight line in Figure 56 to pH = 0, the slope being = 0.99. The pK_{H_3Sfl} value of the acid dissociation constant determined by solubility measurements, 21.4 and 2.12, is in a good agreement with that, 2.20, determined by absorbance measurements, Chapter V.

The experimental and calculated points needed to evaluate the intrinsic solubility and the value of pK_{H_3Sfl} are given in Table 18 in which the slopes and the intercepts were calculated using the least-squares method.

Figure 55. Determination of the intrinsic solubility, S_i, and the first dissociation constant K_{H_3Sfl} of 6-sulfofluorescein measurements.

$S_o \times 10^5$ moles/liter vs. $\frac{1}{[H^+] \cdot 10^2}$

Intercept $(\frac{1}{[H^+]} = 0) = 1.32$

$S_i = 1.32 \times 10^{-5}$ moles/liter

Slope $= S_i \cdot K_{H_3Sfl} = 9.56 \times 10^{-8}$ moles^2/liter^2

$pK_{H_3Sfl} = 2.14$

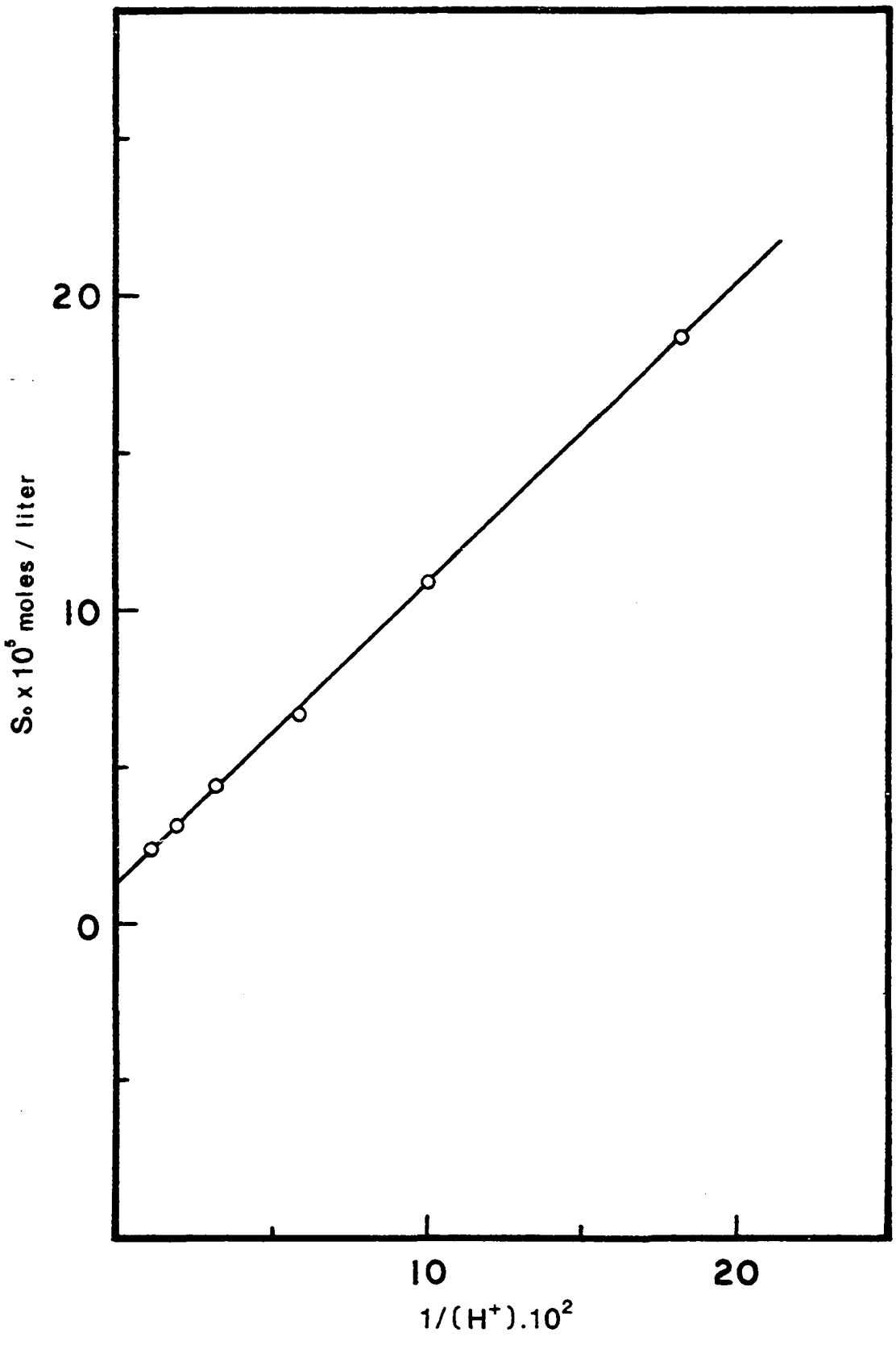

20
10
0
$S_o \times 10^5$ moles / liter
10
20
$1/(H^+).10^2$

Figure 56. Determination of the first acid dissociation constant of 6-sulfofluorescein from solubility measurements.

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

$S_i = 1.32 \times 10^{-5}$ moles/liter

Intercept (pH = 0) = -2.12

$pK_{H_3Sfl} = 2.12$

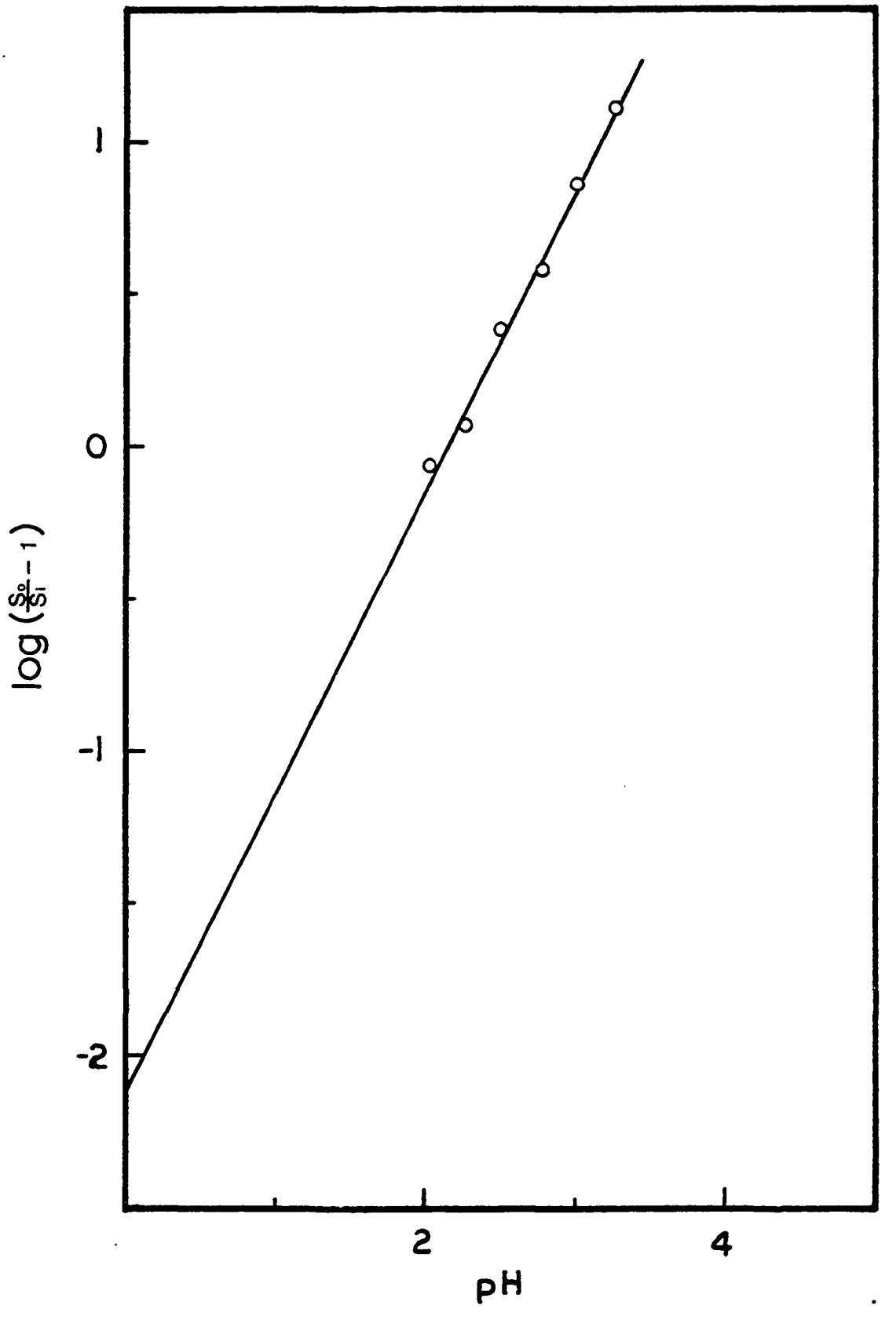

1
0
-1
-2
log ($\frac{S_e}{S_i}$ - 1)
2
4
pH

Table 18. Solubility of 6-sulfofluorescein as a function of pH

pH	$S_o \times 10$ moles/liter	$\frac{1}{[H^+] \cdot 10^2}$	$\log (\frac{S_o}{S_i} - 1)$	
2.02	2.44	1.05	-0.175	$\mu = 0.1$
2.27	2.86	1.86	0.067	$S_i = 1.32 \times 10^{-5}$ moles/liter
2.50	4.46	3.16	0.376	$pK_{H_3Sfl} = 2.12$
2.77	6.27	5.88	0.574	slope = 0.99
3.00	10.9	10.0	0.861	
3.26	18.7	18.20	1.119	

F. Discussion

The solubility of 4-sulfofluorescein in aqueous solution is lower that that of the β-isomers and the change with pH through the pH range 2 to 4 quite different. This difference is attributed to the conversion of a considerable portion of the monoanion of 4-sulfofluorescein to the lactone form; presumably the lactone is much less polar than the zwitter ion form and less soluble.

The solubility of the β-isomers, that is, 5- and 6-sulfofluorescein is different, and this is the only real

difference observed in their physical properties. Although 5-sulfofluorescein is somewhat more soluble than 6-sulfofluorescein in the pH range 1 to 3, the latter is considerably more soluble in the pH range 3.5 to 4. This difference in solubility made it possible to separate the β-isomers by fractional crystallization as discussed in Chapter II.

It has been observed (3) that the solubility of both the yellow and the red forms of fluorescein exhibits a minimum at pH about 3.4. The explanation has been offered in terms of the amphoteric nature of fluorescein, that is, at pH less than 3.4 the neutral molecule, H_2Fl, is protonated to form the monocation H_3Fl^+, and at a pH higher than 3.4 the neutral molecule dissociates to form the monoanion, HFl^-. Both the monocation and the monoanion are more soluble in water than the neutral molecule.

Another factor which may contribute to the low solubility of fluorescein at about pH 3.4 is that a portion of the fluorescein dissolved may be present in the lactone form in addition to the zwitter ion-pyrylium ring form.

VIII. POTENTIOMETRIC TITRATIONS OF THE SULFOFLUORESCEINS IN WATER

A. Apparatus and Procedure

A Hach Model 8596 pH meter with a Beckman high alkalinity glass electrode was used. The pH meter was standardized against two standard buffer solutions: pH 4.01, prepared from potassium hydrogen phthalate, and pH 6.86, prepared from potassium dihydrogen phosphate and disodium hydrogen phosphate. Both were prepared according to the specifications of the National Bureau of Standards as directed in Diehl (ref. 25, p. 58).

A weighed amount of about 0.100 g of purified and dried sulfofluorescein in the free acid form was added to about 250 ml of 0.1 M potassium chloride solution, the ionic strength being maintained at $\mu = 0.1$. The dissolution was effected by heating on a hot plate and stirring with a magnetic bar. The solution was cooled and titrated with 0.1 N sodium hydroxide. Throughout the course of titration the expanded scale of the pH meter was used and the reading was recorded when the pH meter reached equilibrium after each addition of alkali.

B. Titration of 4-Sulfofluorescein

The titration curve of 4-sulfofluorescein in aqueous solution of ionic strength, $\mu = 0.1$, is presented in Figure

57. Two points of inflection in vertical parts of the curve are present, at one equivalent and at three equivalents of base added per mole of compound. The first end-point at pH 4.08 marks the complete neutralization of the proton of the carboxylic acid group on the phthalate ring, Figure 57, and the second end-point, at pH 8.65, marks the complete neutralization of the two protons of the phenolic groups on the xanthene ring. The shape of the titration curve with breaks at one and at three equivalents of base added differs from those of the β-isomers in aqueous solution; this difference and similarities which appear when the titrations are made in 50 per cent ethanol are of great interest.

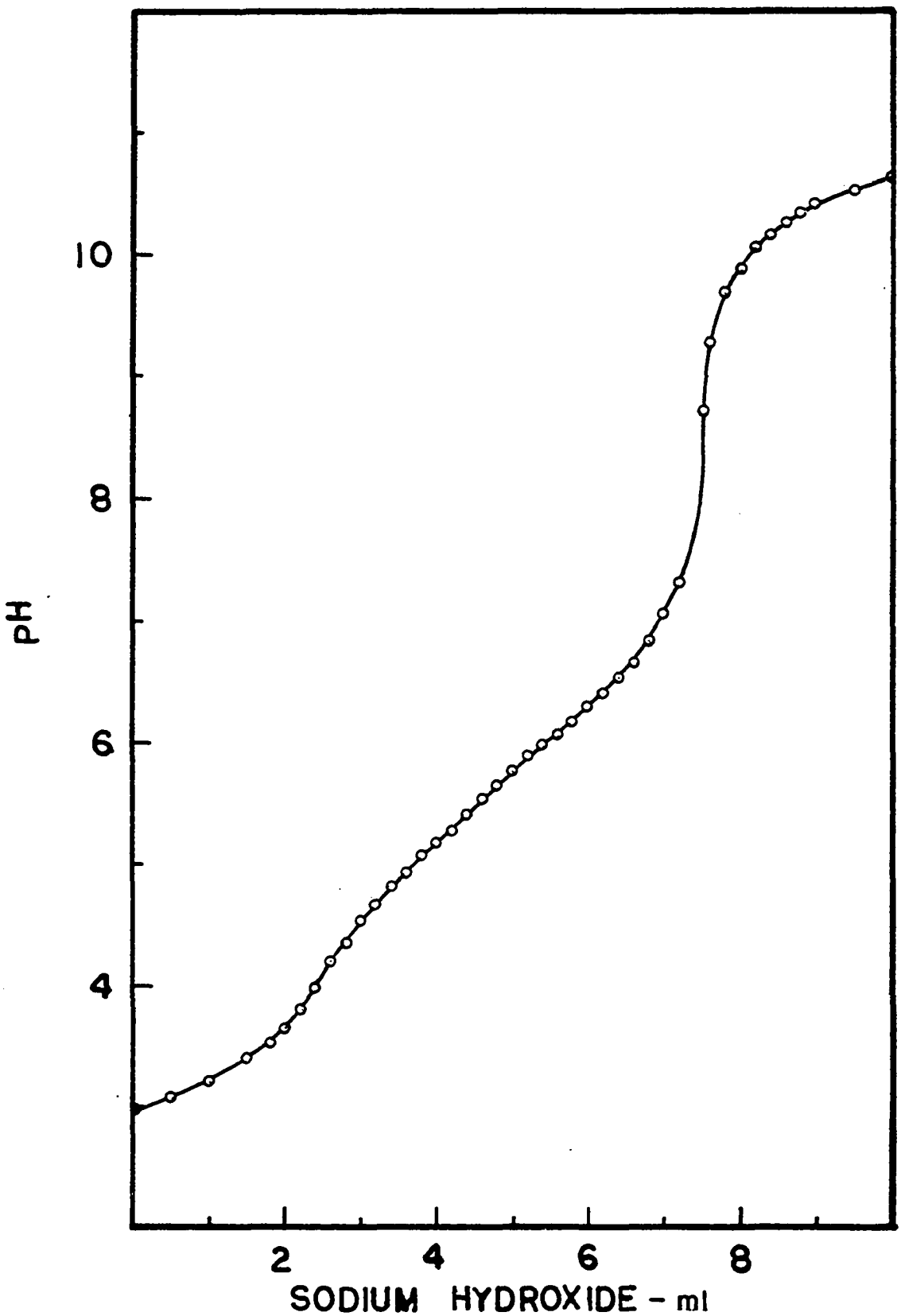
The fact that the first replaceable hydrogen atom is titrated separately is an indication that the difference $pK_{H_2Sfl^-} - pK_{H_3Sfl} > 3$, and because the second and the third protons are titrated together, the difference $pK_{HSfl^{2-}} - pK_{H_2Sfl^-} < 3$.

The first mid-point could not be used to evaluate pK_{H_3SF} because the analytical concentration, C_{H_3SF}, of the acid is in the order of 10^{-3} M, and smaller in magnitude than the dissociation constant, $pK_{H_3Sfl} = 1.2$, as determined by measurement of absorbance and of solubility as a function of pH. Under these conditions the approximation that pK_{H_3SF} = pH value at the mid-point does not hold. The second mid-point, that between the first and second points

Figure 57. Potentiometric titration of 4-sulfofluorescein in water.

4-Sulfofluorescein: 0.1038 g

Titrant: 0.1004 N sodium hydroxide


10
8
6
4
pH
2
4
6
8
SODIUM HYDROXIDE - ml

of inflection, does, however, yield a value for the second dissociation constant. Using the relation

$$pH = \frac{pK_{H_2Sfl^-} + pK_{HSfl^{2-}}}{2} \qquad (7)$$

the value of pH at the second mid-point (5.75, Figure 57), and the value $pK_{HSfl^{2-}} = 6.22$ found from the measurements of absorbance and fluorescence, the value of $pK_{H_2Sfl^-}$ was calculated to be 5.28. This value differs from that determined by absorbance measurements, 4.85. The lower value found from the measurement of absorbance is related to the overlap of the ionic species. The fraction $\frac{[HSfl^{2-}]}{C_{H_3Sfl}}$ at pH about 5.8, at which it reaches maximum value, is less than 0.6, thus the absorption of the conjugate base $A_{HSfl^{2-}}$ is by no means the real value when substituted in the term $\log \frac{A_m - A_{H_2Sfl^-}}{A_{HSfl^{2-}} - A_m}$ and plotted <u>versus</u> pH to determine $pK_{H_2Sfl^-}$.

Furthermore, to confirm the validity of equation (7) and to evaluate the second and third acid dissociation constants, a different approach was taken.

In obtaining the value of $pK_{HSfl^{2-}}$ from the pH value at the second end-point, the following equation is used

$$pH_{end\text{-}pt.} = (pK_w + pK_{HSfl^{2-}} + \log [Sfl^{3-}])/2 \qquad (15)$$

the value of pH at the second end-point, Figure 57, is 8.65, and the calculated concentration of the trianion, Sfl^{3-}, is 1.007×10^{-3}, the value of $pK_{HSfl^{2-}} = 6.30$ is calculated, a value in good agreement with that determined by absorbance measurements, $pK_{HSfl^{2-}} = 6.21$, and with that determined by fluorescence measurement, $pK_{HSfl^{2-}} = 6.24$.

The value of $pK_{H_2Sfl^-}$ is obtained by using the general equation which applies to a polybasic acid at the end-point

$$[H^+] = \left(\frac{K_1K_2[HA^-] + K_1K_w}{[HA^-] + K_1}\right)^{\frac{1}{2}} \qquad (16)$$

in the case of 4-sulfofluorescein, H_2Sfl, $K_1K_w << K_1K_2[H_2Sfl^-]$ and $K_1 = 10^{-1.20}$ and $[H_2Sfl] \simeq 1.00 \times 10^{-3}$. Thus equation (16) is easily converted to

$$[H^+] = (K_{H_2Sfl^-}[H_2Sfl^-])^{\frac{1}{2}} \qquad (17)$$

Taking the negative logarithm yields

$$pH_{end\text{-}pt.} = (pK_{H_2Sfl^-} - \log [H_2Sfl^-])/2 \qquad (18)$$

Using equation (18), the pH value 4.08 at the first end-point, Figure 57, and the value $\log [H_2Sfl^-] = -3.00$, the value $pK_{H_2Sfl^-} = 5.16$ was calculated. This is close to the

value 5.28 calculated by equation (7) above. Furthermore, using equation (7), the value $pK_{HSfl^{2-}} = 6.30$ and the value $pK_{H_2Sfl^{2-}} = 5.16$, the pH value 5.73 is found, this value is in excellent agreement with the experimental pH value 5.75 at the second mid-point.

C. Titration of 5-Sulfofluorescein

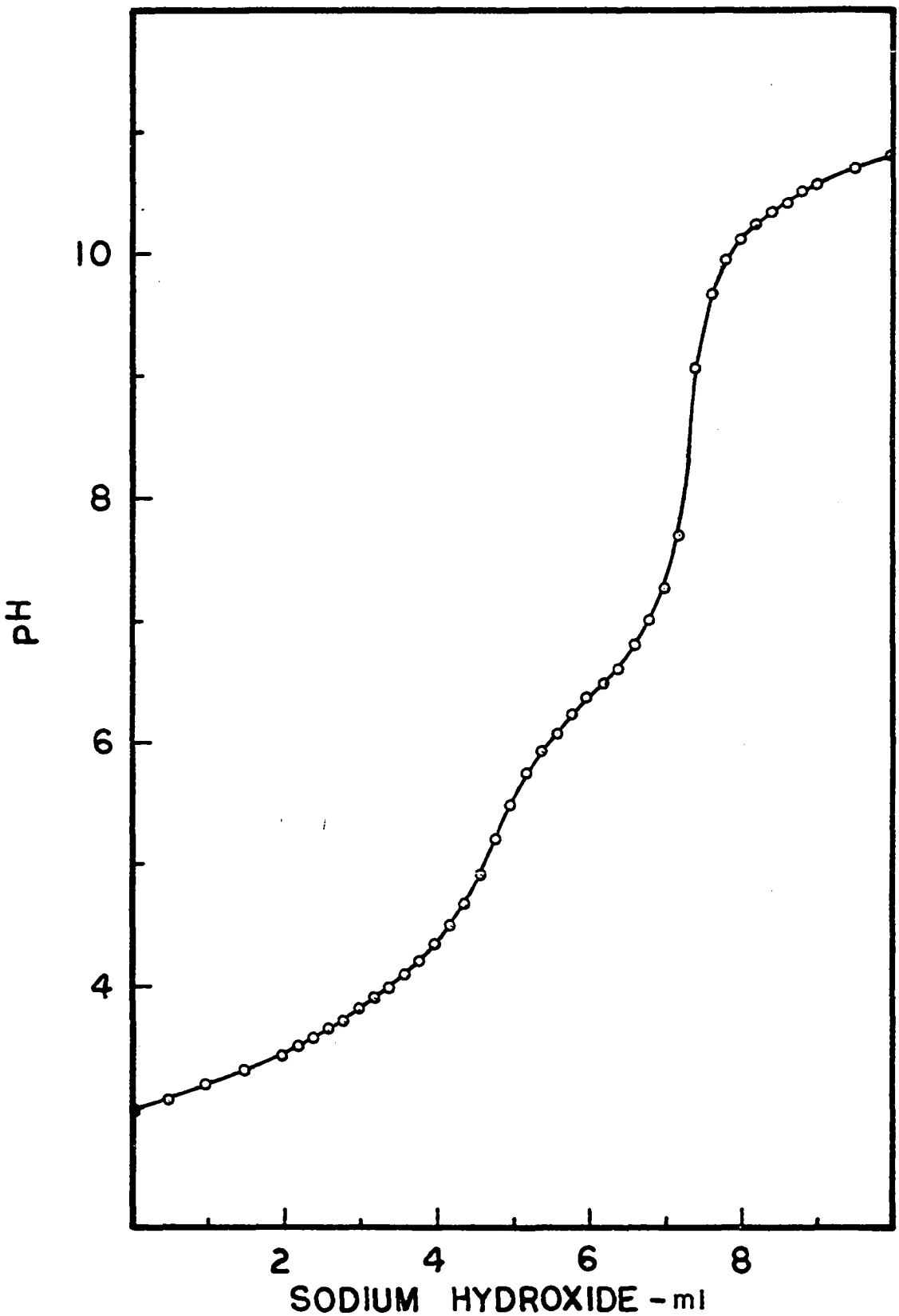
The titration curve of 5-sulfofluorescein in aqueous solution of ionic strength value $\mu = 0.1$ is presented in Figure 58. Two points of inflection are present, at two and at three equivalents of base added per mole of compound. The first end-point, at pH 5.33, marks the complete neutralization of the hydrogen atom of the carboxylic acid group on the phthalate ring and one of the phenolic hydrogen atoms of the xanthene ring. The second end-point, at pH 8.70, marks the neutralization of the second phenolic hydrogen atom on the xanthene ring. The difference $pK_{H_2Sfl^-} - pK_{H_3Sfl}$ must be less than 3 inasmuch as the first two replaceable hydrogen atoms are neutralized together, a not unexpected finding inasmuch as solubility and absorbance measurements yielded for pK_{H_3SF} 2.13 and 2.19 and for $pK_{H_2SF^-}$ 4.48.

The second mid-point, that is between the first and second break of the titration curve, occurs at pH 6.46 and

Figure 58. Potentiometric titration of 5-sulfofluorescein in water.

5-Sulfofluorescein: 0.1020 g

Titrant: 0.1004 sodium hydroxide


pH
10
8
6
4
2
4
6
8
SODIUM HYDROXIDE - ml

corresponds to $pK_{HSfl^{2-}}$. Using equation (7) above, the value $pK_{HSfl^{2-}} = 6.46$ and the first end-point value, pH = 5.33, the value of $pK_{H_2Sfl^-} = 4.20$ is found.

The value $pK_{HSfl^{2-}} = 6.46$ is not very different from that determined by absorbance and fluorescence measurements, 6.22; the deviation is attributed mainly to the error inherent in the approximations necessary in evaluating $pK_{HSfl^{2-}}$ and $pK_{H_2Sfl^-}$ from titration data obtained on low concentrations of the acid titrated. Thus, it is not surprising that when the value $pK_{HSfl^{2-}} = 6.22$ is used in equation (7), the $pK_{H_2Sfl^-}$ is calculated to be 4.44 and in excellent agreement with that determined by fluorescence measurements, $pK_{H_2SF^-} = 4.48$.

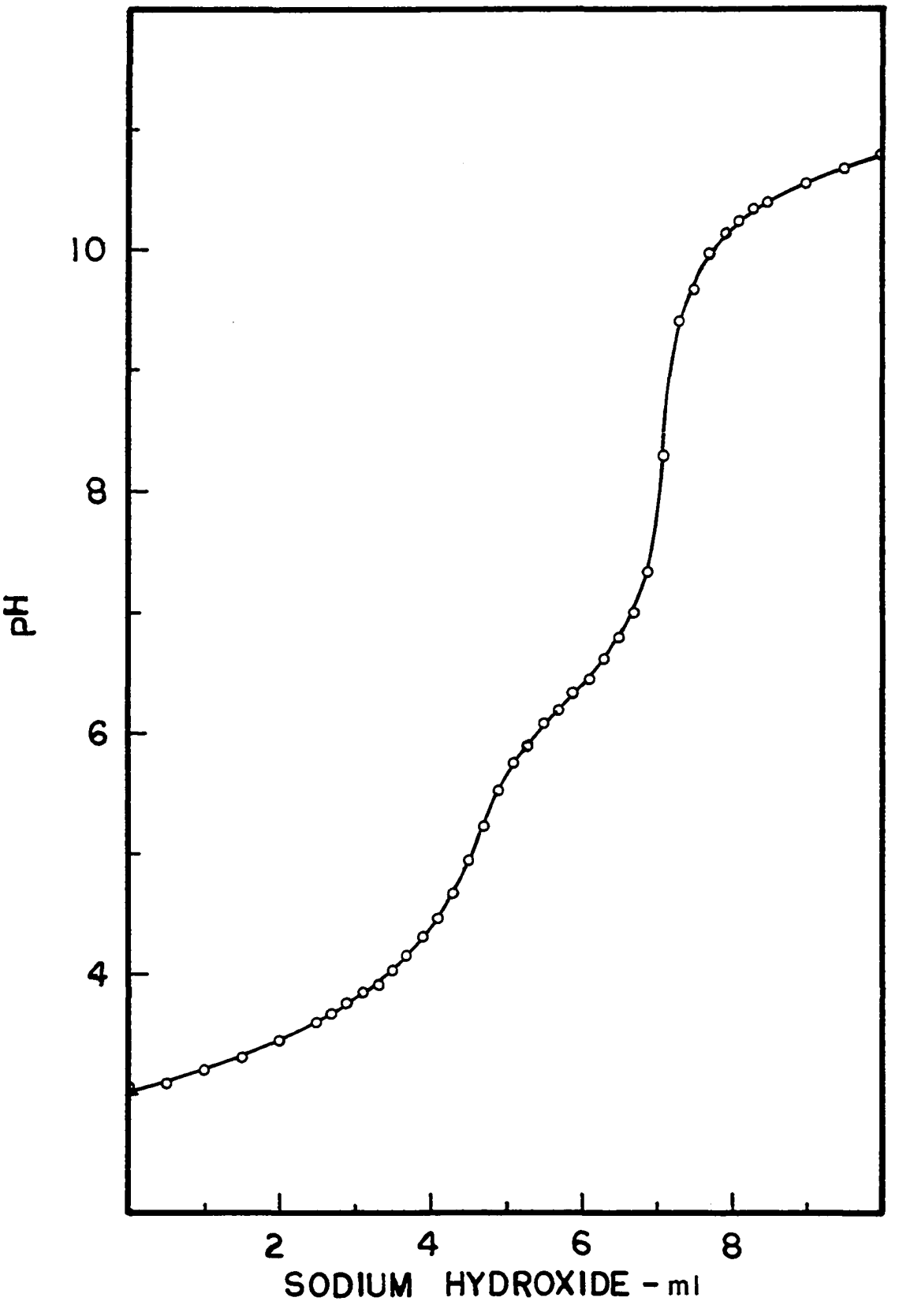
D. Titration of 6-Sulfofluorescein

The titration curve of 6-sulfofluorescein in aqueous solution of ionic strength value, $\mu = 0.1$, is presented in Figure 59. The titration curve is almost identical with that of 5-sulfofluorescein. Two points of inflection are present, at two equivalents and at three equivalents of base added per mole of the compound. The first end-point at pH 5.34 marks the neutralization of the replaceable hydrogen atoms of the carboxylic group and one of the

Figure 59. Potentiometric titration of 6-sulfofluorescein in water.

6-Sulfofluorescein: 0.1023 g

Titrant: 0.1004 N sodium hydroxide


pH
10
8
6
4
2
4
6
8
SODIUM HYDROXIDE - ml

phenolic groups. The second end-point, at pH 8.60, marks the neutralization of the second phenolic hydrogen atom on the xanthene ring.

A value for the third dissociation constant, $pK_{HSfl^{2-}} = 6.35$, was obtained from the mid-point between the first and the second breaks of the titration curve; this value not greatly different from those obtained by absorbance and fluorescence measurements, 6.16 and 6.25, respectively.

Using equation (7), the value $pK_{HSfl^{2-}} = 6.35$, and the pH value 5.34 at the first end-point, the value $pK_{H_2Sfl^-} = 4.33$ was calculated; this is close to the value 4.45 determined by fluorescence measurements.

E. Conclusions

The titration curves of 5- and 6-sulfofluoresceins confirm again the very similar acid-base properties of these two isomers, and the difference in properties of these isomers from those of 4-sulfofluorescein. The acid dissociation constants of 4-, 5-, and 6-sulfofluorescein as determined in this work, are summarized in Tables 19, 20 and 21.

The corresponding acid dissociation constants of 5-, and 6-sulfofluorescein and of fluorescein are almost

Table 19. The acid dissociation constants of 4-sulfofluorescein (H_3Sfl)

Dissociation Constant as Negative Logarithm	Reaction	Method
pK_{H_3Sfl}	$H_3Sfl = H_2Sfl^- + H^+$	
1.23		solubility
1.19		solubility
1.18		absorbance
1.20 average		
$pK_{H_2Sfl^-}$	$H_2Sfl^- = HSfl^{2-} + H^+$	
5.28		potentiometric titration
$pK_{HSfl^{2-}}$	$HSfl^{2-} = Sfl^{3-} + H^+$	
6.21		absorbance
6.24		fluorescence
6.30		potentiometric titration
6.25 average		

Table 20. The acid dissociation constants of 5-sulfofluorescein (H_3Sfl)

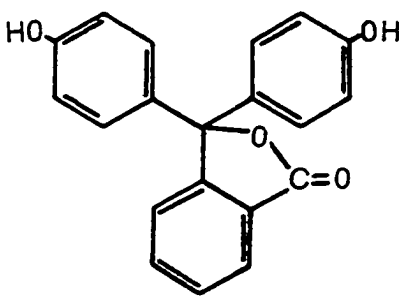
Dissociation Constant as Negative Logarithm	Reaction	Method
pK_{H_3Sfl}	$H_3Sfl = H_2Sfl^- + H^+$	
2.13		solubility
2.10		solubility
2.19		absorbance
2.14 average		
$pK_{H_2Sfl^-}$	$H_2Sfl^- = HSfl^{2-} + H^+$	
4.48		fluorescence
4.20		potentiometric titration
4.34 average		
$pK_{HSfl^{2-}}$	$HSfl^{2-} = Sfl^{3-} + H^+$	
6.13		absorbance
6.22		fluorescence
6.46		potentiometric titration
6.27 average		

Table 21. The acid dissociation constants of 6-sulfofluorescein (H_3Sfl)

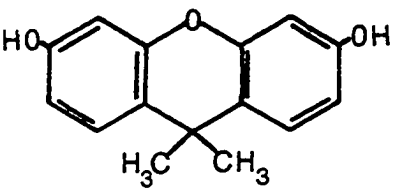
Dissociation Constant as Negative Logarithm	Reaction	Method
pK_{H_3Sfl}	$H_3Sfl = H_2Sfl^- + H^+$	
2.12		solubility
2.14		solubility
2.20		absorbance
2.15 average		
$pK_{H_2Sfl^-}$	$H_2Sfl^- = HSfl^{2-} + H^+$	
4.45		fluorescence
4.33		potentiometric titration
4.39 average		
pK_{HSfl^-}	$HSfl^{2-} = Sfl^{3-} + H^+$	
6.16		absorbance
6.25		fluorescence
6.35		potentiometric titration
6.25 average		

identical; the first and second dissociation constants of 4-sulfofluorescein differ markedly from those of fluorescein and the β-isomers; this has been explained as being the result of the existence of the monoanion of 4-sulfofluorescein in major part in the lactone form.

The second and third dissociation constants of all four compounds are far greater than expected for phenols, the acid dissociation constants of which are normally in the range 10^{-9} to 10^{-10}, too low to permit titrations in water. This has been demonstrated by Markuszewski (3), who attempted unsuccessfully to titrate compounds closely related to the lactone form of fluorescein, namely phenolphthalein, Structure XXV, and 3,6-dihydroxy-9,9-dimethylxanthene, Structure XXVI. Markuszewski explained the enormous



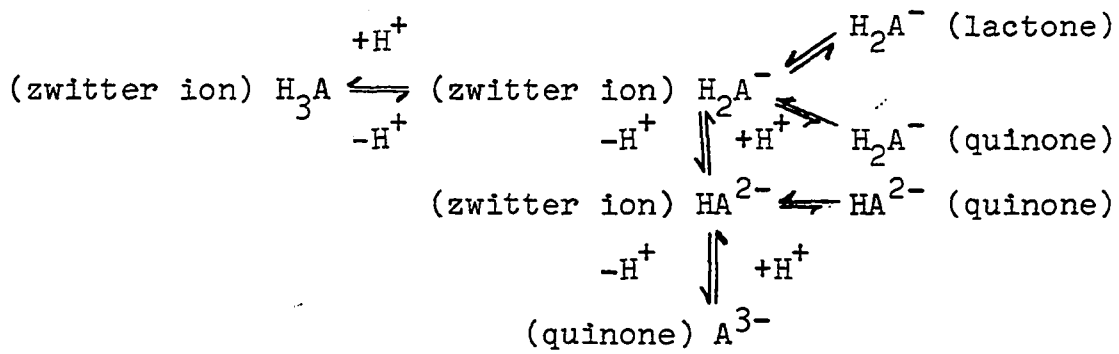
XXV. Phenolphthalein



XXVI. 3,6-Dihydroxy-9,9-dimethylxanthene

enhancement in the acidic character of the phenolic groups in fluorescein as being the result of the electron withdrawing property of the positive charge on the pyrylium ring.

Adopting Markuszewski's proposal, supplemented with my deductions based on absorbance, fluorescence and solubility studies and on potentiometric titration, I propose the following dissociation steps for the monocation of fluorescein and for the neutral molecule of sulfofluorescein. Using the symbol H_3A for convenience,



The large value of the first dissociation constant of 4-sulfofluorescein, $pK_1 = 1.2$, as compared with those of the β-isomers of sulfofluorescein and of fluorescein, $pK_1 = 2.2$, was explained above in terms of the tendency of the monoanion of 4-sulfofluorescein to form the lactone more readily than the other three compounds. Thus, the equilibrium of the first dissociation step is shifted favoring the ionization of the neutral molecule. Furthermore, the existence of the monoanion in large part in the lactone form, Structure XVc, the first dissociation constant of 4-sulfofluorescein will approach that of a sulfonic acid.

Again, the scheme presented above accounts for the smaller value of the second dissociation constant of

4-sulfofluorescein, $pK_2 = 5.3$, as compared with those of the β-isomers of sulfofluorescein and of fluorescein, $pK_2 = 4.4$. Two reasons are offered; first, the fact that the monoanion of 4-sulfofluorescein favors the lactone form, the enhancement of the positive charge on the pyrylium ring is expected to be less, leading to a smaller acid dissociation constant; second, the formation of the lactone will shift the equilibrium of the second dissociation step by decreasing the degree of ionization of the monoanion, again leading to a smaller acid dissociation constant.

The third dissociation constant of the four compounds is of the same magnitude, $pK_3 = 6.3$. For all the four compounds have the same type equilibrium involved in the third dissociation step, as presented above.

F. Distribution of the Ionic Forms of the Sulfofluoresceins as a Function of pH

Each isomer of the sulfofluoresceins is a tribasic acid H_3Sfl, and which could exist in different ionic species in solution, namely H_2Sfl^-, $HSfl^{2-}$, and Sfl^{3-}. To obtain an overall picture of the distribution of each species in solution as a function of pH, and to correlate these distributions to the absorbance, fluorescence, solubility studies and to potentiometric titration, the following equations were derived.

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

$$C = [H_3Sfl] + [H_2Sfl^-] + [HSfl^{2-}] + [Sfl^{3-}]$$

and the fraction α_n, where (n) is the number of replaceable hydrogen atoms associated with each of the species in solution, can be defined as:

$$\alpha_3 = \frac{[H_3Sfl]}{C} \text{ and } \frac{1}{\alpha_3} = 1 + \frac{[H_2Sfl^-]}{[H_3Sfl]} + \frac{[HSfl^{2-}]}{[H_3Sfl]} + \frac{[Sfl^{3-}]}{[H_3Sfl]}$$

$$\alpha_2 = \frac{[H_2Sfl^-]}{C} \text{ and } \frac{1}{\alpha_2} = \frac{[H_3Sfl]}{[H_2Sfl^-]} + 1 = \frac{[HSfl^{2-}]}{[H_2Sfl^-]} + \frac{[Sfl^{3-}]}{[H_2Sfl^-]}$$

$$\alpha_1 = \frac{[HSfl^{2-}]}{C} \text{ and } \frac{1}{\alpha_2} = \frac{[H_3Sfl]}{[HSfl^{2-}]} + \frac{[H_2Sfl^-]}{[HSfl^{2-}]} + 1 + \frac{[Sfl^{3-}]}{[HSfl^{2-}]}$$

$$\alpha_0 = \frac{[Sfl^{3-}]}{C} \text{ and } \frac{1}{\alpha_0} = \frac{[H_3Sfl]}{[Sfl^{3-}]} + \frac{[H_2Sfl^-]}{[Sfl^{3-}]} + \frac{[HSfl^{2-}]}{[Sfl^{3-}]} + 1$$

The above ratios can be expressed in terms of the hydrogen ion concentration $[H^+]$ and the acid dissociation constants, by using the following equations:

$$K_1 = K_{H_3Sfl} = \frac{[H_2Sfl^-][H^+]}{[H_3Sfl]} \text{ and } \frac{[H_2Sfl^-]}{[H_3Sfl]} = \frac{K_1}{[H^+]}$$

$$K_2 = K_{H_2Sfl^-} = \frac{[HSfl^{2-}][H^+]}{[H_2Sfl^-]} \text{ and } \frac{[HSfl^{2-}]}{[H_2Sfl^-]} = \frac{K_2}{[H^+]}$$

$$K_3 = K_{HSfl^{2-}} = \frac{[Sfl^{3-}][H^+]}{[HSfl^{2-}]} \quad \text{and} \quad \frac{[Sfl^{3-}]}{[HSfl^{2-}]} = \frac{K_3}{[H^+]}$$

Substitution and rearrangements yield:

$$\alpha_3 = \frac{[H^+]^3}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

$$\alpha_2 = \frac{K_1[H^+]^2}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

$$\alpha_1 = \frac{K_1K_2[H^+]}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

$$\alpha_0 = \frac{K_1K_2K_3}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

1. 4-Sulfofluorescein

By using the average values for the acidsdissociation constants of 4-sulfofluorescein, Table 22, the fractions of each species can be calculated and plotted as a function of

$pK_{H_3Sfl} = 1.20$ $\quad K_{H_3Sfl} = 6.31 \times 10^{-2}$

$pK_{H_2Sfl^-} = 5.28$ $\quad K_{H_2Sfl^-} = 5.25 \times 10^{-6}$

$pK_{HSfl^{2-}} = 6.25$ $\quad K_{HSfl^{2-}} = 5.62 \times 10^{-7}$

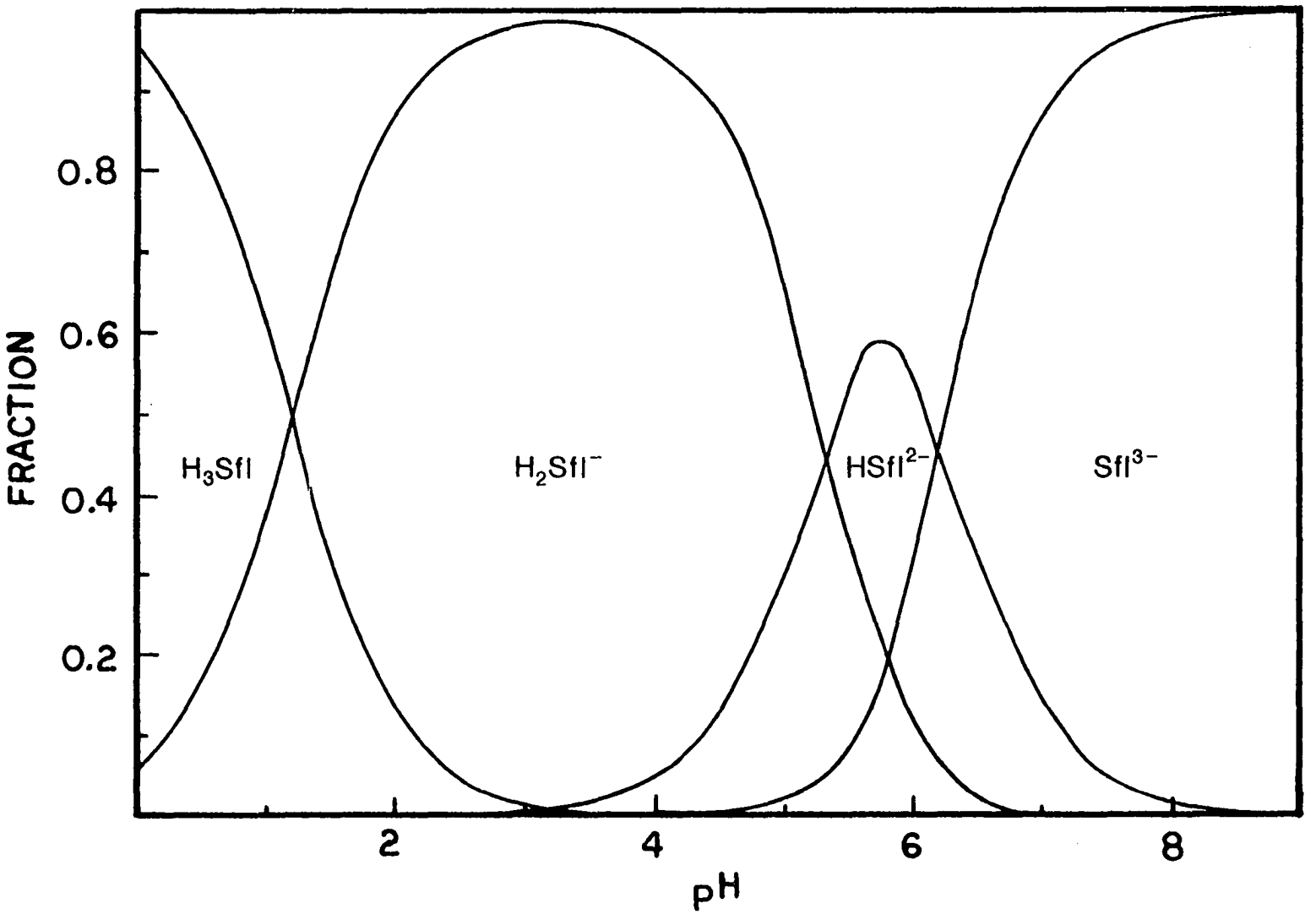
Table 22. The acid dissociation constants of fluorescein and the sulfofluoresceins

	pK_1	pK_2	pK_3
Fluorescein[a]	2.14	4.73	6.55
4-Sulfofluorescein	1.20	5.28	6.25
5-Sulfofluorescein	2.14	4.34	6.27
6-Sulfofluorescein	2.15	4.39	6.25

[a]Average values determined and reported by Markuszewski (3).

hydrogen ion concentration. The graph is presented in Figure 60. There are definite correlations between this figure and the different measurements in the previous sections; at pH about 3.2 at which the fraction of H_3Sfl is minimum and that of H_2Sfl^- is maximum, corresponds to the minimum absorbance, Figure 26, and to the minimum solubility, Figure 48. Also, the maximum concentration of $HSfl^{2-}$ occurs at pH about 5.8 and corresponds to the maximum absorbance, Figure 26. Again, the maximum fraction of Sfl^{3-} occurs at pH about 8.0 and above, corresponds to the leveling off in absorbance, Figures 25 and 27, in fluorescence, Figure 40, and the second end-point on the titration curve, Figure 57.

Figure 60. Relative distribution of the prototropic forms of 4-sulfofluorescein (H_3Sfl, H_2Sfl^-, $HSfl^{2-}$ and Sfl^{3-}) as a function of pH.


FRACTION
0.8
0.6
0.4
0.2
H_3Sfl
H_2Sfl^-
$HSfl^{2-}$
Sfl^{3-}
2
4
6
8
pH

Finally, the rise in the concentration of Sfl^{3-} at pH 4.5 and above is almost superimposable on the increase in fluorescence as a function of pH, Figure 40. Again, the decrease in absorbance, Figure 26, at pH 0 and above is almost superimposable on the decrease of H_3Sfl as a function of pH.

2. 5-Sulfofluorescein

By using the average values for the acid dissociation constants of 5-sulfofluorescein, Table 22, the fractions of

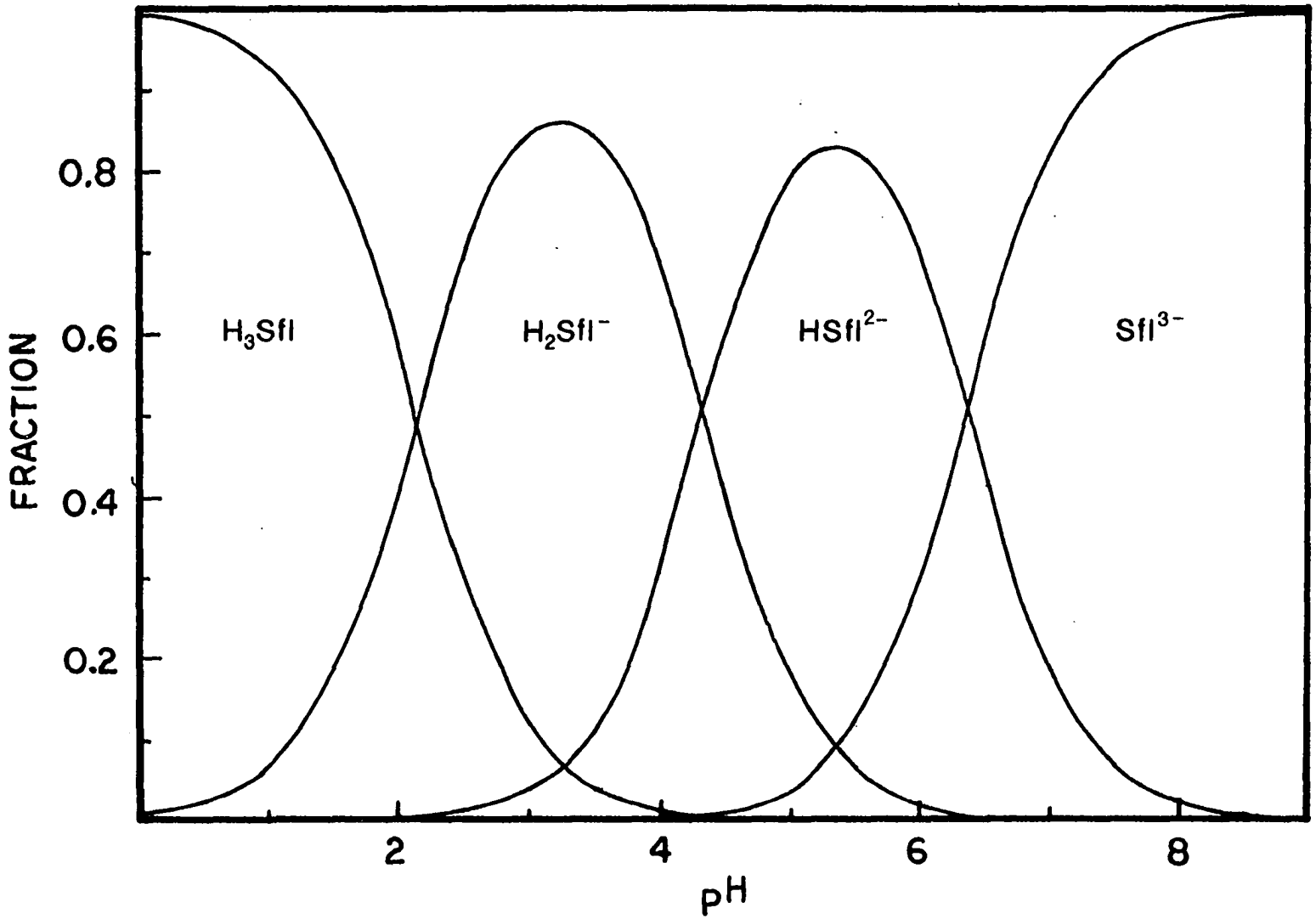
$$pK_{H_3Sfl} = 2.14 \qquad K_{H_3Sfl} = 7.24 \times 10^{-3}$$

$$pK_{H_2Sfl^-} = 4.34 \qquad K_{H_2Sfl^-} = 4.57 \times 10^{-5}$$

$$pK_{HSfl^{2-}} = 6.27 \qquad K_{HSfl^{2-}} = 5.37 \times 10^{-7}$$

each species can be calculated and plotted as a function of hydrogen ion concentration. The graph is presented in Figure 61. The relative concentrations are correlated with the previous observations; the maximum concentration of H_2Sfl^- at pH about 3.4 corresponds to the minimum absorbance, Figure 31, the maximum concentration of $HSfl^{2-}$ at pH about 5.5 corresponds to the first end-point on the titration curve, Figure 58, finally, the maximum concentration of Sfl^{3-} at pH about 8.0 and above, corresponds to the second end-point on the titration curve, Figure 58, to the leveling

Figure 61. Relative distribution of the prototropic forms of 5-sulfofluorescein (H_3Sfl, H_2Sfl^-, $HSfl^{2-}$ and Sfl^{3-}) as a function of pH.


FRACTION
0.8
0.6
0.4
0.2
H_3Sfl
H_2Sfl^-
$HSfl^{2-}$
Sfl^{3-}
2
4
6
8
pH

off in absorbance, Figures 31 and 33, and to that in fluorescence, Figure 43. Again, the rise in relative concentration of Sfl^{3-} at pH about 4.0 and above is almost superimposable on the increase of fluorescence as a function of pH, Figure 43.

3. 6-Sulfofluorescein

Again by using the average values for the acid dissociation constants of 6-sulfofluorescein, Table 22, the

$$pK_{H_3Sfl} = 2.15 \qquad K_{H_3Sfl} = 7.08 \times 10^{-3}$$

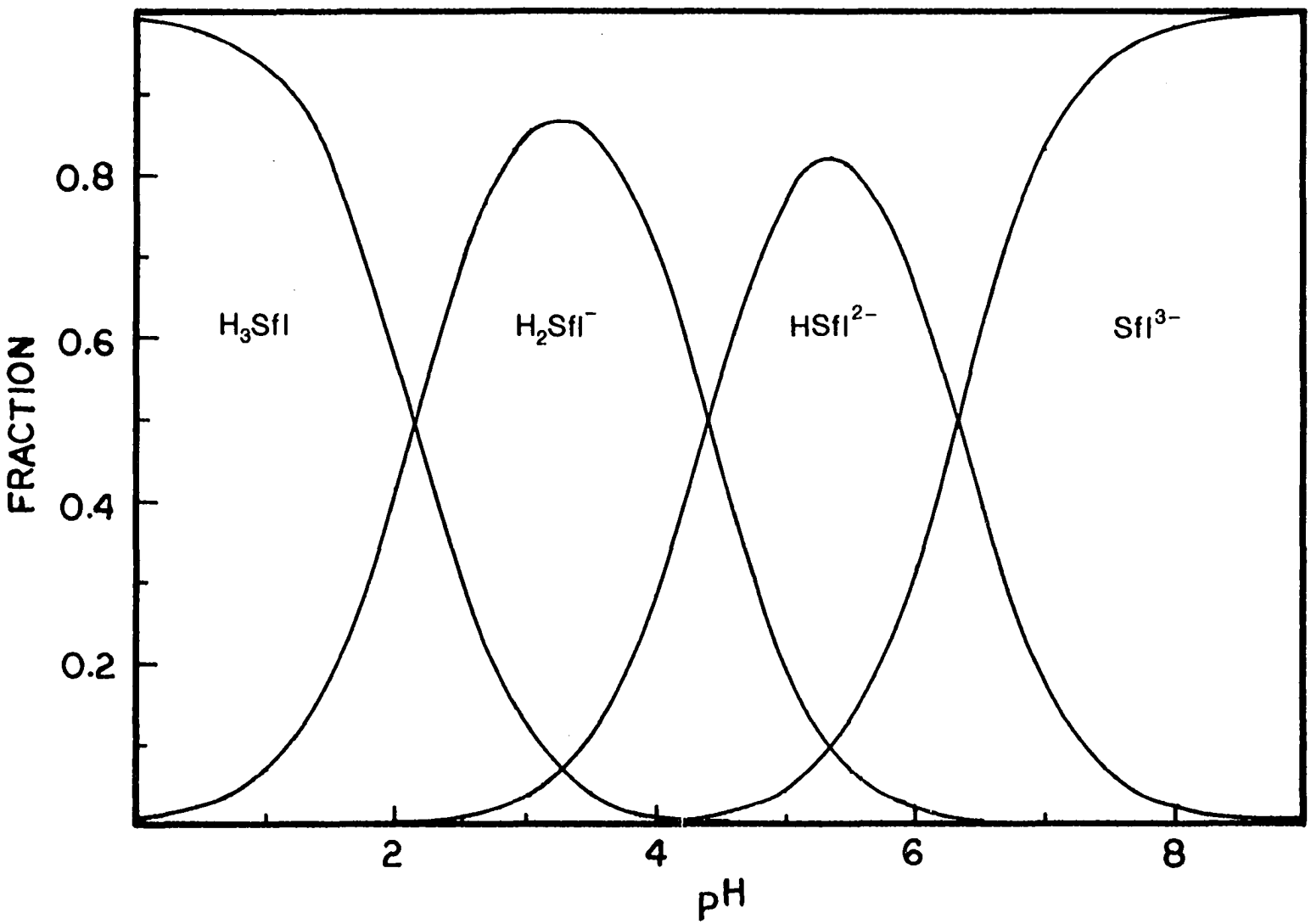
$$pK_{H_2Sfl^-} = 4.39 \qquad K_{H_2Sfl^-} = 4.07 \times 10^{-5}$$

$$pK_{HSfl^{2-}} = 6.25 \qquad K_{HSfl^{2-}} = 5.62 \times 10^{-7}$$

fractions of each species can be calculated and plotted as a function of the hydrogen ion concentration. The graph is presented in Figure 62, and it is almost identical, as expected, to Figure 61 representing the distribution curves of 5-sulfofluorescein as a function of pH.

The correlations between Figure 62 and the previous measurements are; the maximum concentration of H_2Sfl^- at pH about 3.4 corresponds to the minimum absorbance, Figure 32, the maximum concentration of $HSfl^{2-}$ at about pH 5.5 corresponds to the first end-point on the titration curve, Figure 59. Finally, the maximum concentration of Sfl^{3-} at

Figure 62. Relative distribution of the prototropic forms of 6-sulfofluorescein (H_3Sfl, H_2Sfl^-, $HSfl^{2-}$ and Sfl^{3-}) as a function of pH.


FRACTION
0.8
0.6
0.4
0.2
H_3Sfl
H_2Sfl^-
$HSfl^{2-}$
Sfl^{3-}
2
4
6
8
pH

about pH 8.0 and above corresponds to the second end-point on the titration curve, Figure 59, and to the leveling off in absorbance, Figures 32 and 34, and to that in fluorescence, Figure 46. Again, the increase in fluorescence as a function of pH at pH about 4.0 and above, Figure 46, is almost superimposable on the rise in relative concentration of Sfl^{3-}.

IX. FLUORESCEIN AND THE SULFOFLUORESCEINS IN ETHANOL-WATER SOLVENT

A. Introduction

In my thesis for the M.S. degree (11), I reported that a marked difference exists between the acid-base properties of the sulfofluoresceins in water and in 50 per cent ethanol. In the titration curve of β-sulfofluorescein (a mixture of the isomers, 5-sulfofluorescein and 6-sulfofluorescein) titrated potentiometrically in 50 per cent ethanol, two points of inflection appeared, at one equivalent and at three equivalents of base added per mole of compound. In the curve of the same isomeric mixture, titrated potentiometrically in water, two points of inflection appeared, at two equivalents and at three equivalents of base added.

Once the isomeric mixtures, α-sulfofluorescein (4-sulfofluorescein and 7-sulfofluorescein) and β-sulfofluorescein (5-sulfofluorescein plus 6-sulfofluorescein) were resolved and the pure 4-, 5-, and 6-sulfofluoresceins made available, it became possible to study the acid-base chemistry of each in water, and in 50 per cent ethanol. Information from potentiometric titrations and absorbance as a function of pH was brought to bear on the problem.

B. Potentiometric Titration in 50 Per Cent Ethanol

1. Apparatus and procedure

A weighed amount, about 0.11 g, of the purified and dried sulfofluorescein in the acid form was dissolved in about 150 ml of 50 per cent ethanol and titrated with 0.1003 N sodium hydroxide. Throughout the course of the titration, the expanded scale of the pH meter was used and the reading was recorded after equilibrium had been reached. A Hach Model 8596 pH meter was used. It was calibrated against two standard buffer solutions prepared according to the specifications of the National Bureau of Standards.

2. 4-Sulfofluorescein

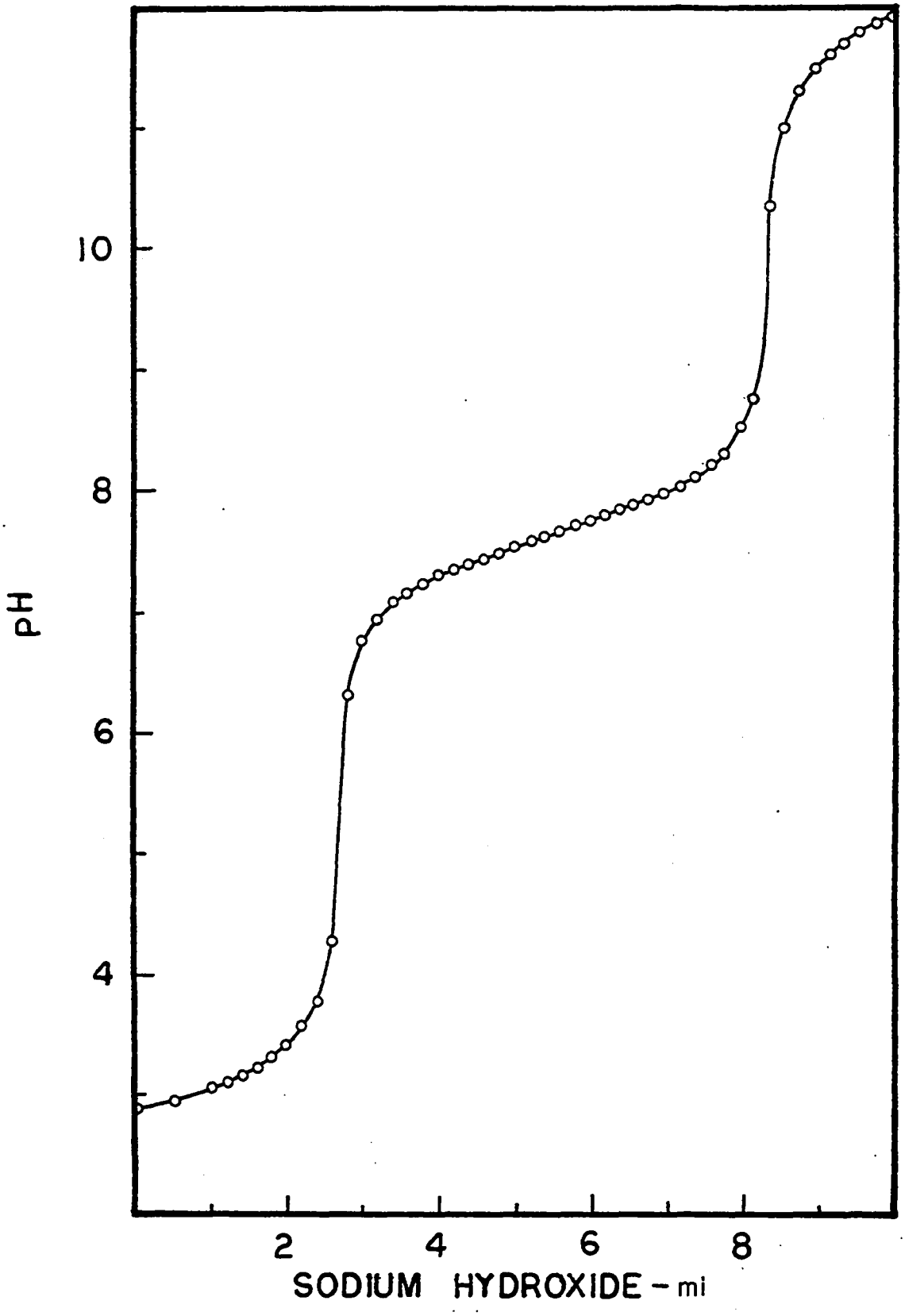
The titration curve of 4-sulfofluorescein is presented in Figure 63. Two sharp end-points appear, at one and at three equivalents of base per mole of compound. The curve differs markedly from that obtained in water solution, Figure 57. The end-points in both titration curves appear at one and at three equivalents of base added per mole of compound but the pH at the mid-points and end-points are very different:

	Water	50 Per Cent Ethanol
First end-point (at one equivalent)	4.08	5.35
Mid-point	5.75	7.65
Second end-point (at three equivalents)	8.65	10.00

Figure 63. Potentiometric titration of 4-sulfofluorescein in 50 per cent ethanol.

4-Sulfofluorescein: 0.1159 g

Titrant: 0.1003 N sodium hydroxide


10
8
6
4
pH
2
4
6
8
SODIUM HYDROXIDE - mi

On the basis of elementary equilibrium theory, and when the magnitude of the first acid dissociation constant is larger than the analytical concentration of the acid titrated, Chapter VIII, Part B, the pH value at the second mid-point could be used to estimate the sum of pK_2 and pK_3 divided by 2. In the titration curve of the compound in 50 per cent ethanol, the second mid-point occurs at pH 7.65. This value is in contrast to that of the second mid-point of the titration curve in water, Figure 57, pH 5.75. This is an indication that the second and the third protons are weaker acids in 50 per cent ethanol than in water. The effect of ethanol is further emphasized in following material on the absorption spectrum. This effect of changing the solvent from water to 50 per cent ethanol is far greater than that experienced in making such a change with a simple monobasic or polybasic acid for which such a change in solvent changes the value of pH by only a few tenths of a unit. In any case, it is apparent that the second proton has become a far weaker acid in 50 per cent ethanol and the third proton has also become a weaker acid. Such big changes probably result from some shift in the proportion of the structural forms, lactone and zwitter ion-pyrylium ring, present of a given prototropic form.

3. 5-Sulfofluorescein

The titration curve is presented in Figure 64. Two points of inflection in vertical portions of the curve are present, at one equivalent and at three equivalents of base added per mole of the compound. The first point of inflection appears at pH 4.55, the second at pH 10.05, and the second mid-point at pH 6.85.

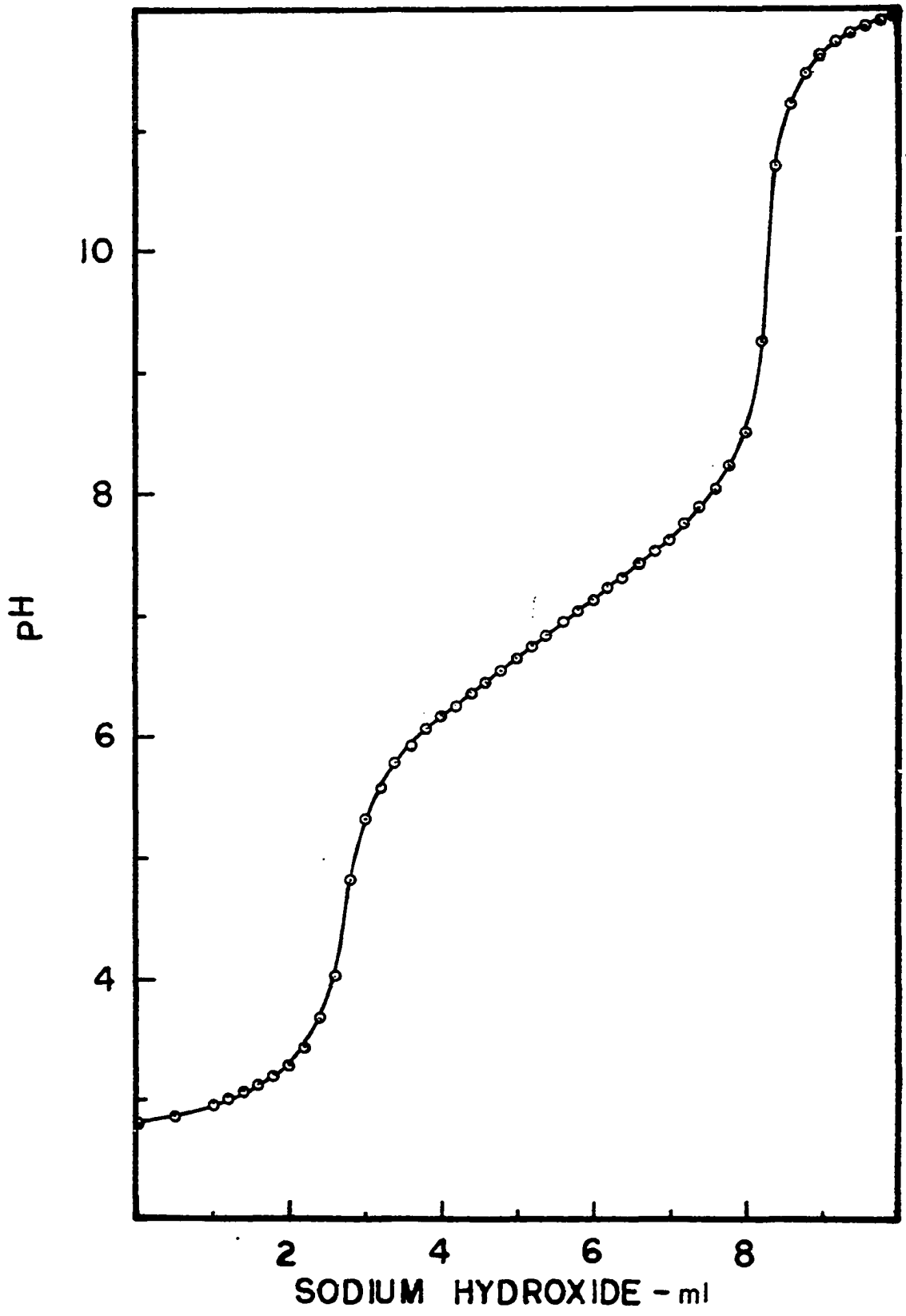
A significant difference appears in the curves obtained by titrating the acid in water, Figure 58, and in 50 per cent ethanol, Figure 64. In the titration curve in water, two points of inflection appeared, at two and at three equivalents of base added per mole of compound. The first end-point marked the neutralization of the carboxylic proton and one of the phenolic protons, the second end-point marked the neutralization of the second phenolic proton. The titration curve of 5-sulfofluorescein in 50 per cent ethanol, Figure 64, is similar to that of 4-sulfofluorescein in water, Figure 57. Two points of inflection are present, at one and at three equivalents of base added. The first end-point marks the neutralization of the carboxylic proton separately, and the second end-point marks the neutralization of the two phenolic protons.

The observations stated above indicate that the first replaceable hydrogen atom of 5-sulfofluorescein, that is the carboxylic proton, is a stronger acid in 50 per cent

Figure 64. Potentiometric titration of 5-sulfofluorescein in 50 per cent ethanol.

5-Sulfofluorescein: 0.1155 g

Titrant: 0.1003 N sodium hydroxide


pH
10
8
6
4
2
4
6
8
SODIUM HYDROXIDE - ml

ethanol than in water, and the second and third replaceable hydrogen, that is the phenolic protons, are weaker acids in 50 per cent ethanol than in water. The fact that the first two replaceable hydrogen atoms are titrated separately in 50 per cent ethanol indicates that the difference, pK_2-pK_1, is greater than 3. This is in contrast to the difference, pK_2-pK_1, is less than 3 in water. Furthermore, the second mid-point, between the first and the second inflection points on the titration curve in 50 per cent ethanol occurs at pH 6.85, and corresponds to the sum of pK_2 and pK_3 divided by 2. This value is in contrast to that of the first end-point of the titration curve in water, at pH 5.33.

The reason for this marked difference in the acid-base properties of 5-sulfofluorescein, as the solvent is changed from water to 50 per cent ethanol, will become evident when the absorbance spectra of the compound in 50 per cent ethanol are presented in the following material.

4. 6-Sulfofluorescein

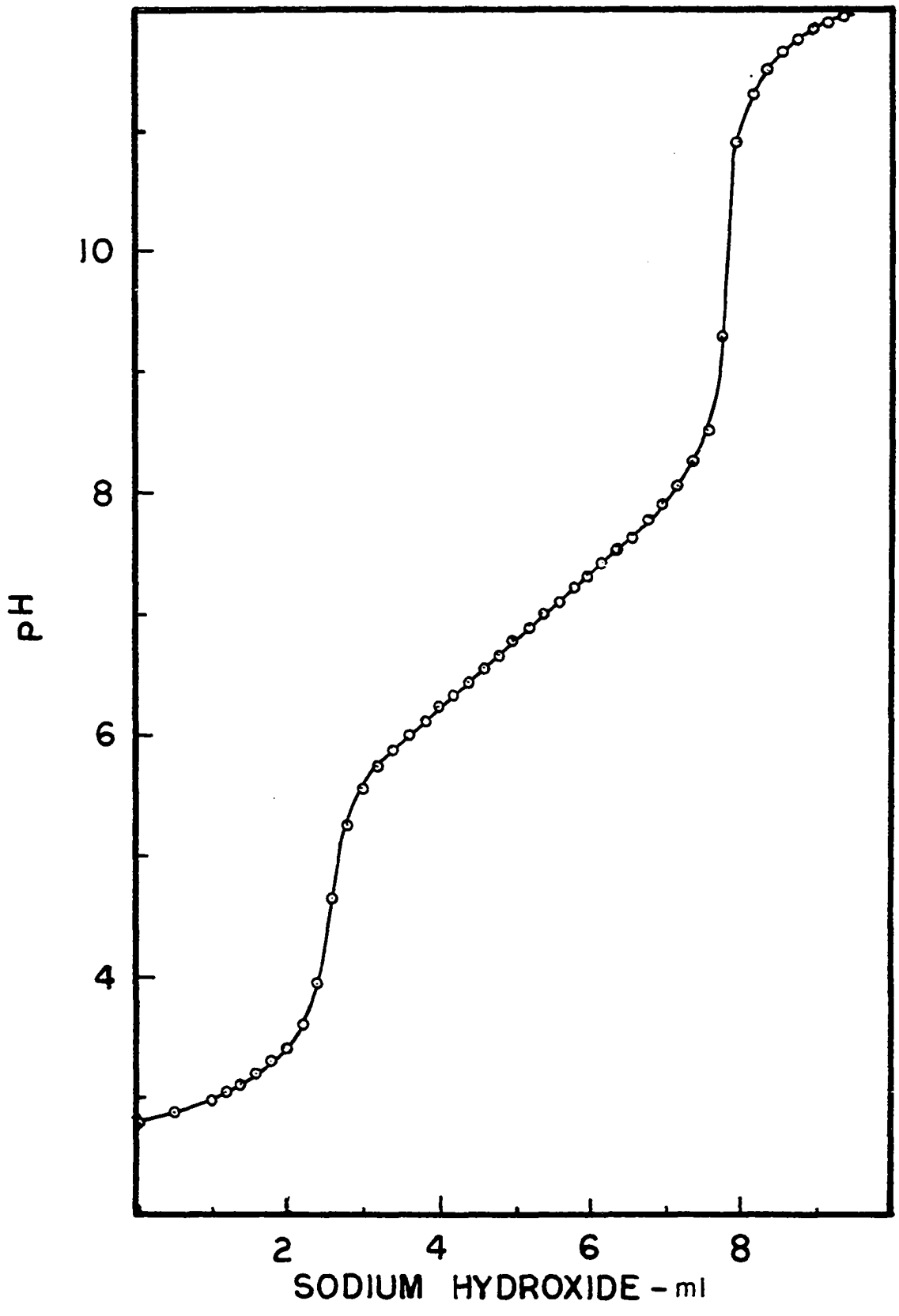
The titration curve of 6-sulfofluorescein is presented in Figure 65. Two points of inflection are present, at one and at three equivalents of base added per mole of compound, the first occurring at pH 4.65, the second at pH 10.00, and the second mid-point at pH 6.84.

Comparison of the curves obtained by titrating the acid in water, Figure 59, and in ethanol-water, Figure 65, shows

Figure 65. Potentiometric titration of 6-sulfofluorescein in 50 per cent ethanol.

6-Sulfofluorescein: 0.1131 g

Titrant: 0.1003 N sodium hydroxide


10
8
6
4
pH
2
4
6
8
SODIUM HYDROXIDE - ml

the same difference in behavior as described in the preceding section. The first point of inflection in water corresponds to the titration of the first and the second replaceable hydrogen atoms, but in 50 per cent ethanol the first point of inflection corresponds to the titration of the first replaceable hydrogen atom only.

In the titration curve of the compound in 50 per cent ethanol, the second mid-point occurs at pH 6.84, and corresponds to the sum of pK_2 and pK_3 divided by 2. This is in contrast to the first end-point in the titration curve in water, Figure 59, at pH 5.34.

5. Fluorescein

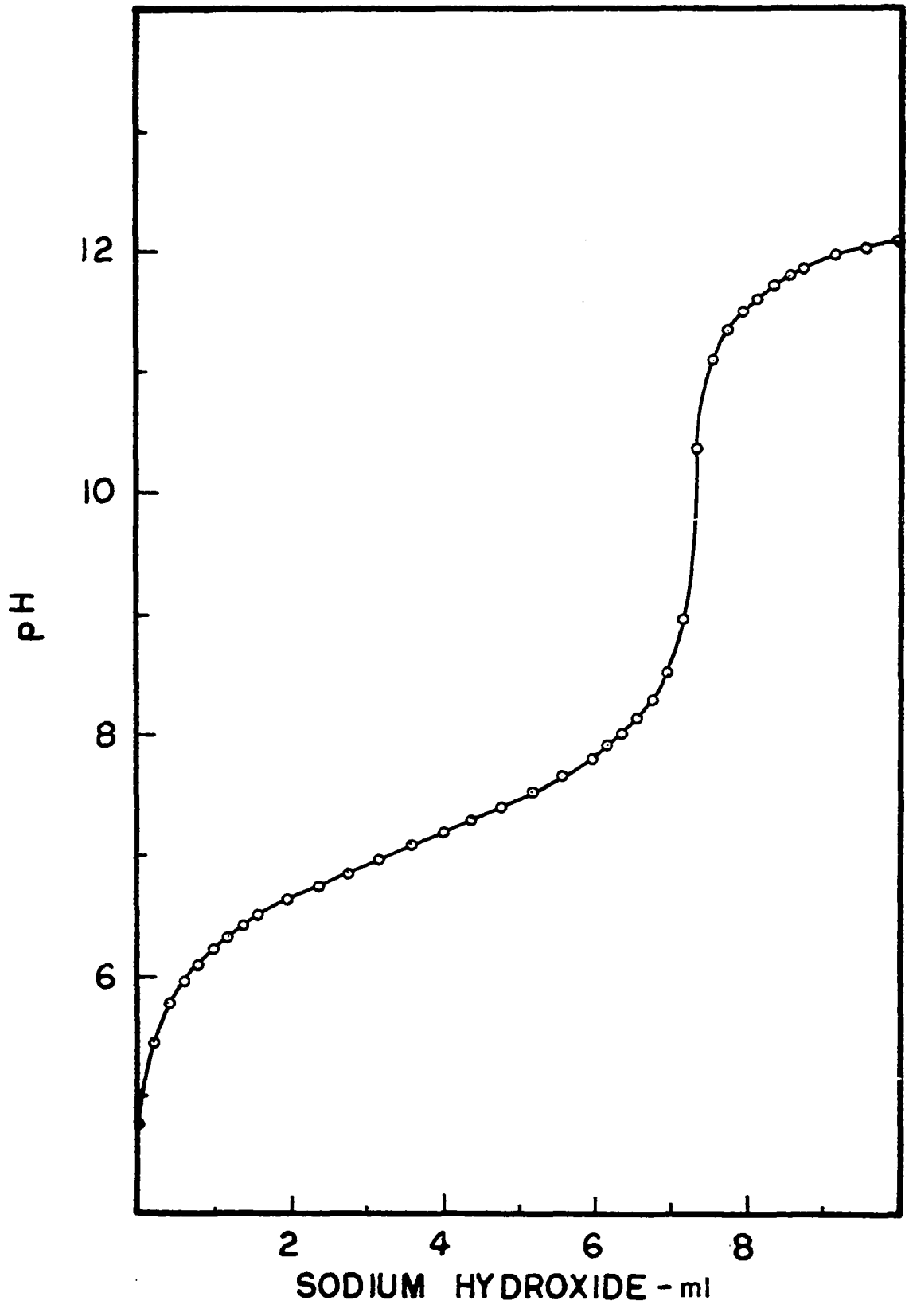
An appropriate amount of yellow fluorescein, purified through the diacetyl derivative, was dissolved in about 150 ml of 50 per cent ethanol and titrated with 0.1003 N sodium hydroxide.

The titration curve is presented in Figure 66. The only point of inflection present occurs at pH 10.00 and corresponds to the addition of two equivalents of base per mole of compound. Interestingly enough, this point of inflection occurs in the same region in which the second point of inflection occurs in the titration of the sulfofluoresceins. The titration curve also begins in the same pH region, 4.7, in which the first points of inflection for 5- and 6-sulfofluorescein occur. This is not surprising

Figure 66. Potentiometric titration of yellow fluorescein in 50 per cent ethanol.

Yellow fluorescein: 0.1230 g

Titrant: 0.1003 N sodium hydroxide


12
10
8
6
pH
2
4
6
8
SODIUM HYDROXIDE - ml

because the acid dissociation constants of fluorescein and the β-isomers of sulfofluorescein in aqueous solution are almost identical.

6. Discussion

The titration curves of 4- and 5-sulfofluorescein, Figures 64 and 65, in 50 per cent ethanol are similar to the titration curve of 4-sulfofluorescein in water, Figure 57; the first replaceable hydrogen atom is titrated separately from the second and the third replaceable hydrogen atoms. In a previous discussion, the marked difference between 4-sulfofluorescein and the β-isomers was attributed to the existence of the monoanion, H_2Sfl^-, of 4-sulfofluorescein in the lactone form; it was explained how this leads to a greater value for the first dissociation constant, and to a smaller value for the second dissociation constant, so much that the difference pK_2-pK_1 became greater than 3.

Inasmuch as the β-isomers in 50 per cent ethanol behave similarly to 4-sulfofluorescein in water, the same conclusion is drawn, that is, the major proportion of the monoanion of the β-isomers in 50 per cent ethanol exist as the colorless lactone. This conclusion is to be confirmed in the following section by the measurements of the absorbance of the sulfofluorescein and of fluorescein in ethanol-water solvent.

C. Absorption Spectra as a Function of pH

1. Apparatus and procedure

Stock solution of purified and dried yellow fluorescein and of each of the isomers of the sulfofluorescein were prepared by dissolving the appropriate amount of the compound in about 30 ml of 0.1 M potassium hydroxide and diluting exactly to one liter with deionized water. The concentration of fluorescein was 4.0×10^{-4} M, and that of the three sulfofluoresceins was 5.0×10^{-4} M. A 2.00-ml aliquot of the stock solution was placed in 100-ml volumetric flasks and diluted with the appropriate buffer. The final concentration of fluorescein was brought to 8.0×10^{-6} M, and that of the sulfofluoresceins to 1.0×10^{-5} M.

The buffer solutions were prepared by using 50 per cent ethanol solutions of 0.1 M hydrochloric acid, potassium hydrogen phthalate, potassium chloride, potassium hydroxide and boric acid. The apparent pH of the ethanol-water buffer solutions were measured before and after the addition of the 2.00 ml from the stock solutions, using a Hach Model 8594 pH meter set on the expanded scale, and calibrated against standard buffer solutions. A Cary 14 Recording Spectrometer was used to obtain the absorption spectra for each of the compounds under study.

2. 4-Sulfofluorescein

The absorption spectra of 4-sulfofluorescein obtained at pH 1.10, 3.74, 5.51, 7.33 and 9.49. These curves are presented in Figure 67. One absorption band, of very low intensity, is present at 450 nm, indicating that the predominant form is the monoanion, H_2Sfl^-, in the lactone form, Structure XVc. As the pH increases to 3.74 and 5.51, the absorption decreases to zero, again these colorless solutions contain the same species in the lactone form. As the pH increases to pH 7.33 and finally to 9.49, the absorption increases drastically with the formation of the dianion, $HSfl^{2-}$, and the trianion, Sfl^{3-}, and the maximum absorption bands at 503 nm are due to the quinone forms, Structure XVIa and XVIIa, respectively.

A comparison of the absorption spectra in water, Figure 18, and those in ethanol-water, Figure 67, all solutions have the same concentration, reveals that at pH about 1.0 the intensity of absorbance in ethanol-water, 0.04, is much lower than that in water, 0.32. Such a large difference could not be attributed only to the change in the molar absorptivity of the neutral molecule, H_3Sfl, but rather to the relative concentration of the colored neutral molecule and of the colorless monoanion, H_2Sfl^-. The fact that the relative concentration of the neutral molecule, H_3Sfl, is minimal at pH 1.0, in ethanol-water, but the monoanion,

Figure 67. Absorption spectra of 4-sulfofluorescein in ethanol-water solvent.

Concentration: 1.0×10^{-5} M

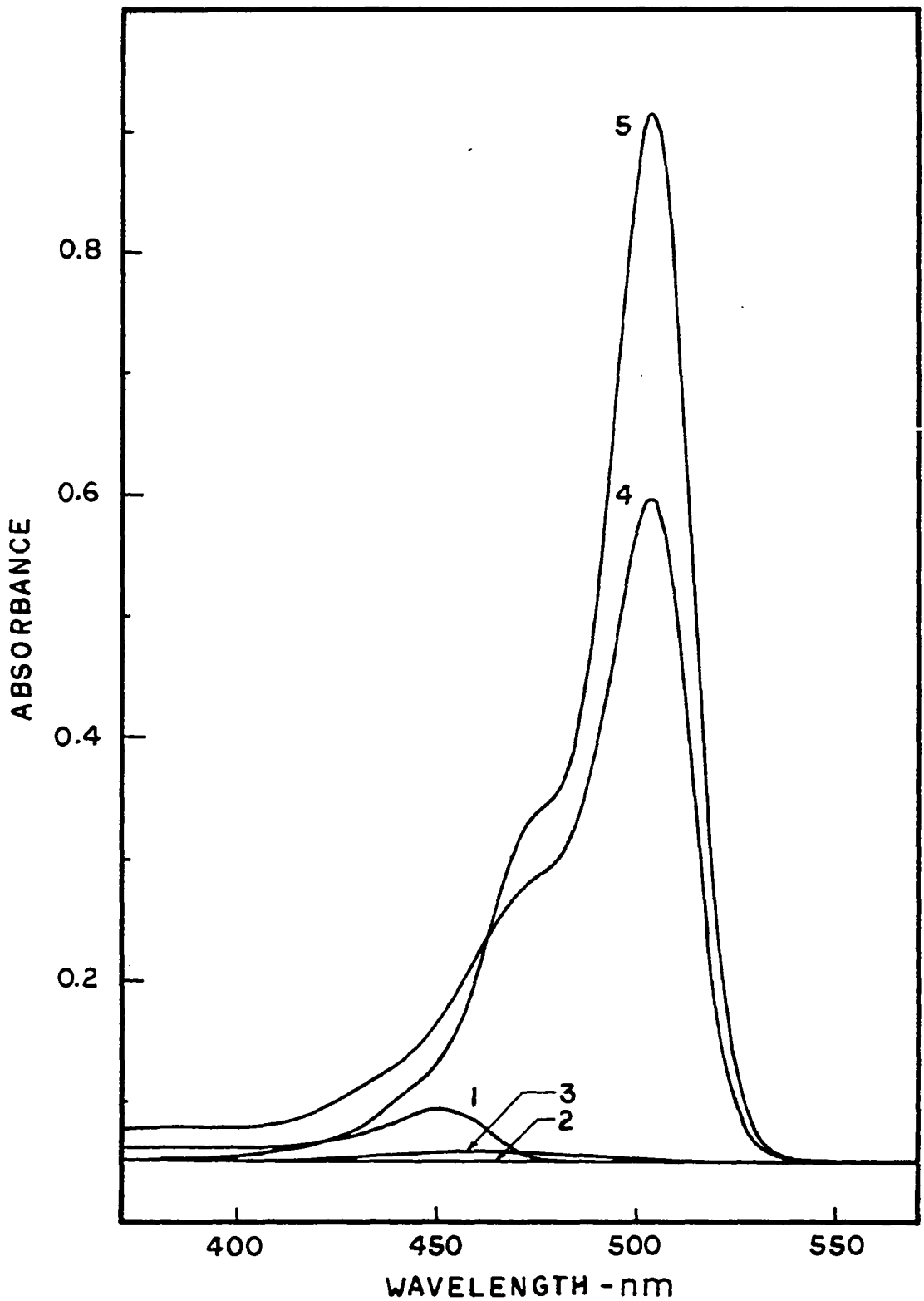
Curve 1: pH 1.10

Curve 2: pH 3.74

Curve 3: pH 5.51

Curve 4: pH 7.33

Curve 5: pH 9.49


ABSORBANCE
0.8
0.6
0.4
0.2
400
450
500
550
WAVELENGTH - nm
1
2
3
4
5

H_2Sfl^-, is maximal, leads to the conclusion that the value of pK_1, for the reaction, $H_3Sfl = H_2Sfl^- + H^+$, in ethanol-water is much less than 1.10. This value is in contrast to that of the $pK_1 = 1.2$ in water. Again, the absorption of the dianion, $HSfl^{2-}$, at pH 5.20 in water is 0.2, much more intense than the absorption in ethanol-water, which is practically zero, at pH 5.51. This observation leads to the conclusion that the relative concentration of the colored dianion is practically zero, but that of the colorless monoanion, H_2Sfl^-, is approaching 1.0. In terms of the second dissociation constant, the value of pK_2 for the reaction, $H_2Sfl^- = HSfl^{2-} + H^+$, in ethanol-water is greater than 5.51. This value is in contrast to the value of $pK_2 = 5.28$ in water. This is consistent with the conclusion based on the comparison of potentiometric titration of 4-sulfofluorescein, in water and in ethanol-water solvent.

3. 5-Sulfofluorescein

The absorption spectra of 5-sulfofluorescein were obtained at pH 1.08, 3.73, 5.50, 7.33 and 9.48. These curves are presented in Figure 68. One absorption band is present at 445 nm in the spectrum at pH 1.08. The absorption of the solution at pH 3.73 is practically colorless, confirming the formation of the lactone, as the pH increases to 5.50 two absorption bands appear at 455 and 480 nm. In the basic region at pH 7.33 and 9.48 there is a drastic

Figure 68. Absorption spectra of 5-sulfofluorescein in ethanol-water solvent.

Concentration: 1.0×10^{-5} M

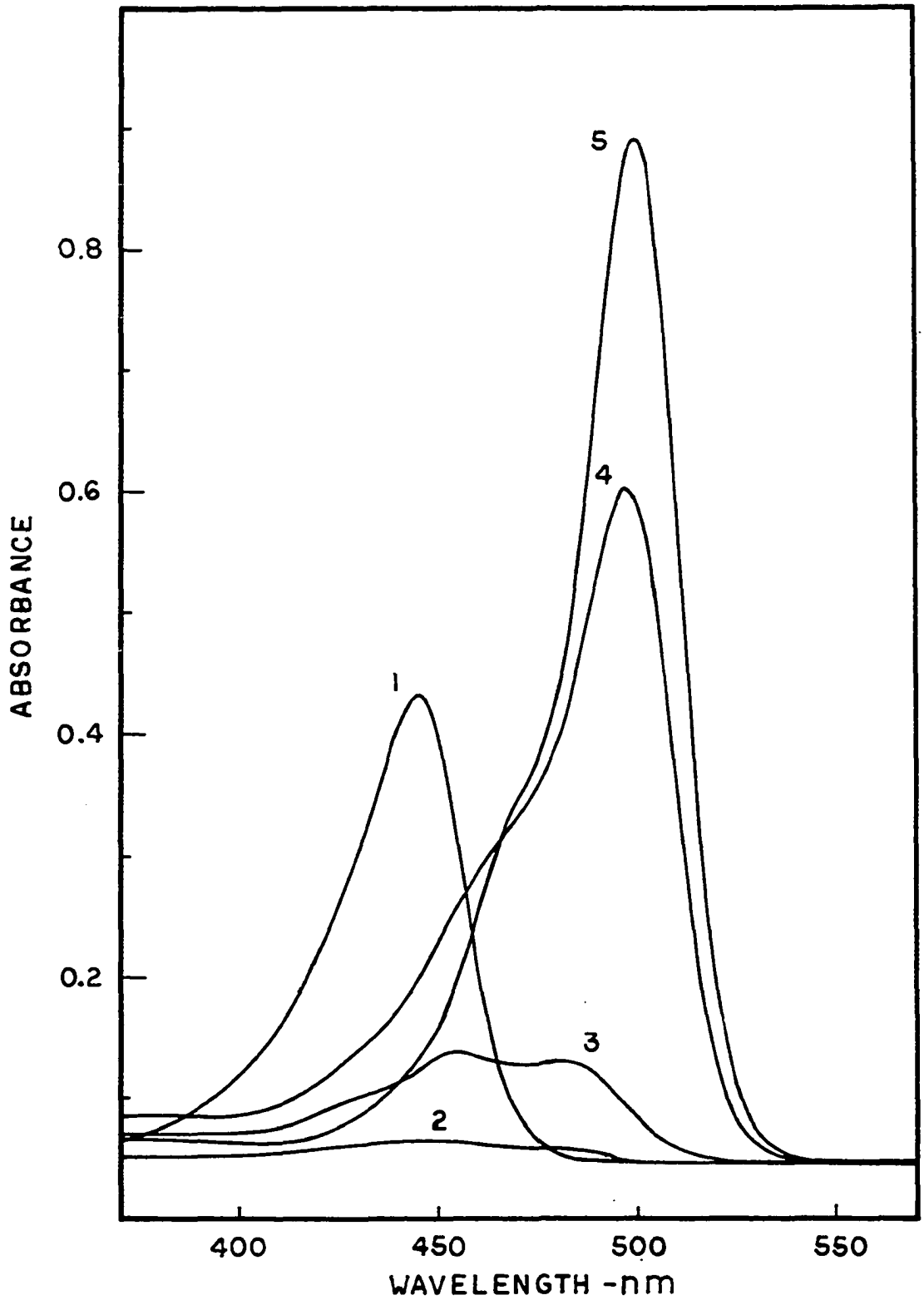
Curve 1: pH 1.08

Curve 2: pH 3.73

Curve 3: pH 5.50

Curve 4: pH 7.33

Curve 5: pH 9.49


ABSORBANCE
0.8
0.6
0.4
0.2
400
450
500
550
WAVELENGTH -nm
1
2
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5

change in the absorbance intensity with a maximum absorbance band at 496 and 498 nm, respectively.

A comparison of the absorption spectra in water, Figure 19, and in ethanol-water, Figure 68, reveals that there is a definite change in the absorbance intensity in the acidic region pH 1.0-5.5, especially that all the solutions have the same analytical concentration with respect to the compound under study. An inspection of the absorbance curve of 5-sulfofluorescein in water as a function of pH, at 443 nm, Figure 31, reveals that the absorbance decreases to reach a minimum at pH about 3.4, at which point the monoanion, H_2Sfl^-, predominates, but never approaches zero. Yet, it is evident that the absorbance in ethanol-water approaches zero at pH about 3.7, Figure 68. Thus, the formation of the lactone by the monoanion is much more favored in ethanol-water solvent than in water.

The decrease in the absorption intensity at the pH values 1.08, 3.73 and 5.50 in ethanol-water, as compared to that in water at the same pH region, is attributed to a higher relative concentration of the colorless monoanion, H_2Sfl^-. Again, by applying the arguments presented above, the same conclusion is drawn, that the apparent values of pK_1 is smaller and pK_2 is larger in ethanol-water than in water. This is consistent with the conclusion based on the potentiometric titrations of the compound as the solvent is changed from water to ethanol-water.

Another observation should be pointed out, that the inspection of the absorption spectra of 5-sulfofluorescein in ethanol-water, Figure 68, in the acidic region, pH 1.0-5.5 reveals close similarity to the absorption spectra of 4-sulfofluorescein in water, Figure 18, with respect to absorbance intensities. Again, this is consistent with the similarity between the titration curves, Figures 64 and 57, with respect to the two end-points in relation to the number of equivalents of base added.

4. 6-Sulfofluorescein

The absorption spectra for 6-sulfofluorescein were obtained at pH 1.09, 3.74, 5.52, 7.33 and 9.48. These curves are presented in Figure 69 and almost identical to the absorption curves of 5-sulfofluorescein, Figure 68. A maximum absorption band is present at 445 nm at pH 1.09. The absorption is practically zero at pH 3.74. As the pH increases to pH 5.52, two bands appear at 455 and 482 nm. And in the basic region at pH 7.33 and 9.48, there is a drastic change in the absorbance intensity at which the maximum absorption bands are at 498 and 500 nm, respectively.

Again, the formation of the lactone at pH 3.74 is evident, and the comparison of the absorption spectra in ethanol-water, Figure 69, and the absorption spectra of 6-sulfofluorescein as a function of pH at 443 nm, Figure 32, it becomes apparent that the lactone is more readily formed

Figure 69. Absorption spectra of 6-sulfofluorescein in ethanol-water solvent.

Concentration: 1.0×10^{-5} M

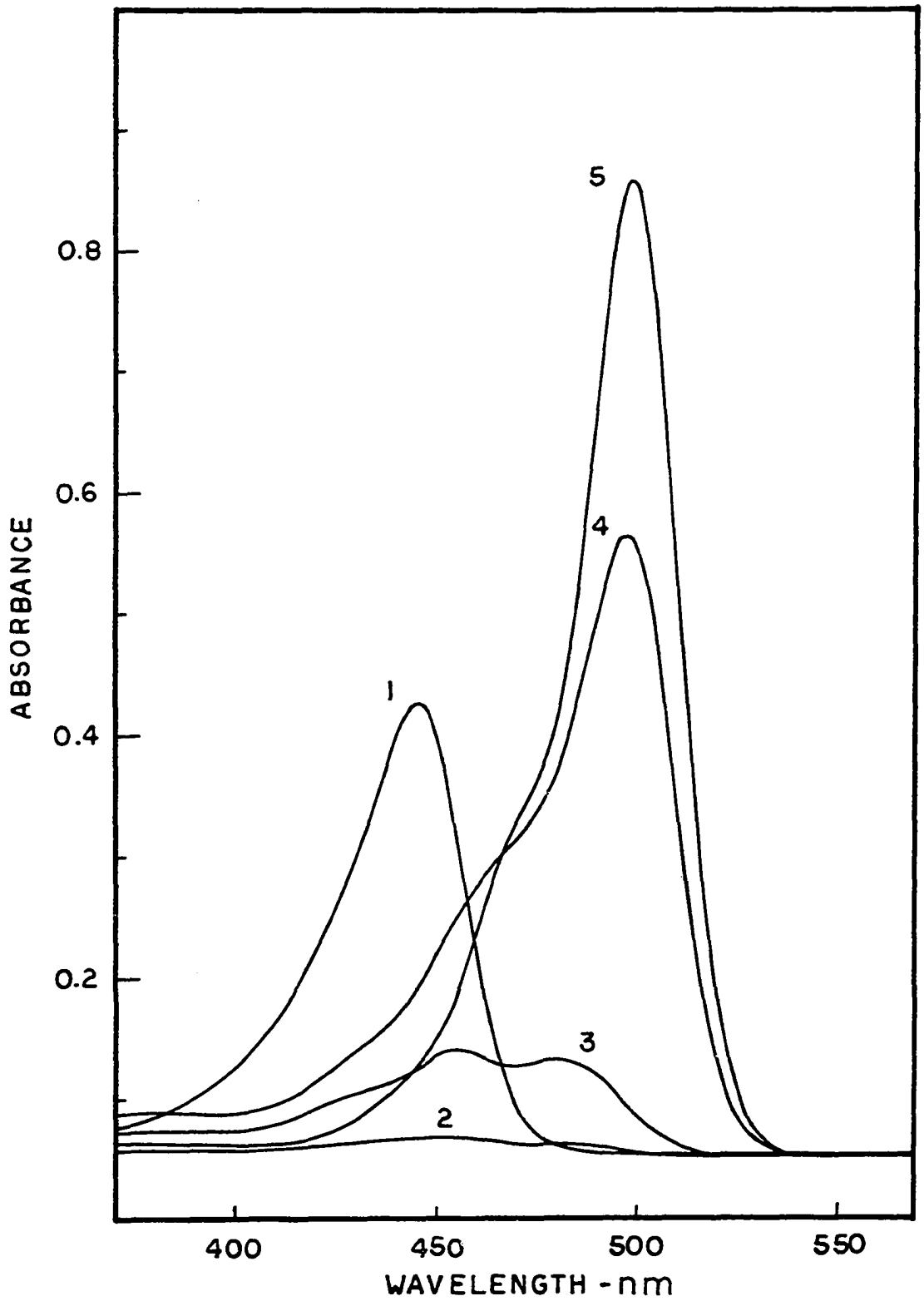
Curve 1: pH 1.09

Curve 2: pH 3.74

Curve 3: pH 5.52

Curve 4: pH 7.33

Curve 5: pH 9.48


ABSORBANCE
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0.4
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WAVELENGTH - nm

by the monoanion, in ethanol-water solvent. And by using the same arguments presented in the previous section, one would come to the same conclusion, that the value of pK_1 is smaller, and of pK_2 is larger in magnitude in ethanol-water than in water solvent. And this difference accounts for the change in the titration curves, as the solvent is changed from water to ethanol-water solvent.

5. Fluorescein

It was of interest to examine the absorbance of fluorescein in ethanol-water solvent to check if the parent compound behaves similarly to the sulfofluorescein, especially since fluorescein is reported to form colorless solution in nonaqueous solvents such as dioxane (3,11,17), and acetone. Yet, the tolerance of these colorless solutions for water is very minimal, upon the addition of a few drops of water, the colorless solution turns yellow.

The absorption spectra of fluorescein in ethanol-water solvent were obtained at pH 1.07, 3.71, 5.53, 7.34 and 9.49. These curves are presented in Figure 70. At pH 1.07, a maximum absorption band is present at 443 nm. At pH 3.71 and 5.53, the absorbance is practically zero. As the pH increases to 7.34 and 9.49, the absorbance increases drastically and the maximum absorption band is at 495 nm.

A comparison of the absorption spectra of the compound in water, Figures 17 and 21, and in ethanol-water, Figure 70,

Figure 70. Absorption spectra of fluorescein in ethanol-water solvent.

Concentration: 8.0×10^{-6} M

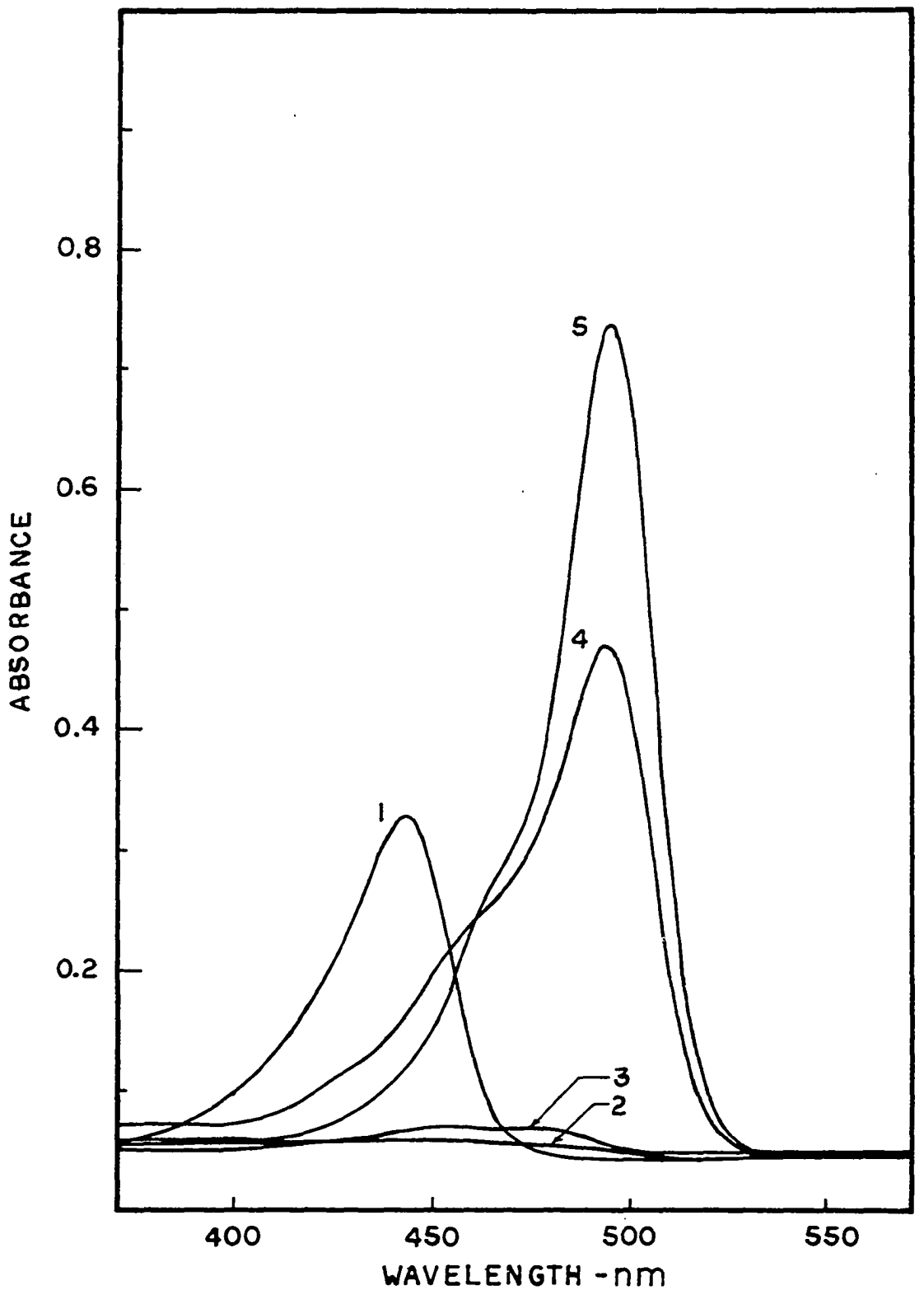
Curve 1: pH 1.07

Curve 2: pH 3.71

Curve 3: pH 5.53

Curve 4: pH 7.34

Curve 5: pH 9.49


0.8
0.6
0.4
0.2
ABSORBANCE
5
4
1
3
2
400
450
500
550
WAVELENGTH - nm

reveals that fluorescein forms the lactone more readily in ethanol-water solvent, at the pH region in which the neutral molecule predominates.

For convenience the absorption bands of fluorescein and the three sulfofluoresceins, in 50 per cent ethanol, are presented in Table 23. The expected predominant species of each of the compounds at the different pH values are indicated. Examination of Table 23 and the absorption spectra of the compounds, Figures 67, 68, 69 and 70, reveals that 4-sulfofluorescein favors the formation of the colorless lactone more than the other three compounds. The same behavior was observed in water.

Table 23. Absorption bands of fluorescein and the sulfofluoresceins in 50 per cent ethanol

Compound	pH	Absorption-band, nm	Predominant Species
Fluorescein	1.07	443	H_3Fl^+, H_2Fl
4-Sulfofluorescein	1.10	450	H_2Sfl^-
5-Sulfofluorescein	1.08	445	H_3Sfl, H_2Sfl^-
6-Sulfofluorescein	1.09	445	H_3Sfl, H_2Sfl^-
Fluorescein	3.71	Colorless	H_2Fl
4-Sulfofluorescein	3.74	Colorless	H_2Sfl^-
5-Sulfofluorescein	3.73	Colorless	H_2Sfl^-
6-Sulfofluorescein	3.74	Colorless	H_2Sfl^-

Table 23. (Continued)

Compound	pH	Absorption-band, nm	Predominant Species
Fluorescein	5.53	Colorless	H_2Fl
4-Sulfofluorescein	5.51	Colorless	H_2Sfl^-
5-Sulfofluorescein	5.50	445, 480	H_2Sfl^-, $HSfl^{2-}$
6-Sulfofluorescein	5.52	445, 482	H_2Sfl^-, $HSfl^{2-}$
Fluorescein	7.34	496	HFl^- and Fl^{2-}
4-Sulfofluorescein	7.33	503	$HSfl^{2-}$ and Sfl^{3-}
5-Sulfofluorescein	7.33	496	$HSfl^{2-}$ and Sfl^{3-}
6-Sulfofluorescein	7.33	498	
Fluorescein	9.49	495	Fl^{2-}
4-Sulfofluorescein	9.49	503	Sfl^{3-}
5-Sulfofluorescein	9.48	498	Sfl^{3-}
6-Sulfofluorescein	9.48	500	Sfl^{3-}

6. Results and discussion

The study of the absorption in the visible of fluorescein and of the sulfofluoresceins in ethanol-water has confirmed the proposal advanced in previous sections that these compounds over a certain range of pH, at which the

neutral molecule of fluorescein, and the monoanion of the sulfofluorescein predominate, do assume the colorless lactone form even in aqueous solutions. In the past, the green-yellowish color of fluorescein in water has concealed the fact that part of the compound is in the colorless form.

In previous sections an argument was advanced explaining the unusual acid-base behavior of 4-sulfofluorescein in water, and this behavior was related to the formation of the lactone by the monoanion of the compound. In the case of the β-isomers of sulfofluorescein and fluorescein once the equilibrium shifted to favor the formation of the lactone in 50 per cent ethanol, the net result was a drastic change in the acid-base properties of the compounds. This change was manifested in the difference between the titration curves of the compounds in water and in 50 per cent ethanol as explained above. Thus, the effect of the formation of the lactone on the acid dissociation constants of the sulfofluoresceins is confirmed further.

The chemistry of fluorescein and of the sulfofluorescein in ethanol-water should receive a special attention. Based on empirical observations, the solubility of fluorescein and of the sulfofluoresceins in either water or in absolute alcohol is much less than the solubility in ethanol-water. The ethanol-water solvent proved effective in separating the β-isomers, 5- and 6-sulfofluorescein. The presence of

ethanol had a drastic effect on the absorbance and acid-base properties of fluorescein and on the three isomers of sulfofluoresceins. All these observations and others could be related to the different acid-base chemistry and in turn to the structures assumed by fluorescein and its derivative in ethanol-water solutions.

A series of experiments could prove very beneficial in correlating and explaining further the chemistry of fluorescein in solution, that is, to study acid-base behavior, solubility, absorbance and fluorescence of fluorescein as a function of per cent ethanol in water and of pH.

X. PREPARATION AND CHARACTERIZATION OF β-SULFOCALCEIN

A. Synthesis

1. Introduction

Calcein was first prepared by Diehl and Ellingboe (1) in 1956, by the Mannich condensation of fluorescein, iminodiacetic acid and formaldehyde in alkaline water solution. The procedure did not yield a product of definite composition. Hefley (6) in 1965 modified the synthesis, and by starting with a pure, metal-free fluorescein and employing glacial acetic acid as solvent, obtained a purer product. This procedure, however, gave a low yield and was plagued by slow filtration and deterioration and loss during recrystallization. The synthesis was further improved by Martin (4) who used water as the solvent for the Mannich condensation and effected the precipitation from a solution by salting out with a large amount of potassium chloride which reduced the solubility of the product and prevented peptization. The yield was increased to 32 per cent and the purity was improved to 92 per cent.

In the present work, Martin's procedure has been modified and adapted to the preparation of Sulfocalcein; the yield has been increased to about 75 per cent and the purity to better than 99 per cent. The salting out procedure has been eliminated and the yield increased by the addition of a

large volume of absolute ethanol. The product obtained is powdery, not in any way gelatinous, is easy to filter, and is free of heavy metals introduced as impurities in potassium chloride used for salting out. Extraction of the final product in a Soxhlet extractor with ethanol has provided a method of removing any unreacted sulfofluorescein.

In the first part of the present work, it has been shown that the β-isomers of sulfofluorescein, 5-sulfofluorescein and 6-sulfofluorescein have practically identical properties with respect to acid-base behavior, absorbance in the visible, and fluorescence. Thus, in preparing the final compound, Sulfocalcein, the β-isomers of sulfofluorescein, in the free acid form, were used without separation; the product is called β-*Sulfocalcein*.

2. Procedure. Isolation as a mixture of potassium salts

A weighed amount of β-sulfofluorescein, in the free acid form, 9.5 g, was mixed with 7.5 g of reagent grade imino-diacetic acid. The mixture was suspended in 100 ml of deionized water in a beaker. Potassium hydroxide was added until, with stirring, all went into solution. The final pH was 6.0. The solution was transferred to a round-bottom flask equipped with a thermometer, magnetic stirrer, and condenser. Then, 10 ml of 37 per cent formaldehyde solution was added. The mixture was heated to 65-70° for 8 hours, with continuous stirring. The hot solution was filtered and

transferred to a beaker, cooled and acidified with 3 M hydrochloric acid, added drop-wise. The pH was brought to 2.5. With stirring, three volumes of absolute ethanol were added slowly, causing a fine yellow precipitate to form. The final pH was 3.2. The mixture was allowed to stand. The precipitate was collected by filtration and washed with a few portions of 85 per cent ethanol. The precipitate was suspended in about 300 ml of 85 per cent ethanol, stirred and filtered. Finally, the product was extracted with 95 per cent ethanol using a Soxhlet extractor, about 12 hours being required. Then, the yellow product was dried under vacuum at about 65° for 24 hours. The weight of product obtained was 13.3 g, a yield of about 74 per cent.

The equivalent weight of the material was determined by potentiometric titration with 0.1 N sodium hydroxide; found 225.6, 225.3, 225.5, average 225.5; theoretical values:

for the free acid, H_7Scal, 5 replaceable hydrogen atoms, 144.37;

for the monopotassium salt, KH_6Scal, 4 replaceable hydrogen atoms, 189.99;

for the dipotassium salt, K_2H_5Scal, three replaceable hydrogen atoms, 266.02.

Apparently a mixture of the monopotassium and dipotassium salts was obtained; this conclusion was supported by further analyses, described below.

B. Conversion of the Potassium Salts of β-Sulfocalcein to the Free Acid. Titration of the Acid

1. Introduction

It was established by Hefley (7) and confirmed by Martin (4) and Markuszewski (3), that the parent compound, Calcein, Structure XXVII, in the free acid form, designated H_6Cal, contains six replaceable hydrogen atoms (protons), only four of which can be titrated in aqueous solution. The fifth and sixth protons, those associated with the two ammonium ions (zwitter ion structure), can be titrated in water only after the addition of an excess of calcium; two atoms of calcium unite with one molecule of Calcein displacing two protons which are then titratable in aqueous solution. The steps in the neutralization processes are:

$$H_6Cal + 4OH^- = H_2Cal^{4-} + 4H_2O$$

$$H_2Cal^{4-} + 2Ca^{2+} = Ca_2Cal^{2-} + 2H^+$$

$$2H^+ + 2OH^- = 2H_2O$$

In the present work, Sulfocalcein was titrated potentiometrically with 0.1 N sodium hydroxide in aqueous solution in a similar manner, first directly and second with calcium chloride added.

2. Apparatus and procedure

A weighed amount of β-Sulfocalcein, 1.0367 g, was dissolved in about 175 ml of deionized water, and passed

through a column of a strong acid cation exchange (Dowex-X8) containing about 75 ml of the wet resin in the hydrogen form. The eluate was collected in 500-ml volumetric flask. Washing of the column with deionized water was continued until the flask was filled to the mark. A complete washing of the column was not necessary as it became apparent in the course of the work.

The eluate was mixed and 50.00-aliquot was titrated potentiometrically with standardized sodium hydroxide, 0.1 N, using a Hach Model 8596 pH meter. The pH meter was standardized against two buffer solutions prepared according to the specifications of the National Bureau of Standards. The titration curve is presented in Figure 71. The concentration of the free acid in the eluate was calculated. Another 50.00-ml aliquot of β-Sulfocalcein was taken, sufficient solution of calcium was added to give a mole ratio of calcium to Sulfocalcein 1:1, and the solution was titrated potentiometrically. This titration was repeated with sufficient calcium added to make the mole ratio 2:1 and 5:1. The titration curves are presented in Figures 72, 73 and 74, respectively.

3. Results and discussion

The titration of β-Sulfocalcein, Figure 71, no calcium added, required 5.30 ml of 0.1115 N sodium hydroxide to reach the end-point. When calcium solution was added to give

calcium to Sulfocalcein mole ratio 1:1, Figure 72, the required volume of sodium hydroxide to reach the end-point was 6.34 ml. The volumes required correspond to a ratio of 5.0:6.0. Expressed alternatively, if the titration of Sulfocalcein in the absence of calcium requires 5.0 equivalents of base per mole to reach the end-point, in the presence of calcium, at the mole ratio 1:1, 6.0 equivalents of base per mole of Sulfocalcein are required.

When calcium solution was added to give a mole ratio of calcium to Sulfocalcein 2:1, Figure 73, 7.35 ml of 0.1115 N sodium hydroxide was required to reach the end-point. That is to say, the break occurred upon the addition of 6.9 equivalents of base per mole of Sulfocalcein.

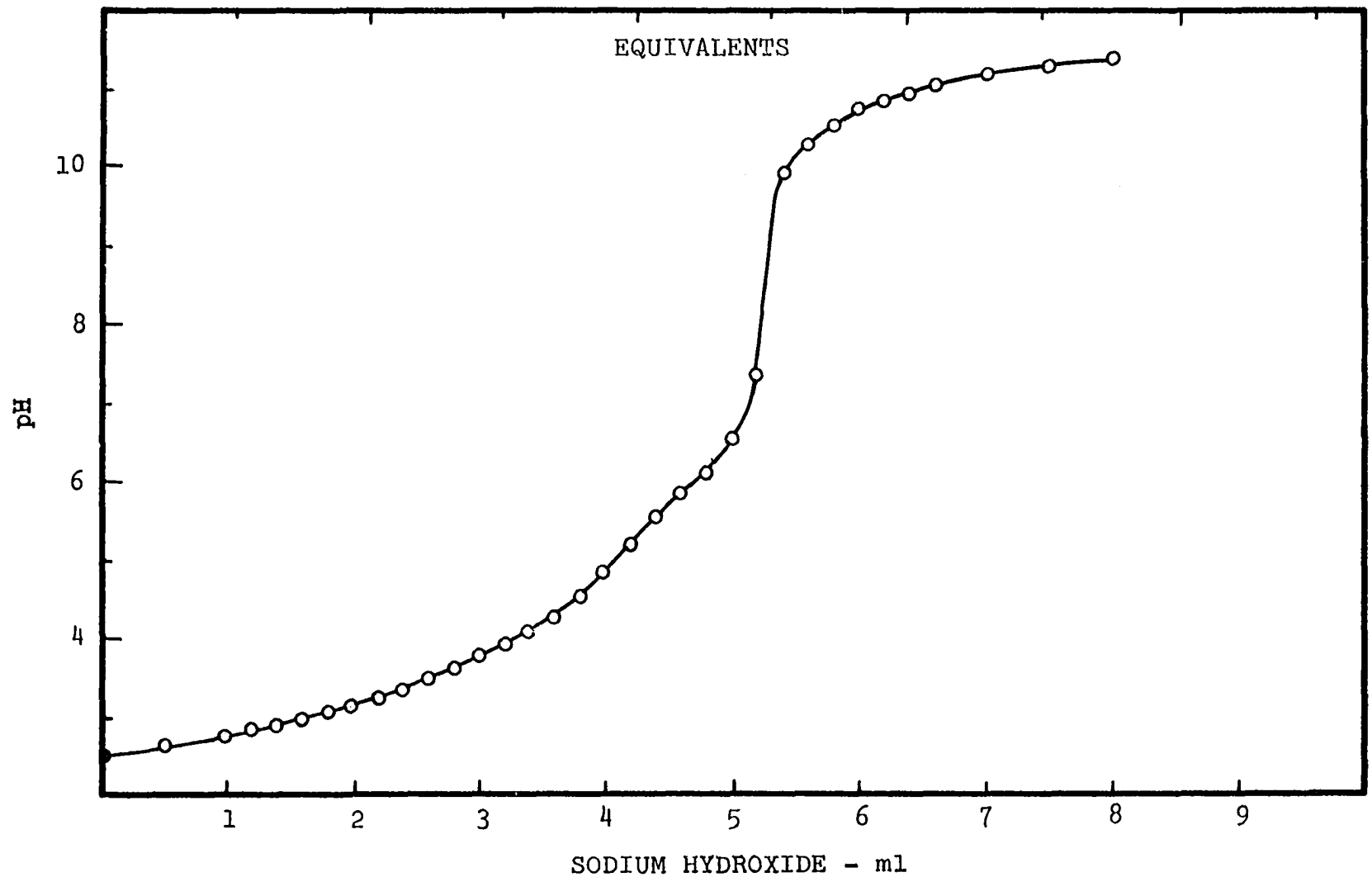
Finally, when excess calcium solution was added to give a mole ratio of calcium to Sulfocalcein 5:1, Figure 74, the end-point occurred at the point at which 7.40 ml of 0.1115 N sodium hydroxide were added. Expressed alternatively, 7.0 equivalents of base per mole of Sulfocalcein were required.

These titrations prove first that Sulfocalcein contains seven protons, and second that Sulfocalcein behaves analogously to the parent compound, Calcein. That is, the last two protons, those associated with the iminodiacetic acid groups of the molecule in the form of zwitter ions, are titratable in water only upon the addition of excess

Figure 71. Potentiometric titration of β-Sulfocalcein in water after passage through Dowex 50-X8 in the hydrogen form.

β-Sulfocalcein: 1.182×10^{-4} moles

Titrant: 0.1115 N sodium hydroxide


EQUIVALENTS
pH
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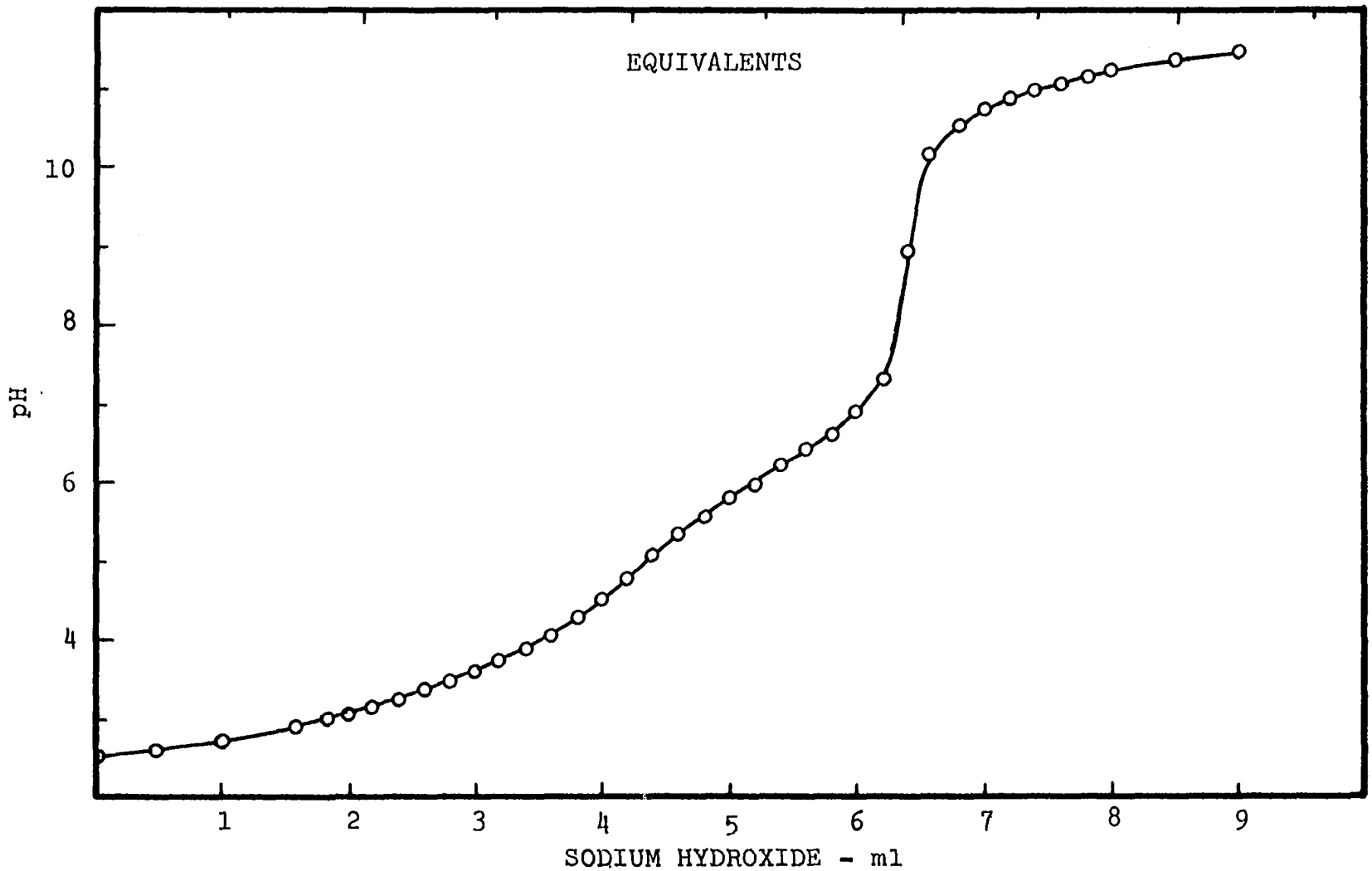
SODIUM HYDROXIDE - ml

Figure 72. Potentiometric titration of β-Sulfocalcein in water after passage through Dowex 50-X8 in the hydrogen form and after the addition of one mole of calcium per mole of β-Sulfocalcein.

β-Sulfocalcein: 1.182×10^{-4} moles

Calcium: 1.182×10^{-4} moles

Titrant: 0.1115 N sodium hydroxide


EQUIVALENTS
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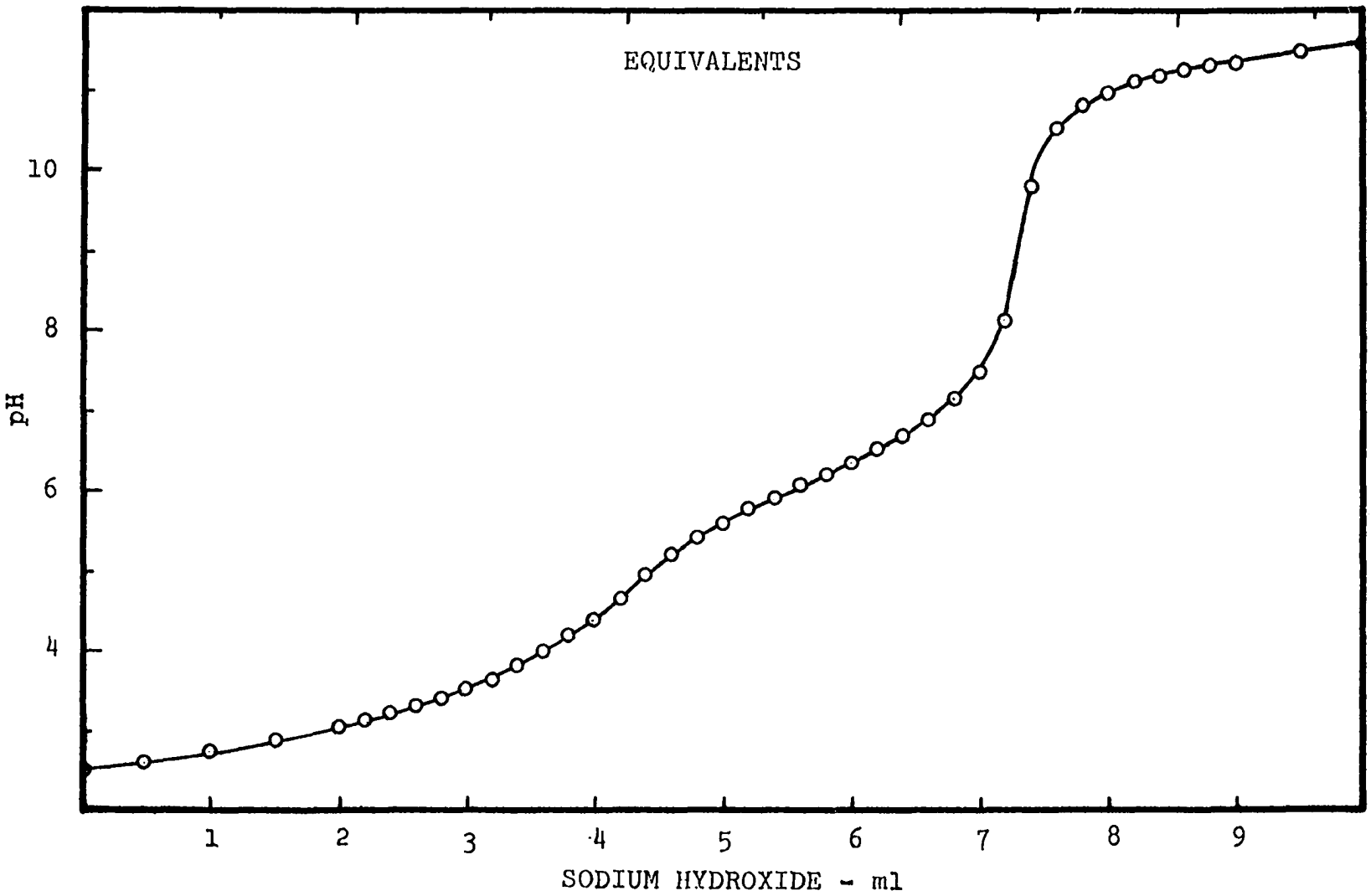
SODIUM HYDROXIDE - ml

Figure 73. Potentiometric titration of β-Sulfocalcein in water after passage through Dowex 50-X8 in the hydrogen form and after the addition of two moles of calcium per mole of β-Sulfocalcein.

β-Sulfocalcein: 1.182×10^{-4} moles

Calcium: 2.365×10^{-4} moles

Titrant: 0.1115 N sodium hydroxide


EQUIVALENTS
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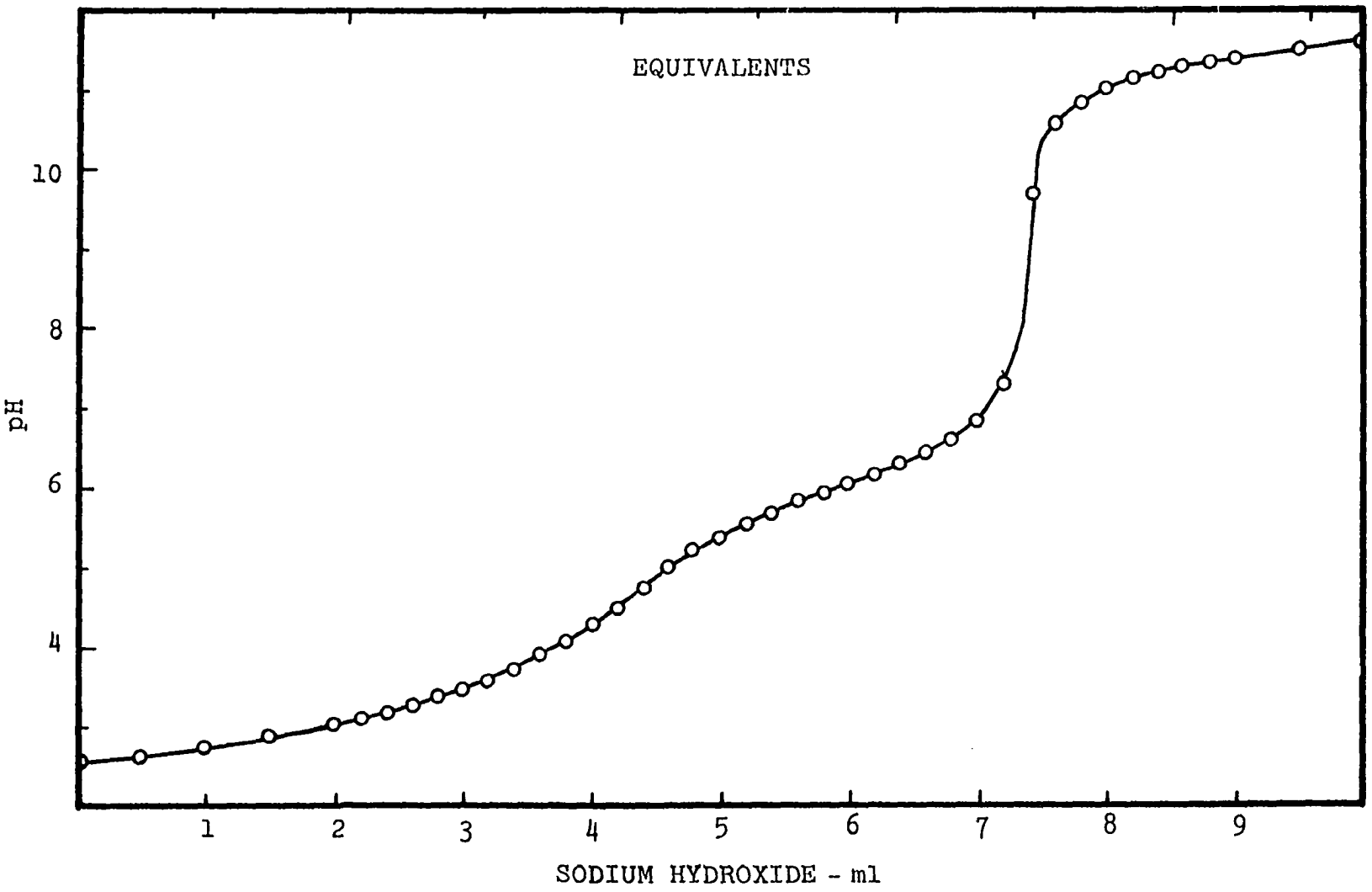
SODIUM HYDROXIDE - ml

Figure 74. Potentiometric titration of β-Sulfocalcein in water after passage through Dowex 50-X8 in the hydrogen form and after the addition of five moles of calcium per mole of β-Sulfocalcein.

β-Sulfocalcein: 1.182×10^{-4} moles

Calcium: 5.912×10^{-4} moles

Titrant: 0.1115 N sodium hydroxide


EQUIVALENTS
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SODIUM HYDROXIDE - ml

calcium ions. The neutralization reactions are represented by:

$H_7Scal + 5OH^- = H_2Scal^{5-} + 5H_2O$

(5 equivalents of base per mole of Sulfocalcein)

$H_2Scal^{5-} + Ca^{2+} = CaHScal^{4-} + H^+$

$H^+ + OH^- = H_2O$

(A total of 6 equivalents of base per mole of Sulfocalcein)

$CaHScal^{4-} + Ca^{2+} = Ca_2Scal^{3-} + H^+$

$H^+ + OH^- = H_2O$

(A total of 7 equivalents of base per mole of Sulfocalcein)

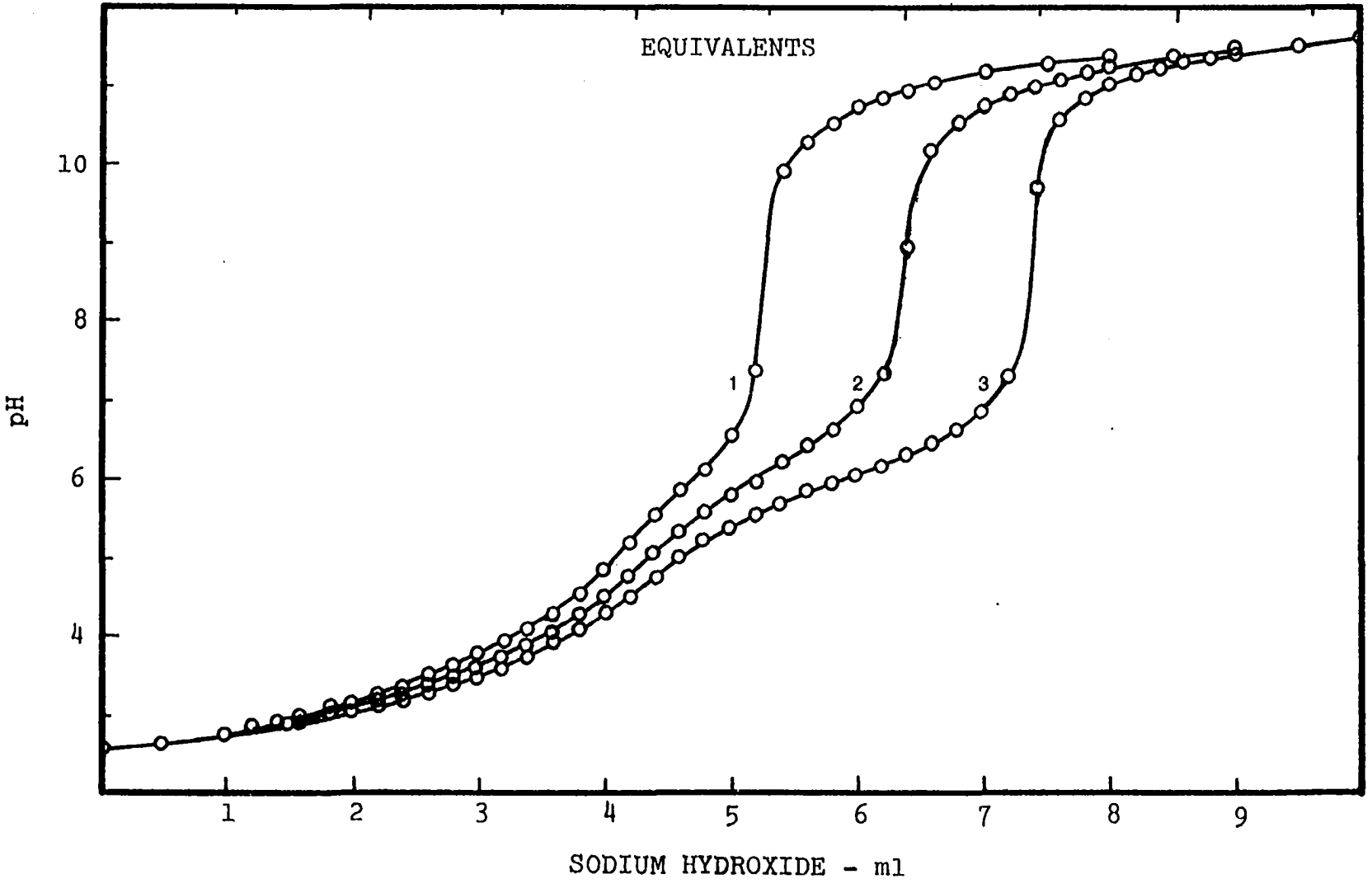
Furthermore, a close examination of the titration curves, Figure 75, indicates that a break occurs at four equivalents of base added per mole of Sulfocalcein. The interpretation of this break will become more significant when the titration curve of Sulfocalcein in aqueous solution is compared to that in 50 per cent ethanol and related to differences in the acid-base behavior of β-sulfofluorescein in aqueous solution and in 50 per cent ethanol.

4. Composition of the mixture of potassium salts obtained from the synthesis

After it had been established, immediately above, that the free acid of β-Sulfocalcein contains seven acidic hydrogen atoms (protons) and that the sixth and seventh protons

Figure 75. Potentiometric titration of β-Sulfocalcein in water after passage through Dowex 50-X8 in the hydrogen form and after the addition of varying amounts of calcium. Data taken from Figures 71, 72, and 74.

1. β-Sulfocalcein
2. One mole calcium per mole of β-Sulfocalcein
3. Five moles calcium per mole of β-Sulfocalcein


EQUIVALENTS
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SODIUM HYDROXIDE - ml

can be titrated in aqueous solution only after the addition of an excess of calcium, this acid-base behavior was utilized to determine the composition of the mixture of the mono-potassium and dipotassium salts of β-Sulfocalcein isolated from the synthesis described above.

A weighed amount, 0.2680 g, of the mixture was dissolved in 150 ml of water; the solution was titrated potentiometrically with 0.1 N sodium hydroxide. After passing the end-point, excess calcium was added to give a calcium to β-Sulfocalcein mole ratio of about 10:1 and the titration continued. Another point of inflection was obtained corresponding to the titration of the sixth and seventh protons.

The titration curve of β-Sulfocalcein as originally isolated in the form of a salt is presented in Figure 76. The volume of 0.1115 N sodium hydroxide to reach the first point of inflection is 10.66 ml. Upon the addition of excess calcium ions the pH of the solution dropped about 5 units, and 6.04 ml of sodium hydroxide was required to neutralize the sixth and seventh protons. Equivalent weight found on the basis of the first end-point, was 225.5. The equivalent weight and the composition of β-Sulfocalcein can be calculated by setting the ratio as follows:

V_1 = 10.66 ml, volume of sodium hydroxide required to reach first end-point.

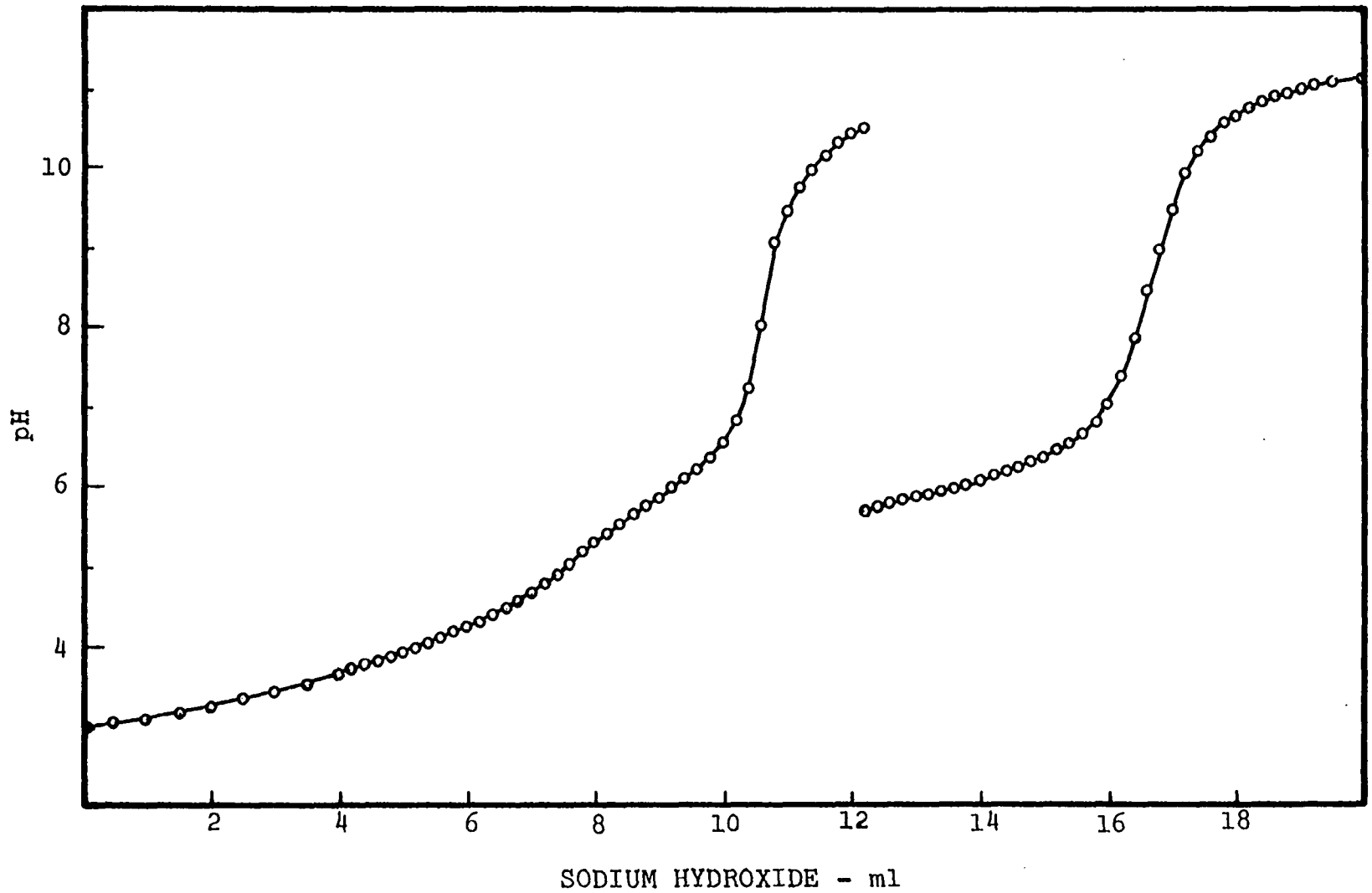
Figure 76. Potentiometric titration of β-Sulfocalcein in water; addition of a 13-fold excess of calcium after passing the first end-point.

β-Sulfocalcein: 0.2680 g

Titrant: 0.1115 M sodium hydroxide

Calcium solution: 0.1819 M

Volume of calcium solution added: 25.00 ml


pH
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SODIUM HYDROXIDE - ml

V_2 = 6.04 ml volume of sodium hydroxide required to titrate the sixth and seventh protons.

Assume

N_1 = mole fraction of β-Sulfocalcein as the monopotassium salt, KH_6Scal.

$1 - N_1$ = mole fraction of β-Sulfocalcein as the dipotassium salt, K_2H_5Scal.

Hence:

$$[4(N_1) + 3(1-N_1)]/2 = 10.66/6.04$$

$$N_1 = 0.5298$$

$$1 - N_1 = 0.4702$$

That is to say, the β-Sulfocalcein salt is composed of 51.8 per cent KH_6Scal and 48.2 per cent K_2H_5Scal.

Equivalent weight of monopotassium salt = 189.99

Equivalent weight of dipotassium salt = 266.02

The apparent equivalent weight of β-Sulfocalcein = (0.5298)(189.99) + (0.4702)(226.02) = 225.7.

The apparent equivalent weight, 225.7, is in excellent agreement with the equivalent weight, 225.5, calculated on the basis of the weight of β-Sulfocalcein titrated, 0.2680 g, and the volume, 10.66 ml, of 0.1115 N sodium hydroxide required to reach first end-point. This agreement is within the experimental error and confirms the assumptions made, first that the isolated salt of β-Sulfocalcein is a double salt, KH_6Scal and K_2H_5Scal, and second that the purity of the compound approaches 100 per cent.

C. Conversion of the Potassium Salts of β-Sulfocalcein to the Free Acid. Titration of the Free Acid in Ethanol-Water Solvent

1. Introduction

As shown in Chapter VIII and IX, above, the titration of β-sulfofluorescein in 50 per cent ethanol follows a quite different course from that in aqueous solution, the end-points in 50 per cent ethanol occurring at one equivalent and three equivalents of base per mole of compound, but those in water at two equivalents and at three equivalents. The same behavior was observed with the individual isomers after the mixture had been separated into the components, 5-sulfofluorescein and 6-sulfofluorescein; the titration curves are shown in Figures 58 and 64 and Figures 59 and 65. The explanation was advanced that this difference results from a large proportion of the compound being present in the lactone form in the 50 per cent ethanol solvent.

It became of interest to convert β-Sulfocalcein to the free acid and to titrate this acid in ethanol-water to learn if β-Sulfocalcein also exists in part in the lactone form.

2. Apparatus and procedure

A solution of β-Sulfocalcein in the acid form was prepared following the procedure described in Chapter X, Part B.2. About 1.0 g of the compound in the salt form was dissolved in 200 ml of dionized water. The solution was

passed through a column of a strong acid cation exchange resin. The eluate was collected and diluted to exactly 500 ml in a volumetric flask. A 50.00-ml aliquot of the eluate was transferred to a beaker and 50.00 ml of absolute ethanol was added. Then, the solution was titrated potentiometrically with 0.1 N sodium hydroxide, using a Hach Model 8596 pH meter. The pH meter was standardized using two standard buffer solutions, prepared according to the specifications of the National Bureau of Standards.

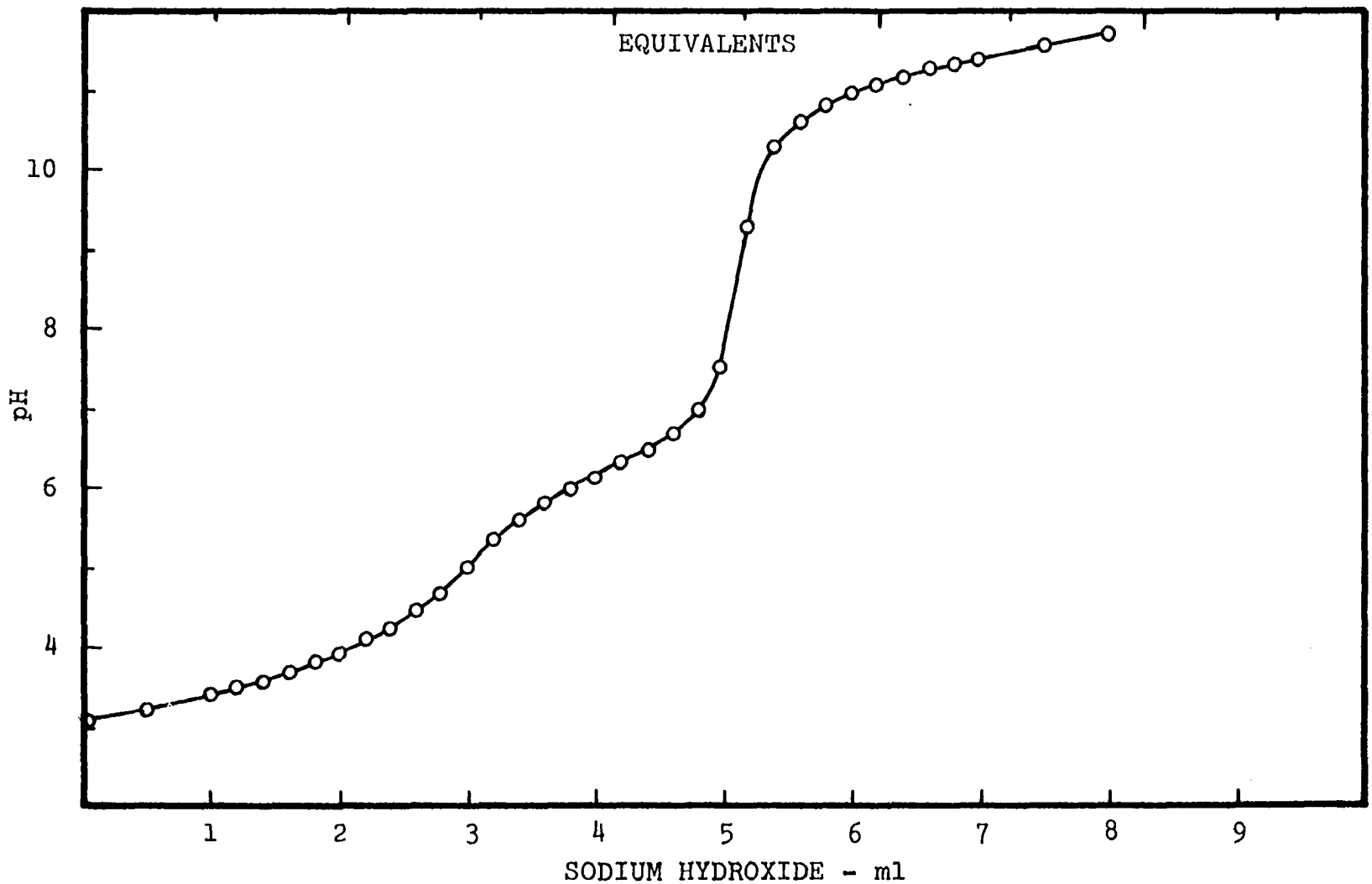
3. Results and discussion

The titration curve of β-Sulfocalcein, free acid, in 50 per cent ethanol solvent is presented in Figure 77. Two points of inflection are present, at three equivalents and at five equivalents of base added per mole of the compound. When β-Sulfocalcein was titrated in water, the two corresponding points of inflection appeared, at four and at five equivalents of base added per mole of the compound. Apparently in 50 per cent ethanol, the acidity of the fourth proton is lower than in water and this proton is titrated concurrently with the fifth.

Figure 77. Potentiometric titration of β-Sulfocalcein in 50 per cent ethanol after passage through Dowex 50-X8 in the hydrogen form.

β-Sulfocalcein: 1.159×10^{-4} moles

Titrant: 0.1114 N sodium hydroxide


EQUIVALENTS
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SODIUM HYDROXIDE - ml

D. The Dissociation Constants and Acid Functions of Fluorescein, Calcein, β-Sulfofluorescein and β-Sulfocalcein

Values for the dissociation constants of Calcein, Structure XXVII, were determined by Hefley (7) using various methods: potentiometric titration, solubility as a function of pH, absorption in the ultraviolet as a function of pH, and fluorescence as a function of pH. On the basis of these measurements and comparison between Calcein and related compounds, Hefley (7) assigned the successive protons to specific acidic groups.

In Calcein, the two iminodiacetic acid groups are present in the zwitter ion form and the positive charge on each of the ammonium ions makes the two remaining carboxylic acid groups strong acids, $pK_1 = 2.5$, $pK_2 = 3.5$. The protons of the ammonium ions are very weak acids and can be titrated only by displacement (as ammonium chloride is titrated by sodium hydroxide); these protons are those numbered five and six. The remaining protons are those on the phenolic groups, $pK_3 = 4.6$ and $pK_4 = 6.2$, and are remarkably strong for phenolic groups for which the dissociation constants are normally in the range 10^{-9} to 10^{-10}.

Markuszewski (3) explained the unusual strength of the two phenolic groups in fluorescein, $pK_2 = 4.7$ and $pK_3 = 6.6$, as caused by the extensive conjugation in the molecule and

the presence of the positive charge on the pyrylium ring. This argument, of course, carries over to Calcein, the corresponding dissociation constants being practically identical. The argument carries over also to β-sulfofluorescein, $pK_2 = 4.4$ and $pK_3 = 6.3$, and to β-Sulfocalcein.

The presence of the sulfonic acid group in β-sulfofluorescein and in β-Sulfocalcein complicates the picture somewhat, but in understandable fashion. The sulfonic acid group is a very strong acid and the pyrylium ring-zwitter ion structure is formed by the movement of the hydrogen ion from the sulfonic acid group to the carboxyl group on the phthalate ring. As will be noted from the dissociation constants tabulated in Table 24, the carboxylic acid group on the phthalate ring in protonated fluorescein, and in β-sulfofluorescein are somewhat stronger acids than those of the iminodiacetic acid (zwitter ion) groups in Calcein.

The corresponding acid functions are thus easily traced through the three compounds, Table 24. By analogy the various ionic forms of β-Sulfocalcein are presented in Figure 78.

β-Sulfofluorescein and β-Sulfocalcein undergo the same change in acid-base behavior as the solvent is changed from water to 50 per cent ethanol. The most striking change is in the strength of the first phenolic proton which in water is titrated with the carboxylic acid proton but in 50 per

Table 24. The acid dissociation constants of fluorescein, Calcein, and β-sulfofluorescein. Assignment to specific acidic groups

Fluorescein H_3Fl^+	Calcein H_6Cal	β-Sulfofluorescein H_3Sfl	β-Sulfocalcein H_7Scal
			Successive Protons
pK_1 = 2.1 (C*)	pK_1 = 2.5 (C)	pK_1 = 2.1 (C*)	1 = (C*)
pK_2 = 4.7 (P)	pK_2 = 3.5 (C)	pK_2 = 4.4 (P)	2 = (C)
pK_3 = 6.6 (P)	pK_3 = 4.6 (P)	pK_3 = 6.3 (P)	3 = (C)
	pK_4 = 6.2 (P)		4 = (P)
	pK_5 = 9.9 (A)		5 = (P)
	pK_6 = 11.6 (A)		6 = (A)
			7 = (A)

C = Carboxylic acid group associated with the iminodiacetic acid group.

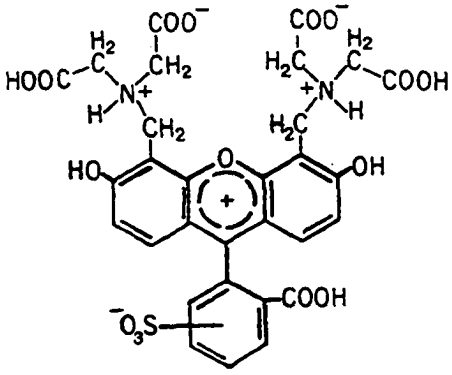
C* = Carboxylic acid group located on the phthalate portion of the molecule. Note in the case of fluorescein that this is the protonated form of the molecule.

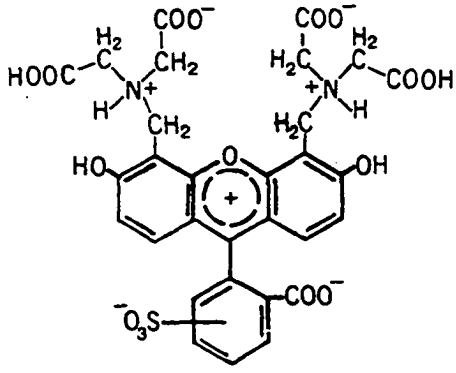
P = Phenolic group.

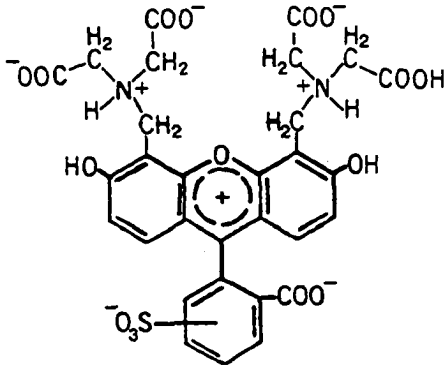
A = Ammonium ion (part of the zwitter ion structure of the iminodiacetic acid group).

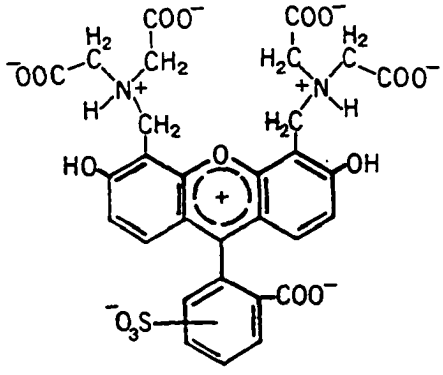
Figure 78. Structural forms of the ionic species of Sulfocalcein.

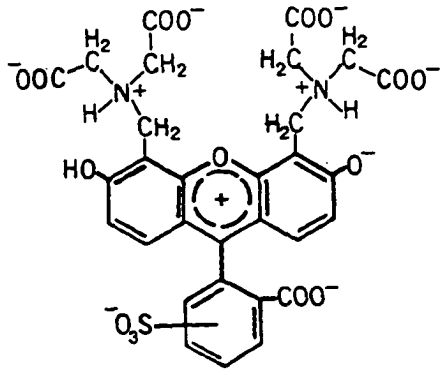
a. H_7Scal (neutral molecule)
b. H_6Scal^- (first carboxyl group ionized)
c. H_5Scal^{2-} (second carboxyl group ionized)
d. H_4Scal^{3-} (third carboxyl group ionized)
e. H_3Scal^{4-} (first phenolic group ionized)
f. H_2Scal^{5-} (second phenolic group ionized)
g. $HScal^{6-}$ (first proton from ammonium-type nitrogen atom ionized)
h. $Scal^{7-}$ (second proton from ammonium-type nitrogen atom ionized)

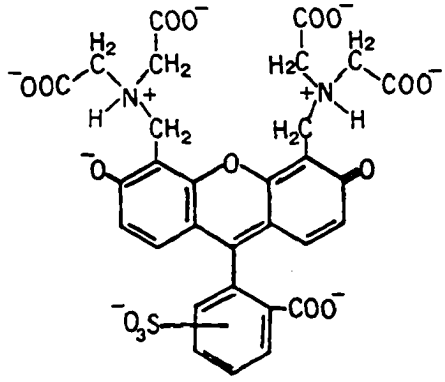


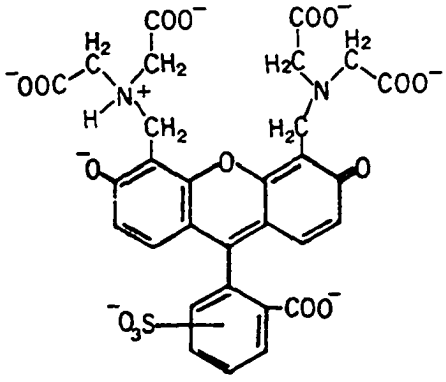


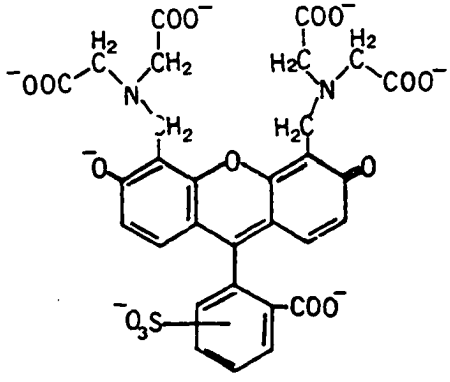












cent ethanol is a weaker acid and titrated concurrently with the second phenolic proton. This change in acidity cannot be attributed to a difference in leveling effect of the solvent inasmuch as the water content of both solvents is high; the best explanation, advanced in the discussions following the various experiments on β-sulfofluorescein (solubility measurements as a function of pH, potentiometric titrations, and absorption in the visible as a function of pH) that the monoanion of β-sulfofluorescein exists in large measure in the lactone form in 50 per cent ethanol. β-Sulfocalcein as the trianion, H_4Scal^{3-}, exhibits the same behavior. This is further borne out in the studies of absorption as functions of pH being reported in the following chapter.

Because of the low solubility of Calcein, a potentiometric titration with sodium hydroxide does not yield precise information about the first four acid dissociation constants; addition of excess standard alkali and back titration with standard acid gives a satisfactory titration curve with a single point of inflection, at four equivalents of base per mole of compound. Hefley (7) observed that the forward titration could be made in 50 per cent ethanol, two points of inflection appearing, at two and at four equivalents of base added per mole of compound. This was confirmed by Markuszewski (3) and by Martin (5) although

none of the three investigators offered an explanation. It is apparent, however, that the behavior observed is another manifestation of the phenomenon observed in the present work with fluorescein, β-sulfofluorescein, and β-Sulfocalcein and that the same explanation applies. That is, the dianion of Calcein, H_4Cal^{2-}, must exist in large measure in the lactone form in 50 per cent ethanol.

XI. THE ABSORPTION AND FLUORESCENCE SPECTRA OF β-SULFOCALCEIN

β-Sulfocalcein has been synthesized with the objective of providing an improved reagent for the fluorometric determination of calcium and other metals. The fluorescence of the reagent is thus of prime importance and it is essential to learn how the fluorescence changes with pH and how it is altered in the presence of calcium and other metals. Fluorescence occurs, of course, only after the absorption of light and thus the absorption spectra of a fluorescence reagent is also of fundamental importance.

A. The Absorption Spectrum of β-Sulfocalcein as a Function of pH

1. Apparatus and procedure

A Cary Model 14 Recording Spectrophotometer was used to obtain the absorption spectra. More exact, single-point measurements of absorbance was made on a Hach DR-2 Spectrophotometer supplied with a flow-through cell.

A stock solution of β-Sulfocalcein, 5.0×10^{-4} M, was prepared by dissolving the appropriate amount of the compound in exactly one liter of deionized water. A 2.00-ml aliquot of this solution was transferred to a 100-ml volumetric flask, 2 ml of 0.01 M EDTA was added, and the solution was diluted to the mark with the appropriate buffer. The final

concentration of each solution was 1.0×10^{-5} M. These solutions were used to obtain the absorption spectra, that is, the absorbance as a function of wavelength, at different pH values. To another solution of β-Sulfocalcein, 1.0×10^{-5} M in 0.1 M potassium hydroxide was added sufficient calcium to give a mole ratio of calcium to β-Sulfocalcein of 10:1 and the spectrum of this solution was obtained.

A second stock solution of β-Sulfocalcein, 2.4×10^{-4} M, was prepared by dissolving the appropriate amount of the compound in exactly one liter of dionized water. Then a 2.00-ml aliquot of this solution was transferred to a 100-ml volumetric flask, 2.00 ml of 0.01 M EDTA added, and the solution diluted to 100.0 ml with appropriate buffer, and mixed. The final concentration of β-Sulfocalcein in each of these solutions was 4.8×10^{-6} M. A total of forty such buffered solutions was prepared, the intervals between the solutions being 0.25 to 0.50 pH unit. The absorbance of these solutions was measured on the Hach DR-2 Spectrophotometer at a wavelength of 500 nm.

2. Preparation of buffers

The buffers were prepared from 0.1 M solutions of hydrochloric acid, potassium hydroxide, potassium hydrogen phthalate (KHP), potassium chloride, and boric acid in 0.1 M potassium chloride solution. The pH of the buffer solutions

was measured before and after the addition of the stock solution of β-Sulfocalcein. A Hach Model 8594 pH meter, set on the expanded scale, and a Beckman high-alkalinity glass electrode were used. The pH meter was calibrated against standard buffer solutions prepared according to the specifications of the National Bureau of Standards.

3. Results and discussion

The absorption spectra of β-Sulfocalcein at pH 1.20, 3.05, 5.10 and 7.02 are presented in Figure 79. The absorption spectra of β-Sulfocalcein in 0.1 M potassium hydroxide, pH 12.98, without added calcium and with a 10-fold excess of calcium, are presented in Figure 80.

A maximum in the absorption spectrum of β-Sulfocalcein at pH 1.20 occurs at 445 nm. The absorbance at 445 nm is of the same magnitude at pH 3.05, higher at pH 5.10 (appearing as a shoulder), and finally lower at pH 7.02. A second maximum in the spectrum appears, at pH 1.20, as a shoulder centered at 475 nm, which at higher pH becomes an absorption maximum of greatly increased intensity and with the maximum shifting to longer wavelength with rising pH (at 498 nm at pH 7.2). This second maximum appears at 506 nm in the spectrum of the 0.1 M potassium hydroxide solution, pH 12.98, and in the presence of a large excess of calcium at 498 nm. The variation of the absorbance with pH of this second

Figure 79. Absorption spectra of β-Sulfocalcein, 1.0×10^{-5} M, at various pH.

Curve 1: pH = 1.20

Curve 2: pH = 3.05

Curve 3: pH = 5.10

Curve 4: pH = 7.02

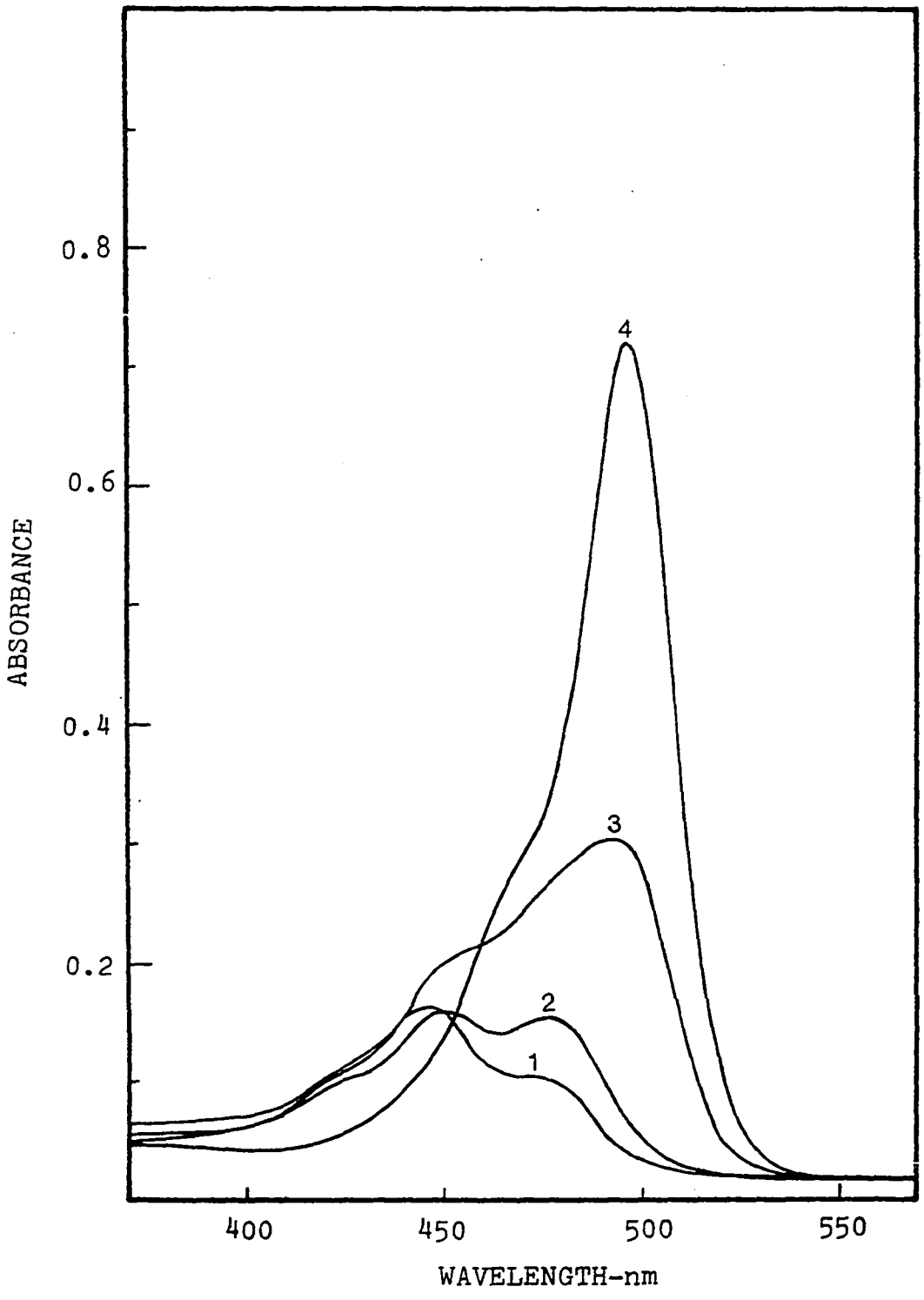
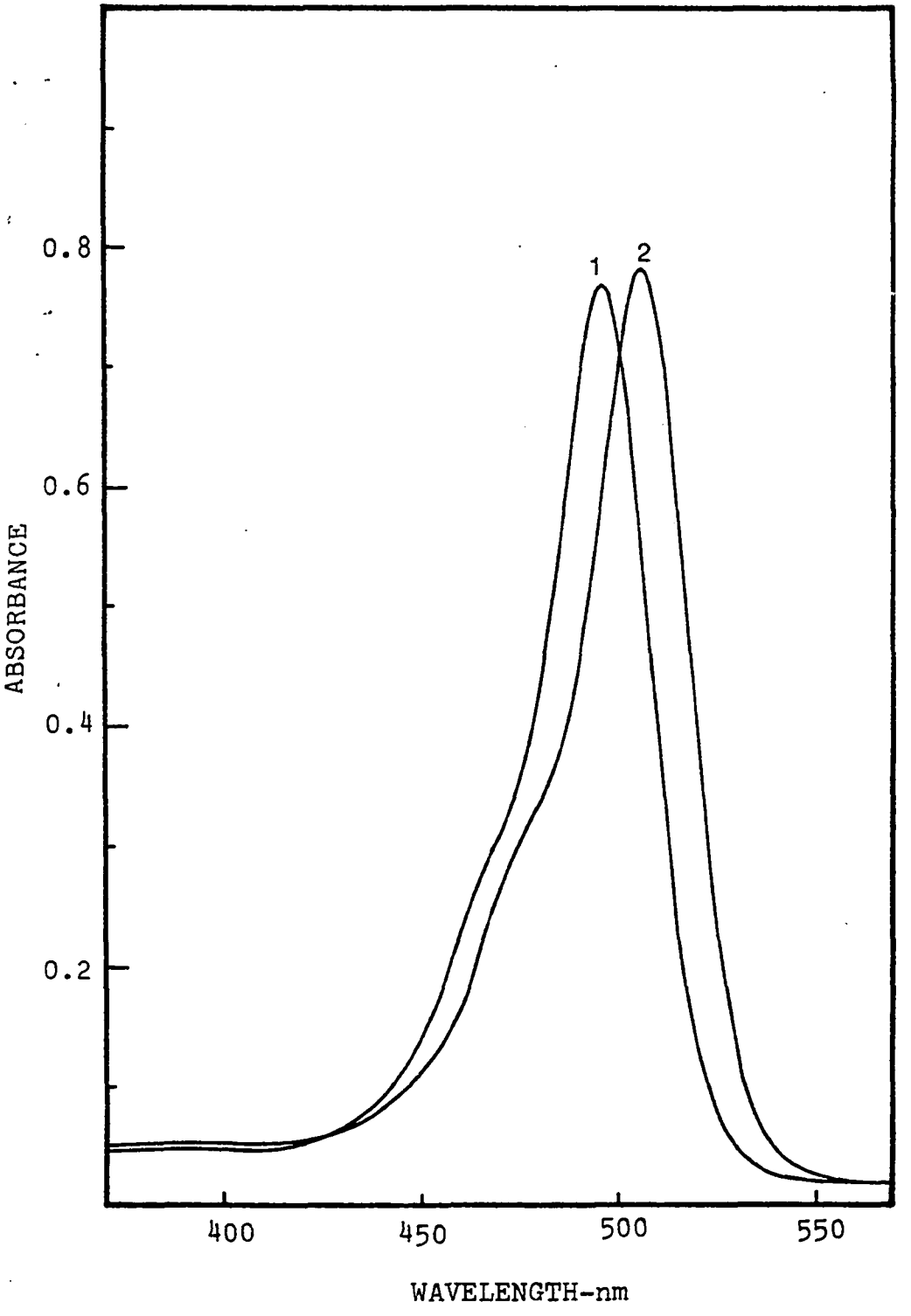

0.8
0.6
0.4
0.2
ABSORBANCE
4
3
2
1
400
450
500
550
WAVELENGTH-nm

Figure 80. Absorption spectra of β-Sulfocalcein, 1.0×10^{-5} M, in 0.1 M potassium hydroxide.

Curve 1: β-Sulfocalcein in the presence of 10-fold excess of calcium chloride solution

Curve 2: β-Sulfocalcein alone


0.8
0.6
0.4
0.2
ABSORBANCE
1
2
400
450
500
550
WAVELENGTH-nm

maximum is shown in Figure 81; all of the absorbance measurements were made at 500 nm.

In an earlier chapter of the present work, Chapter V, the absorption band at 442 nm of β-sulfofluorescein (that is, of the mixture of 5- and 6-sulfofluorescein) was assigned to the zwitter ion form of the compound and the band at 470 nm (shifting with rising pH to 495 nm) to the quinone form.

It would appear straightforward to make similar assignments of the corresponding bands in the spectra of β-Sulfocalcein; however, some significant differences appear. At pH 1, the intensity of the absorption band at 445 nm of β-Sulfocalcein, Figure 80, is considerably less than that of β-sulfofluorescein, Figures 19 and 20, (the concentration being the same, 1.0×10^{-5}, in all solutions. The shoulder at 475 nm in the spectrum of β-Sulfocalcein does not appear in the spectrum of β-sulfofluorescein. The lower intensity is interpreted as evidence that an appreciable fraction of the β-Sulfocalcein must be present in the lactone form and the shoulder as evidence that some is present in the quinone form. That is, in the acidic region, β-Sulfocalcein is present in all three forms simultaneously. The position of the two absorption maxima at a various pH are summarized in Table 25.

The spectra of β-Sulfocalcein are very similar to those obtained by Hefley (7) for the parent compound. Calcein. In the absorption spectrum of Calcein at pH 1.09, two low

Figure 81. Absorption spectrum of β-Sulfocalcein, 4.8×10^{-6} M, as a function of pH at wavelength of 500 nm.

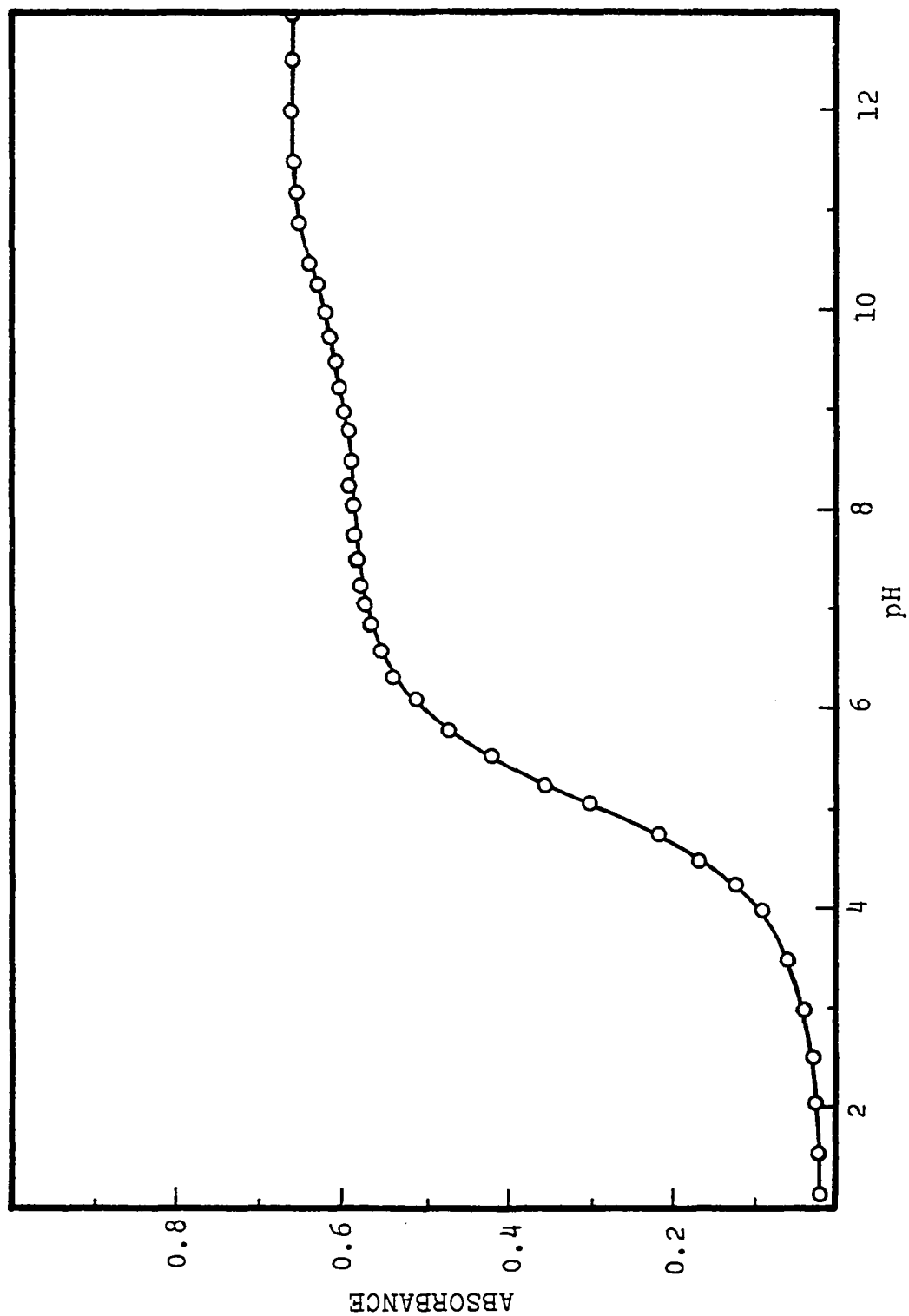
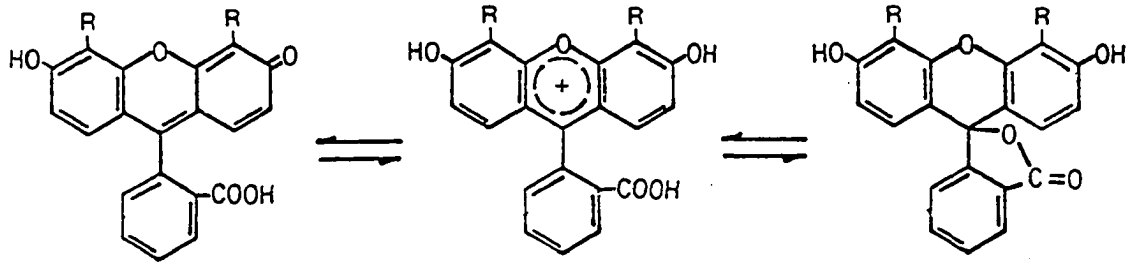

ABSORBANCE
0.8
0.6
0.4
0.2
2
4
6
8
10
12
pH

Table 25. Absorption bands of the β-Sulfofluoresceins and of β-Sulfocalceins

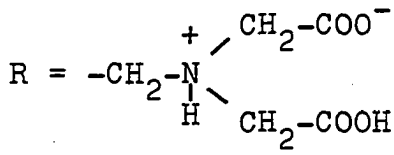
Compound	pH	Absorption Band-nm	Absorbance	Predominant form(s)
5-Sulfofluorescein	1.0	442	0.51	zwitter ion
6-Sulfofluorescein	1.0	443	0.50	zwitter ion
β-Sulfocalcein	1.2	445,475sh	0.15,0.09	lactone, zwitter ion, quinone
5-Sulfofluorescein	3.1	443,480sh	0.24,0.07	lactone, zwitter ion, quinone
6-Sulfofluorescein	3.1	443,480sh	0.26,0.07	lactone, zwitter ion, quinone
β-Sulfocalcein	3.0	450,475sh	0.14,0.13	lactone, zwitter ion, quinone
5-Sulfofluorescein	5.0	458,480	0.29,0.30	zwitter ion, quinone
6-Sulfofluorescein	5.0	458,483	0.29,0.30	zwitter ion, quinone
β-Sulfocalcein	5.1	455sh,495	0.19,0.28	quinone, zwitter ion
5-Sulfofluorescein	8.1	495	0.84	quinone
6-Sulfofluorescein	8.1	496	0.83	quinone
β-Sulfocalcein	7.2	498	0.70	quinone
β-Sulfocalcein	13.0	506	0.76	quinone

sh = shoulder

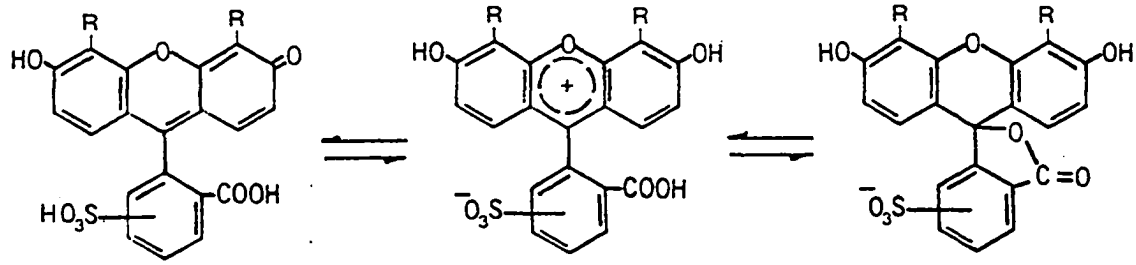
intensity bands are present, at 445 nm and at 468 nm. By the same reasoning, it would appear that Calcein at low pH, pH of 1, is not present as the zwitter ion only but rather is present in all three forms, lactone, zwitter ion, and quinone.



XXVIIa. Quinone XXVIIb. Zwitter ion XXVIIc. Lactone



Calcein



Ia. Quinone Ib. Zwitter ion Ic. Lactone

Sulfocalcein

The intensity of the longer wavelength absorption band of β-Sulfocalcein as a function of pH is shown in Figure 81. The absorbance is low at pH below 3, rises slowly between pH 3.5 and 4.5, rises most rapidly between pH 4.5 and 5.5, reaches a plateau at about pH 7.5, and remains essentially constant to pH 8.5. The absorbance then again increases, reaches a maximum and essentially constant value at pH 11.5. This behavior over the pH range 1 to 8 is essentially the same as that of the β-sulfofluoresceins and the interpretation in terms of the protons neutralized is the same. Thus, between pH 1 and 3.5, three protons are neutralized, between pH 3.5 and 4.5 the first phenolic proton is neutralized and a considerable portion of the compound is converted to the quinone form. Above pH 4.5, the second phenolic proton is neutralized and at pH 7.5 the neutralization of both phenolic protons is complete and the compound is predominantly in the quinone form. Finally at about pH 8.5, the neutralization of the two protons of the ammonium groups begins and the absorption increases reaching a constant value at a pH about 11.5, at which point the neutralization of these two protons is complete.

B. The Fluorescence Spectra of β-Sulfocalcein as a Function of pH

1. Apparatus and procedure

The excitation and emission spectra of β-Sulfocalcein were obtained on the Amino-Bowman Spectrophotofluorometer equipped with a Hewlett Packard X-Y Recorder.

The spectra were obtained on the same buffered solution measured in the preceding section reporting the absorption spectra of β-Sulfocalcein. The solutions were 1.0×10^{-5} in β-Sulfocalcein. A more detailed measure of the intensity of fluorescence as a function of pH was obtained using the same forty buffered solutions, 4.8×10^{-6} M in β-Sulfocalcein, used in the measurements of absorbance.

2. Results and discussion

In preliminary work it was observed that the wavelength of maximum excitation shifted with pH, being a maximum at 475 nm at pH 3.0 and at 496 nm at pH 9.0. This, of course, was expected inasmuch as absorption and fluorescence excitation spectra are always essentially identical. The wavelength of the maximum in the emission spectrum remained constant at 520 nm.

Emission spectra obtained on solutions of varying pH are shown in Figures 82 and 83; for these spectra, the emission monochromator was set at 520 nm and the excitation

Figure 82. Fluorescence emission spectra of β-Sulfocalcein, 1.0×10^{-5} M, at various values of pH. Excitation monochromator set at 475 nm, emission monochromator set at 520 nm.

Curve 1: pH = 9.02

Curve 2: pH = 7.20

Curve 3: pH = 5.10

Curve 4: pH = 3.05

Curve 5: pH = 1.20

Curve 6: pH = 12.98, in 0.1 M potassium hydroxide

Curve 7: in 0.1 M potassium hydroxide plus a 10-fold calcium chloride added

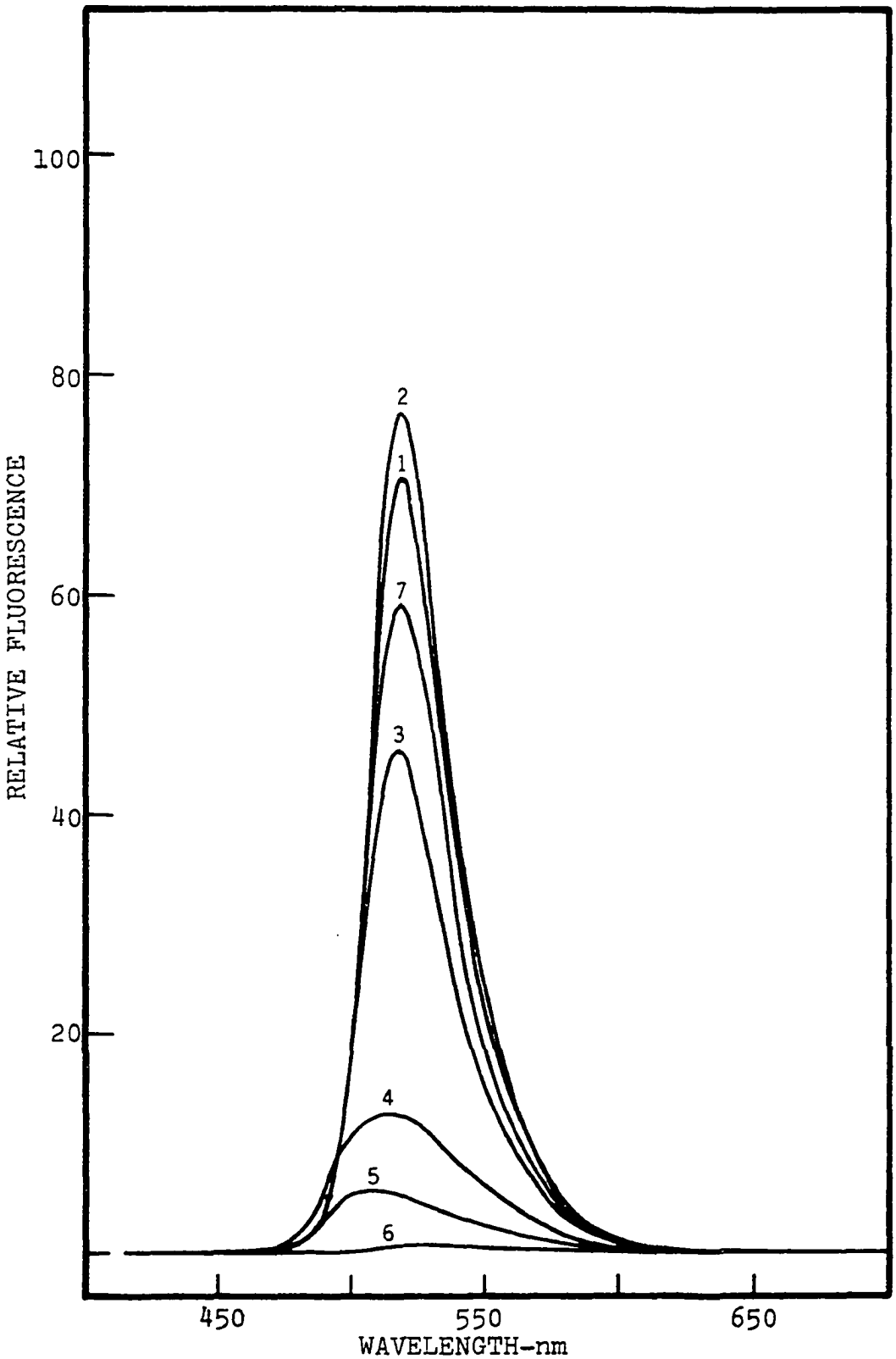

100
80
60
40
20
RELATIVE FLUORESCENCE
2
1
7
3
4
5
6
450
550
650
WAVELENGTH-nm

Figure 83. Fluorescence emission spectra of β-Sulfocalcein, 1.0×10^{-5} M, at various values of pH. Excitation monochromator set at 496 nm, emission monochromator set at 520 nm.

Curve 1: pH = 9.02

Curve 2: pH = 7.20

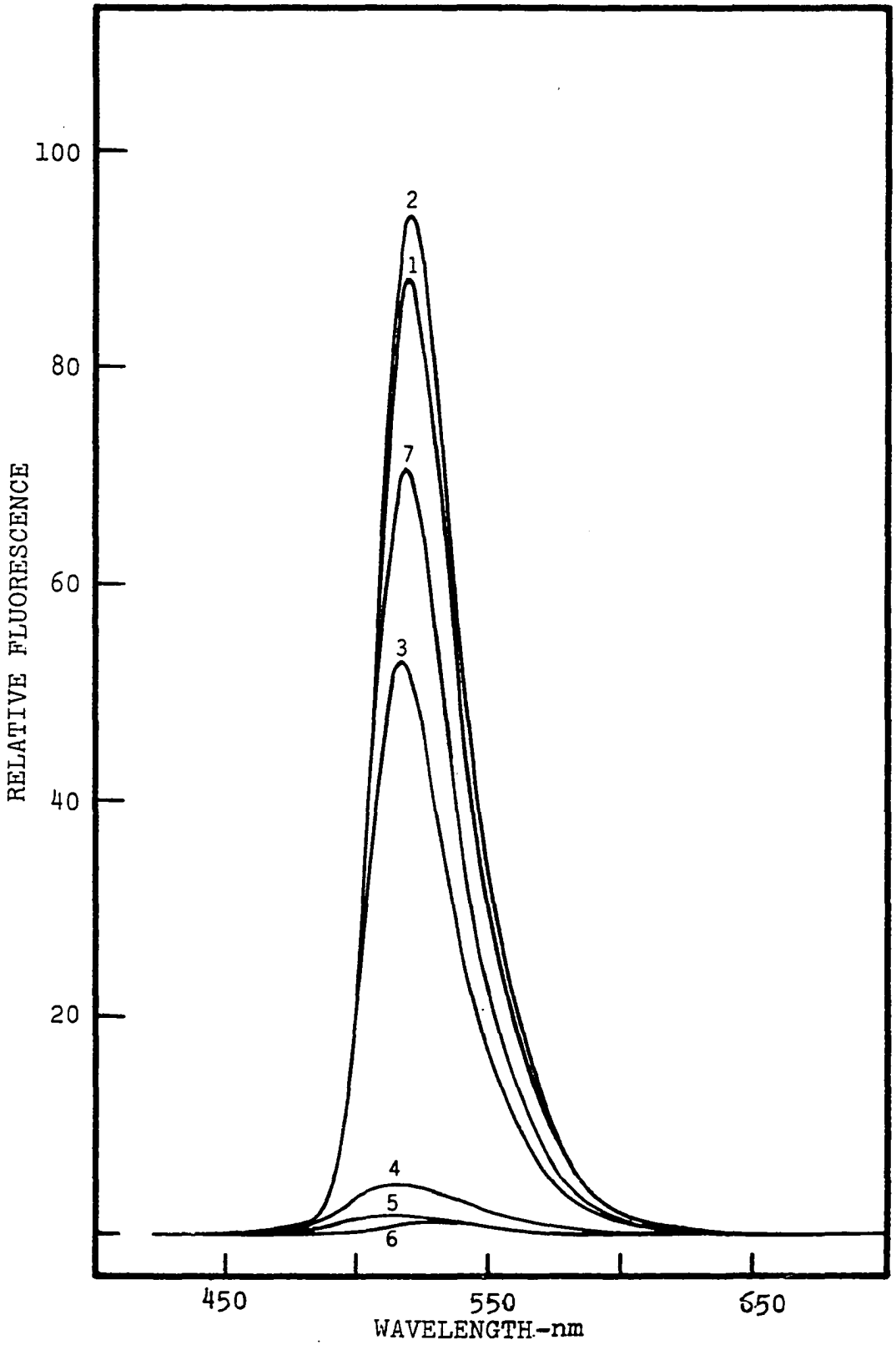
Curve 3: pH = 5.10

Curve 4: pH = 3.05

Curve 5: pH = 1.20

Curve 6: pH = 12.98, in 0.1 M potassium hydroxide

Curve 7: in 0.1 M potassium hydroxide plus a 10-fold calcium chloride added


100
80
60
40
20
RELATIVE FLUORESCENCE
2
1
7
3
4
5
6
450
550
650
WAVELENGTH-nm

monochromator set at 475 nm, Figure 82, and at 496 nm, Figure 83.

The intensity of the fluorescence is low in the low pH region, reaches a maximum at about pH 7, then drops to zero in very basic solution. A more detailed presentation of this data is given in Figure 84.

When calcium is added to the very basic solution, the fluorescence is restored.

In Figure 85 are shown the absorbance and the fluorescence of β-Sulfocalcein, 4.8×10^{-6} M, as a function of pH, the absorbance being measured at 500 nm and (in the fluorescence experiment) the excitation monochromator being set at 500 nm. It is apparent that at pH above 7.5, although the absorbance actually increases, the fluorescence drops; as pointed out previously above pH 8 the protons of the ammonium groups are neutralized. This same phenomenon was observed with Calcein by Hefley (7). Her explanation was the following: below pH 8, the carboxylate group of one of the acetic acid groups is attached to the ammonium through hydrogen bonding. The removal of the proton of the ammonium ion by neutralization destroys this hydrogen bonding and the acetic group is free to rotate about the nitrogen-α-carbon bond. Internal collision of this rotating acetate group with the other acetate group causes a dissipation of the energy obtained by the absorption of light and prevents the emission

Figure 84. Relative fluorescence of β-Sulfocalcein, 4.8×10^{-6} M, as a function of pH.

Excitation monochromator set at 500 nm

Emission monochromator set at 520 nm

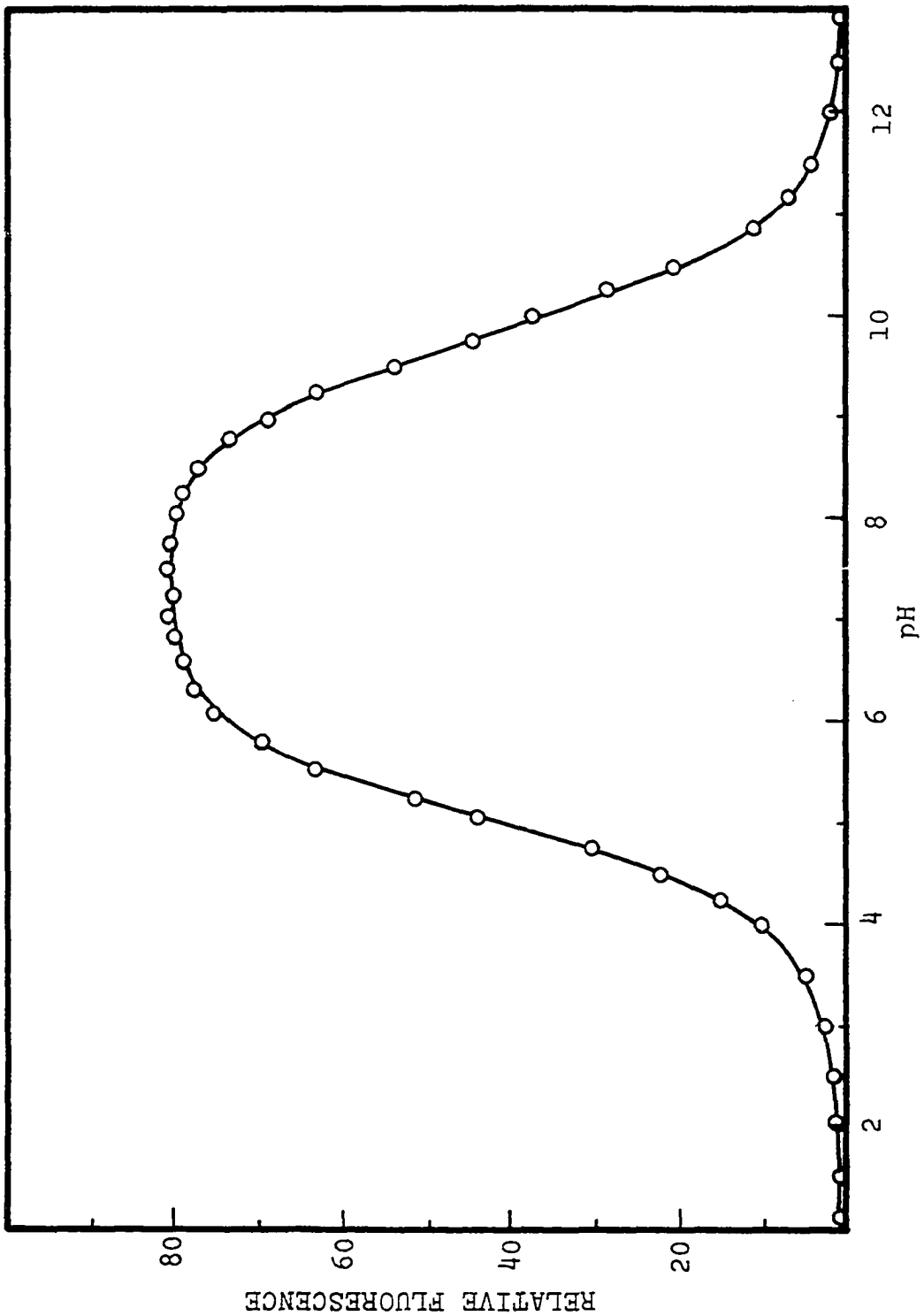
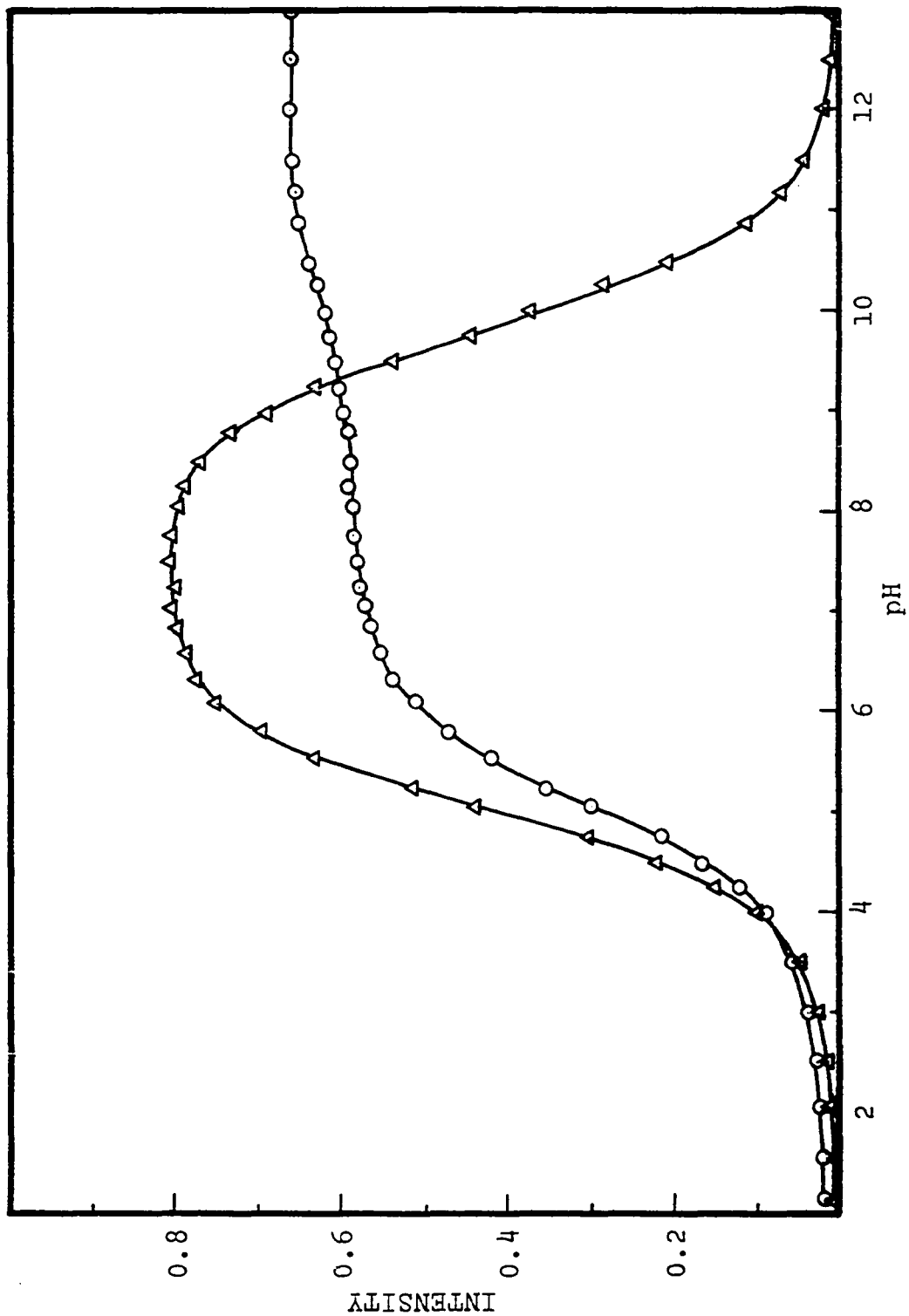

RELATIVE FLUORESCENCE
20
40
60
80
2
4
6
8
10
12
pH

Figure 85. Absorbance and relative fluorescence of β-Sulfocalcein as a function of pH. Data taken from Figures 81 and 84.

Δ: Fluorescence

o: Absorbance


INTENSITY
0.8
0.6
0.4
0.2
2
4
6
8
10
12
pH

of this energy as fluorescence. This occurs with both of the methyleneiminodiacetic acid groups. A point of inflection in the absorbance versus pH curve occurs at pH 10.3, corresponding to the average of the negative logarithm of the sixth and seventh dissociation constants.

C. Purity of β-Sulfocalcein. Fluorometric Determination of β-Sulfofluorescein in β-Sulfocalcein

The presence of β-sulfofluorescein as an impurity affects the quality of β-Sulfocalcein whether used as an indicator in complexometric titrations or for the direct fluorometric determination of calcium. The fluorescence of an impurity of β-sulfofluorescein would constitute a disturbing background and reduce the sensitivity and usefulness of the β-Sulfocalcein as a metallofluorchromic reagent. β-Sulfofluorescein fluoresces at high pH but β-Sulfocalcein does not, except, of course, in the presence of calcium. Two methods were used to determine the presence of β-sulfofluorescein in β-Sulfocalcein.

1. Paper chromatography

A few milligrams of β-Sulfocalcein was dissolved in about 5 ml of 0.1 M potassium hydroxide. In another test tube a few milligrams of β-sulfofluorescein was dissolved in the same manner. Three spots were applied to a strip of

filter paper, Whatman No. 3, about 10 cm wide and 35 cm long. Spot (a) contained β-sulfofluorescein; spot (b) contained β-Sulfocalcein; and spot (c) contained both β-sulfofluorescein and β-Sulfocalcein.

The driving solvent was water buffered at pH 4.0 by dissolving 10.2 g of potassium hydrogen phthalate in one liter of deionized water. After elution, the chromatogram was air dried and examined under an ultraviolet (black light) lamp. Two spots were observed for (a) as expected, with Rf values 0.52 and 0.65 corresponding to 5- and 6-sulfofluorescein, respectively. One spot was observed for (b) with Rf value about 1.0, corresponding to β-Sulfocalcein with no indication at all of other spots. Three spots were observed for (c), corresponding to the sulfofluoresceins and to β-Sulfocalcein.

This experiment indicated that the β-isomers of sulfofluorescein can be separated from β-Sulfocalcein on a paper chromatogram and that the β-Sulfocalcein was free from β-sulfofluorescein.

2. Fluorescence at high pH

A method was developed by Martin (4) to determine quantitatively the amount of fluorescein present as an impurity in Calcein. The method was based on the observation that in a solution of high pH, pH about 13, fluorescein was fluorescent but Calcein was not. The same facts are true of

the sulfonated derivatives and advantage was taken of this for an analogous analytical method.

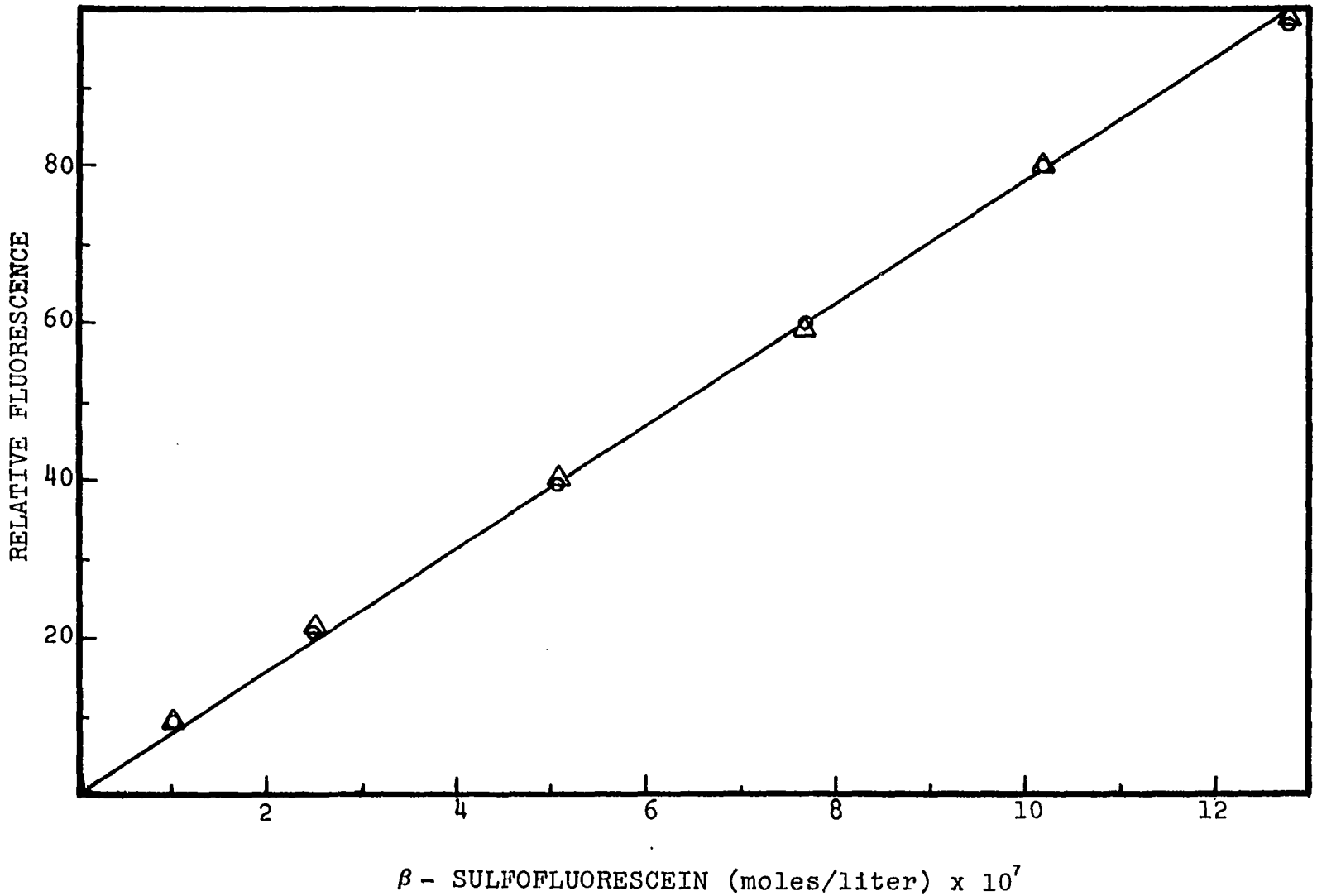
a. Apparatus and procedure All measurements of fluorescence were obtained with a Turner Model 110 Fluorometer. The primary filter was a Corning 5850 glass filter. The secondary filter was a combination of a Corning 2A-15 glass filter and a Wratten 10 per cent neutral density filter.

A stock solution of 5.155×10^{-4} M β-sulfofluorescein was prepared by dissolving 0.2126 g of the compound in exactly 1 liter of 0.1 M potassium hydroxide. An aliquot of 10.00 ml was transferred to a 1-liter volumetric flask, diluted to the mark with 0.1 M potassium hydroxide and the solution was mixed. The final solution was 5.16×10^{-6} M in β-sulfofluorescein. In each of seven 100-ml volumetric flasks was placed 2.0 ml of 0.01 M EDTA. The first solution was diluted to the mark with 0.1 M potassium hydroxide; this solution, without the addition of β-sulfofluorescein, served as a blank to zero the fluorometer. To the remaining flasks were added from 2.00 to 25.00 ml of 5.15×10^{-6} M β-sulfofluorescein. Each solution was diluted to exactly 100.0 ml with 0.1 M potassium hydroxide. The relative fluorescence was measured. The fluorescence was found to be linear over the entire range, 0 to 12.8×10^{-7} M, Figure 86.

Figure 86. Relative fluorescence as a function of the concentration of β-sulfofluorescein in 0.1 M potassium hydroxide.

o: β-Sulfofluorescein alone

Δ: β-Sulfofluorescein plus β-Sulfocalcein, 5.2×10^{-7} M


RELATIVE FLUORESCENCE
80
60
40
20
2
4
6
8
10
12
β - SULFOFLUORESCEIN (moles/liter) x 10⁷

A stock solution of β-Sulfocalcein, 5.158×10^{-4} M, was prepared by dissolving the appropriate amount of the compound in exactly 500.0 ml of deionized water. An aliquot of 10.00 ml was transferred to a 1-liter volumetric flask and diluted to the mark with 0.1 M potassium hydroxide. The final solution was 5.16×10^{-6} M in β-Sulfocalcein.

Another series of seven 100-ml volumetric flasks was prepared. To each flask was added 2.0 ml of 0.01 M EDTA and 10.00 ml of 5.16×10^{-6} M β-Sulfocalcein. To these solutions was added from 0.00 to 25.00 ml of 5.16×10^{-6} M β-sulfofluorescein. All were diluted to the mark with 0.1 M potassium hydroxide and thoroughly mixed. The final solutions were 5.2×10^{-7} M in β-Sulfocalcein, and 0.00 to 12.8×10^{-7} M in β-sulfofluorescein. The relative fluorescence was measured. The resulting curve is presented in Figure 86.

b. Results and discussion The relative fluorescence of β-sulfofluorescein solutions and of β-Sulfocalcein solutions spiked with β-sulfofluorescein are presented in Figure 86. The relative fluorescence curves of both coincide through the entire range measured. Furthermore, the relative fluorescence curve of β-Sulfocalcein spiked with β-sulfofluorescein passes through the origin, which confirms that β-Sulfocalcein does not contain any β-sulfofluorescein as impurity.

The new compound β-Sulfocalcein should prove to be superior than the parent compound Calcein, not only because of the high solubility of the new compound but also because of the absence of the sulfofluorescein as impurity.

XII. THE REACTION OF β-SULFOCALCEIN WITH CALCIUM

A. Introduction

The overall objective of the present work was to provide an improved reagent for the fluorometric determination of calcium. It was thus with a certain trepidation that I faced the final phase of the investigation, to learn if the final product of the long sequence of organic syntheses, Sulfocalcein, indeed reacted with calcium, and if so, if the material could be used to advantage by the analytical chemist. The answers fortunately appear to be yes.

B. The Fluorescence of β-Sulfocalcein as Produced by Added Calcium

1. Reagents

a. Calcium chloride A stock solution of calcium chloride, 0.0100 M, was prepared by weighing out exactly 1.000 g of primary standard calcium carbonate. The compound was transferred directly into a 1-liter volumetric flask. A volume of 6 ml of concentrated hydrochloric acid was added, and after dissolution had occurred, the solution was diluted to the mark with deionized water and thoroughly mixed. An aliquot of 10.00 ml of this solution was transferred to another 1-liter volumetric flask, diluted to

volume with deionized water and the solution thoroughly mixed. The final solution was thus 1.00×10^{-4} M in calcium.

b. β-Sulfocalcein A stock solution, 2.50×10^{-4} M, of β-Sulfocalcein was prepared by transferring the appropriate amount, accurately weighed, of the material to a 1-liter volumetric flask. The flask was filled to the mark with deionized water and the resulting solution was thoroughly mixed.

2. Apparatus and procedure

In each a series of 100-ml volumetric flasks was placed 50.00 ml of 0.2 M potassium hydroxide and 2.0 ml of 2.5×10^{-4} M β-Sulfocalcein. In sequence was added varying volumes of a standard solution of calcium chloride, 1.00×10^{-4} M, such that the ratio of calcium to β-Sulfocalcein varied from zero to 4. Each solution was diluted with deionized water and thoroughly mixed. Two solutions were also prepared which contained no calcium chloride. To one of them was added 2.0 ml of 0.01 M EDTA; this solution was used as a blank to zero the fluorometer. The second of these solutions, containing neither calcium nor EDTA, was used to correct for the amount of calcium present at impurity in the potassium hydroxide and water used.

3. Results and discussion

The relative fluorescence of β-Sulfocalcein with varying amounts of added calcium is presented in Figure 87. As shown, the straight lines placed through the well-defined individual regions of the curve, on extrapolation (dotted lines) yield two points of intersection. These points fall just short of mole ratios of calcium to β-Sulfocalcein of one and two. Thus, calcium combines with β-Sulfocalcein in the ratio of one and of two and the formation of the first compound is essentially complete before the second compound begins to form. The data indicate that both compounds are fluorescent and that the intensity of fluorescence of the second compound is about twice that of the first. The data indicate further that the stability of the calcium-β-Sulfocalcein compounds is high.

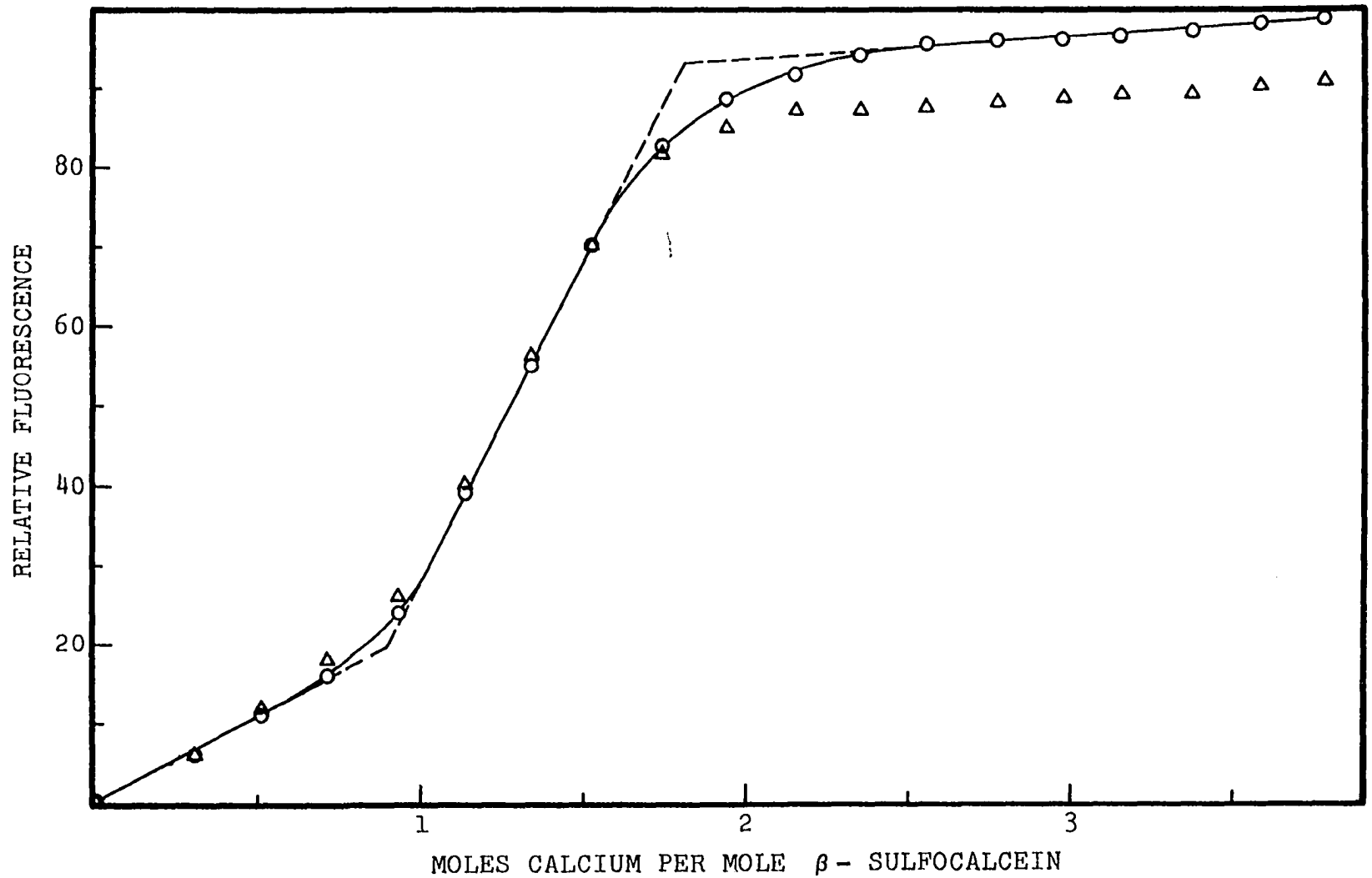
As shown by the data present in Figure 87, some decrease in the intensity of the fluorescence occurs on standing but this is confined to solutions containing an excess of calcium; decrease may well be the result of the precipitation of some calcium-β-Sulfocalcein compound.

It is apparent that β-Sulfocalcein will be suitable for the direct fluorometric determination of calcium and that the most appropriate working range will be that over which the second atom of calcium is added to the β-Sulfocalcein.

Figure 87. Relative fluorescence of β-Sulfocalcein, 5.0×10^{-6} M in 0.1 M potassium hydroxide as a function of the amount of calcium added.

o: Fluorescence immediately after mixing

Δ: Fluorescence after 8 hours


RELATIVE FLUORESCENCE
80
60
40
20
1
2
3
MOLES CALCIUM PER MOLE β - SULFOCALCEIN

C. Application to the Determination of Calcium in Limestone

1. General chemistry involved

It is proposed to employ β-Sulfocalcein as indicator in the EDTA titration of calcium. The chemistry of this titration and the use of the parent compound, Calcein, as indicator is described by Diehl (2,24). Basically, the solution containing the calcium is treated to mask (prevent interference by) any copper, iron and aluminum present and the pH brought to 13 by the addition of concentrated potassium hydroxide. A standard solution of EDTA (designated Na_2H_2Y) is added causing the calcium present to form a slightly dissociated ion:

$$Ca^{2+} + Na_2H_2Y + 2OH^- = CaY^{2-} + 2Na^+ + 2H_2O$$

Prior to the end-point, the small amount of β-Sulfocalcein added as indicator unites with calcium to form a yellow-green, fluorescent compound. At the conclusion of the main reaction, the first small excess of EDTA extracts the calcium from the β-Sulfocalcein-calcium compound and the fluorescence disappears marking the end-point:

$$\beta\text{-Sulfocalcein-Ca} + Na_2H_2Y + 2OH^- = CaY^{2-} + 2Na^+ + H_2O + \beta\text{-Sulfocalcein}$$

Fluorescent — nonfluorescent

The interference of copper and iron is eliminated by reducing these metals to the univalent and bivalent state, respectively, usually by adding ascorbic acid, and then converting the reduced ions to the slightly-dissociated cyanide compounds by the addition of potassium cyanide. Aluminum is masked by the addition of triethanolamine.

Magnesium reacts with EDTA the same way calcium does; however, at pH 13, magnesium is precipitated as magnesium hydroxide which does not react with EDTA. Thus, raising the pH to 13 serves two purposes, to render the magnesium non-titratable and to make the β-Sulfocalcein (in the absence of calcium ions) nonfluorescent. The high pH is obtained by adding 12 M potassium hydroxide, potassium hydroxide rather than sodium hydroxide inasmuch as high concentrations of sodium produce a slight fluorescence with Calcein but high concentrations of potassium do not.

a. Reagents A solution of 0.0100 M disodium dihydrogen ethylenediaminetetraacetate (EDTA) was prepared by dissolving approximately 4 g of the salt in 1 liter of deionized water. The solutions was standardized against a standard solution of 0.0100 M calcium solution prepared from reagnet grade calcium carbonate.

A solution of calcium-free potassium hydroxide, 5 M, was prepared by diluting 50 per cent potassium hydroxide obtained from the Hach Chemical Company, Ames, Iowa.

Solutions of 0.2 per cent ascorbic acid, 2 per cent potassium cyanide, and 5 per cent triethanolamine were prepared.

A solution of β-Sulfocalcein, 0.03 per cent, in water was prepared.

b. Apparatus Titrations were performed in a dark box supplied with a black light. The end-point was observed through an amber glass at a right angle to the light source. Such a "titration box" is described by Diehl (reference 24, page 286).

c. Procedure A sample of 1 g of limestone was weighed accurately and transferred to a 600-ml beaker. About 15 ml of concentrated perchloric acid (70 per cent) was added, a cover glass was placed on the beaker, and the mixture heated on a hot plate for 15 to 20 minutes. At this point only a colorless residue of silica remained as the acid was refluxing gently on the wall of the beaker. The solution was cooled. Deionized water was added and the solution was transferred to a 1-liter volumetric flask. The solution was diluted to the mark and mixed well. The silica was allowed to settle and an aliquot of 25.00 ml was pipetted into a 400-ml conical flask. To the solution was added 5 ml of 0.2 per cent ascorbic acid, 5 ml of 5 per cent potassium cyanide, and 5 ml of 10 per cent triethanolamine. After mixing, 5 ml of 5 M potassium hydroxide was added.

Finally, 5 drops of 0.03 per cent β-Sulfocalcein was added. The solution was titrated with standard ETDA solution. The titration was repeated with a more rapid addition of the standard EDTA to within a 1 to 2 ml of the end-point with vigorous stirring and the titration completed with a regulated flow of EDTA, one drop every four seconds, with continuous stirring. The end-point was taken as the first disappearance or change in fluorescence.

2. Results and discussion

The method was applied to the determination of calcium in two standard samples of the National Bureau of Standards, NBS 1A, Argillaceous Limestone, and NBS 88, Dolomite. The results are reported in Table 26.

The results obtained were excellent. Close attention, however, was necessary at several points in the analysis.

The approach to the end-point should be at a slow and uniform rate with vigorous stirring, preferably mechanical (magnetic stirring bar). This is especially true when large amounts of magnesium are present as in the analysis of a dolomite. It is likely that the precipitate of magnesium hydroxide carries with it some calcium which reacts slowly with the EDTA

The addition of excess amounts of the indicator, about 5 drops of 0.03 per cent of β-Sulfocalcein is advisable, again, especially when large amounts of magnesium are present.

Table 26. Determination of calcium in standard samples of limestone

	Argillaceous Limestone NBS No. 1A	Limestone Dolomite NBS 88
CaO reported, per cent	41.32	30.49
MgO reported, per cent	2.19	21.48
CaO found, per cent	41.27	30.41
	41.30	30.44
	41.22	30.39
	41.23	30.44
	41.31	30.42
	41.28	30.51
	41.26	30.47
	41.34	30.40
	41.21	30.42
Average	41.27	30.43
Range	0.13	0.12
Standard deviation, σ	0.044	0.034
(δ = 1000 σ/Average)	1.07	1.12

XIII. SUMMARY

A survey has been made of our knowledge of the metallofluorochromic indicator *Calcein*. The literature on this reagent is now approaching two hundred papers, the majority of which deal with the use of the reagent in the determination of calcium. In particular, those papers originating at Iowa State University have been reviewed; these include the first paper to describe the reagent and later ones concerned with the synthesis, purity and structure of the compound and with the nature of the reaction of the reagent with calcium and other metals.

It has been proposed to prepare a sulfonated Calcein, the objective being to obtain a reagent of greater solubility, enhanced fluorescence, and altered acid-base and chelating characteristics.

It was proposed for the synthesis of the sulfonated Calcein to follow the route: naphtholsulfonic acid, sulfophthalic acid, sulfofluorescein, sulfocalcein. This synthesis how has been carried through; the product has been designated β-*Sulfocalcein*.

The oxidation of 1-naphthol-5-sulfonic acid to 3-sulfophthalic acid and of 2-naphthol-6-sulfonic acid to 4-sulfophthalic acid with potassium permanganate has been improved and the products isolated as the potassium hydrogen

salts. These preparations, described in the thesis for the M.S. degree, have now been checked on a larger scale.

Conversion of 3-sulfophthalic acid to the isomeric mixture of 4- and 7-sulfofluorescein by condensation with resorcinol, also reported in my M.S. thesis, has been repeated and the synthesis has been improved. The isomeric mixture of these two isomers is referred to as α-sulfofluorescein.

Similarly, 4-sulfophthalic acid has been converted to the isomeric mixture of 5- and 6-sulfofluorescein. This mixture is referred to as β-sulfofluorescein.

For both mixtures, α-sulfofluorescein and β-sulfofluorescein, the product isolated has been shown to be the monopotassium salt; the evidence was derived from direct titration with standard alkali and from conversion to the free acid by passage through a strong acid cation exchange resin in the hydrogen form followed by titration with alkali.

The conversion of the monopotassium salts of α-sulfofluorescein and of β-sulfofluorescein on a large scale has been accomplished by the cation exchange resin method and the free acids isolated in crystalline form and the purities established by titration.

β-Sulfofluorescein has been separated into the component isomers by repeated recrystallization from water-ethanol mixtures. The purity of the component acids,

obtained in about equal amounts, and the effectiveness of the separation were established by paper chromatography, Rf values of 0.51 and 0.64.

α-Sulfofluorescein has been shown to consist almost entirely of one isomer, by paper chromatography, Rf 0.41.

The isomers obtained from β-sulfofluorescein have been shown to be 5-sulfofluorescein (Rf 0.51) and 6-sulfofluorescein (Rf 0.64) by nuclear magnetic resonance (NMR) spectroscopy, the identifications being based on chemical shifts, splitting patterns, integration of the areas under peaks, and comparison with the spectrum of fluorescein and related compounds. Similarly, the isomer isolated in sufficient quantity for study from α-sulfofluorescein has been shown to be 4-sulfofluorescein. The NMR spectra provided no information relative to the replaceable hydrogen atoms and of the structural forms of the respective sulfofluoresceins.

The infrared spectra of the yellow and of the red solid forms of fluorescein and of the yellow solids of 4-, 5-, and 6-sulfofluorescein have been obtained on both thin films and on potassium bromide discs. Well-resolved spectra have been obtained.

The infrared spectra of the yellow and red solid forms of fluorescein confirm the structures assigned by Markuszewski to these compounds, the zwitter ion-pyrylium ring structure to the yellow solid and the quinone-carboxylic acid structure to the red solid. Additional

bands have been found in these spectra not observed by Markuszewski: in the yellow solid two bands, at 1537 cm^{-1} and 1640 cm^{-1}, which correspond to symmetric and asymmetric carbon-oxygen stretching vibrations and further confirm the zwitter ion-pyrylium ring structure; and in the red solid, a band at 1632 cm^{-1} corresponding to the carbon-oxygen stretching vibration of a highly conjugated quinone, further confirming the assignment of the quinone-carboxylic acid structure.

It has been shown that in the process of preparing potassium bromide discs on which to obtain infrared spectra that some interconversion of the red form of fluorescein to the colorless (lactone) form occurs, and also that interconversion of the yellow form to the red and colorless forms occurs.

It has been observed that the three isomers of sulfofluorescein exist only in the yellow form in the solid state. The infrared spectra of these solids have furnished evidence of the presence in each compound; a free carboxylic acid group, a sulfonate group, phenolic groups, and a pyrylium ring. Thus, there has been assigned to the three solid sulfofluoresceins the zwitter ion-pyrylium ring structure bearing a sulfonate group and a free carboxylic acid group.

Values for the acid dissociation constants of 4-, 5-, and 6-sulfofluorescein have been obtained by combining data

from potentiometric titrations with measurements of absorbance, absorbance, fluorescence and solubility as functions of pH. The values obtained, expressed as negative logarithms are:

	Sulfofluorescein			Fluorescein, Yellow
	4-	5-	6-	(H_3Fl^+)
pK_1	1.20	2.14	2.15	2.15
pK_2	5.28	4.34	4.39	4.73
pK_3	6.25	6.27	6.25	6.55

the values for fluorescein being those obtained by Markuszewski.

An explanation has been advanced to account for the values of the first two acid dissociation constants of 4-sulfofluorescein, which differ markedly from the corresponding values for 5- and 6-sulfofluorescein and fluorescein, the latter agreeing closely among themselves. The explanation is based on the assumption that all four compounds exist in acidic solution in the colorless lactone form as well as in the yellow, zwitter ion-pyrylium ring form, the proportion of lactone simply being much greater in solutions of the 4-sulfofluorescein isomer. Supporting evidence for the simultaneous presence of the lactone and zwitter ion forms in solutions of low pH of all of the compounds has been derived from potentiometric titrations in water and in 50 per cent ethanol, the complex and surprising solubility as a function of pH curves of the sulfofluoresceins, and the

absorption spectra of the compounds in water and in 50 per cent ethanol as functions of pH. This understanding of the nature of fluorescein in water solution differs sharply from the recent work of Markuszewski which is based on the belief that the only form of fluorescein, and of Calcein, in water solution of low pH is the zwitter ion-pyrylium ring form.

The absorption spectra of fluorescein, 4-sulfofluorescein, 5-sulfofluorescein and 6-sulfofluorescein have been obtained at accurately measured pH about 1, 3, 5 and 8. The corresponding spectra of the four compounds have been found to be almost identical in the number of absorption bands, the positions of the bands, and the changes of the bands with pH. From a detailed consideration of the two absorption bands which appear in the spectra of fluorescein, the intensities of the bands, and the changes in the bands with pH, it has been concluded that the neutral form of fluorescein when dissolved in water exists in part in the colorless lactone form as well as in the zwitter ion-pyrylium ring form. Similar considerations have been found to apply to 4-, 5-, and 6-sulfofluorescein. 4-Sulfofluorescein in water solution has been found to exist almost entirely in the lactone form; the steric considerations which bring this about have been discussed.

The absorbance of fluorescein and of 4-, 5-, and 6-sulfofluorescein at specific wavelengths in solutions of varying pH in small intervals over the range 1 to 11 has

been measured. The data have been treated mathematically to yield values for the acid dissociation constants of each compound. Assignments have been made of the structural form or mixtures of structural forms in which the various prototropic forms of the four compounds exist.

The fluorescence excitation and emission spectra of 4-, 5-, and 6-sulfofluorescein have been obtained and found to be almost identical and identical with the spectra of fluorescein. The relative intensity of fluorescence and change in this intensity with pH have also been found to be almost identical. The third and the second acid dissociation constants of the three sulfofluoresceins have been calculated from the data of relative fluorescence at appropriate pH. From the mathematical treatment of the fluorescence of 5- and 6-sulfofluorescein, it has been concluded the dianion, $HSfl^{2-}$, of the sulfofluoresceins does fluoresce, and by examining the analogous data of fluorescein, reported by Markuszewski, it has been concluded also that the corresponding prototropic form of fluorescein, HFl^{-}, is fluorescent.

A minimum has been found in the curve of solubility as a function of pH of 4-sulfofluorescein, at pH 3.3, and of 5-sulfofluorescein, at pH 3.4; in this respect these compounds resemble fluorescein, minimum at pH 3.4 (Markuszewski (3)). 4-Sulfofluorescein and 5-sulfofluorescein have been

found to differ from fluorescein in exhibiting a maximum in solubility, at pH 2.2 and 2.7, respectively. It has been found that the solubility of 6-sulfofluorescein displays neither a minimum nor a maximum. An explanation has been offered for the changes in solubility with pH of the sulfofluoresceins in terms of the coexistence of the two structural forms, lactone and zwitter ion-pyrylium ring, for each of the prototropic species involved over the pH range covered, pH 1 to 4. The solubility data has been subjected to a mathematical treatment involving the hypothetical "intrinsic solubility", designated S_i, to yield values for the first acid dissociation constant of each compound:

	Sulfofluorescein		
	4-	5-	6-
S_i (moles/liter) x 10^5	1.96	7.30	1.32
pK_{H_3Sfl}	1.19	2.13	2.14

The three isomers of sulfofluorescein have been titrated in water. From the titration curves, the second and the third dissociation constants of each isomer have been calculated. The potentiometric titration of 5-, and 6-sulfofluorescein has confirmed further the very similar acid-base properties of these two isomers. The titration curve of 4-sulfofluorescein has furnished further evidence that the acid-base properties of this isomer differ markedly from the β-isomers.

The chemistry of fluorescein and of the three isomers of sulfofluorescein in 50 per cent ethanol have been investigated. A correlation between the acid-base properties and the absorption spectra of these four compounds in water and in 50 per cent ethanol solvent has confirmed the following: 1) fluorescein and the sulfofluoresceins do exist in the colorless lactone form in aqueous solution. 2) The acid-base properties of these compounds are very dependent on the equilibria established between the different structural forms in a particular solvent.

β-Sulfofluorescein has been subjected to the Mannich condensation with formaldehyde and iminodiacetic acid, water being used as solvent for the reaction. Details of the synthesis, isolation and purification of the product have been perfected, and yields of 74 per cent obtained. The product, a new metallofluorochromic indicator, has been named β-*Sulfocalcein*.

By means of titration with alkali, titration with alkali after the addition of excess calcium, and passage through a cation exchange resin in the hydrogen form, β-Sulfocalcein has been shown to carry seven replaceable hydrogen atoms and the product actually isolated from the synthesis to be a one-to-one mixture of the monopotassium and dipotassium salts (designated KH_6Scal and K_2H_5Scal).

By paper chromatography β-Sulfocalcein has been shown to be free of β-sulfofluorescein. A method has been

developed for the quantitative determination of an impurity of β-sulfofluorescein in β-Sulfocalcein, taking advantage of the great difference in the properties of the two compounds in solutions of pH 13, β-sulfofluorescein being strongly fluorescent but β-Sulfocalcein completely nonfluorescent. By application of this procedure, the β-Sulfocalcein which has been synthesized in this work, has been shown to be completely free of β-sulfofluorescein.

Potentiometric titrations of β-Sulfocalcein in water and in 50 per cent ethanol show strong resemblances to the acid-base characteristics of fluorescein, β-sulfofluorescein and Calcein. Assignments of the seven replaceable hydrogen atoms (protons) to specific acid groups in the molecule have been made. Certain prototropic forms of β-Sulfocalcein, like to those of β-sulfofluorescein, exist in solution as mixtures of the lactone, zwitter ion-pyrylium ring and quinone structures.

The absorption spectrum of β-Sulfocalcein has been obtained at several pH. The spectrum has been shown to contain at low pH an absorption band with a maximum at 445 nm, which disappears with rising pH, and another band which rises in intensity and shifts to longer wavelengths with rising pH: shoulder at 475 nm at pH 5.10, a maximum at 498 nm at pH 7 to 9, a maximum at 506 nm at pH above 11. These absorption bands have been interpreted in terms of

the prototropic forms present at the various pH and the mixture of coexisting structural forms.

The fluorescence excitation and emission spectra of β-Sulfocalcein have been obtained at various pH over the range 1 to 13. The relative intensity of fluorescence has been measured over appropriate ranges of pH at small intervals of pH and using for excitation radiation the wavelengths of maximum excitation (475 nm at low pH; 500 nm at high pH) and measuring the emitted light at 520 nm, the maximum in the emission spectrum. As with Calcein, the fluorescence of β-Sulfocalcein has been found to reach a maximum at pH 7.5 and at pH above this to decrease to zero at pH 13.

By the appearance and increase of fluorescence with added calcium added at high pH, it has been shown that β-Sulfocalcein reacts sequentially with calcium, adding first one atom of calcium and then a second atom, the rate of increase of fluorescence being twice as great as the second atom of calcium is added as when the first atom is added.

β-Sulfocalcein has been found highly satisfactory as an indicator in the EDTA titration of calcium at high pH. The titration has been applied to the determination of the calcium in two standard samples of limestone of the National Bureau of Standards, No. 1A, Argillaceous Limestone, and No. 88, Dolomite; excellent results have been obtained.

XIV. BIBLIOGRAPHY

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