SYNTHESIS, CHARACTERIZATION AND TLC-FLUORESCENCE ANALYSIS OF THE FLUORESCEINS FROM DICARBOXYLIC ACIDS

By

Zenaida L. Ochotorena

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ABSTRACT

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The reaction of resorcinol with the dicarboxylic acids, succinic, 2,2 dimethylsuccinic, methylsuccinic, suberic, adipic, pimelic and o-phthalic acids were studied so that the formation of their fluoresceins could be utilized in the quantitative determination of these acids by fluorimetry. The fluoresceins of the above mentioned acids were synthesized using as the condensing catalyst, sulfuric acid, polyphosphoric acid, p-toluenesulfonic acid or Dowex 50W x 12. The fluoresceins were characterized by taking their mass spectra, proton and carbon-13 NMR, adsorption and emission behavior, TLC chromatograms and elemental carbon analysis. Yields from the use of the different condensing catalyst were determined and compared.

The optimum conditions to carry out the reactions so that they attained a constant yield or went to completion using PPA as the condensing catalyst were determined. Using the optimum conditions of 1:10 or greater acid to resorcinol ratio, a reaction temperature maintained at

120 to 140° for about 2 hours and an 0.20 ml of PPA and an acid sample of the order of 700 or less ug, succinic acid present singly in a sample was analyzed by measuring the fluorescence intensity of the solution prepared from the reaction mixture. Interference from the resorcinol blank was observed to be negligible at the dilution of the reaction mixture used and the analysis were carried out even with acid content in the sample of less than 5 ug. The average relative error of about 5.5% was obtained when a calibration curve was used and about 1.5% when the sample was compared to a standard succinic acid sample run simultaneously with the samples being analyzed.

Analyses of mixtures of succinic, methylsuccinic and 2,2 dimethylsuccinic, suberic and o-phthalic acids were carried out using a TLC-fluorescence method. TLC chromatograms prepared from the reaction mixture dissolved in methanol and developed with a mixture of chloroform and methanol at low humidity, yielded fluorescence peaks of the separated fluoresceins of the acids. The areas under the fluorescence peaks were linearly related to the concentration of the corresponding acid for acid contents as low as 10^{-8} g. Analyses by using a standard sample mixture, prepared and developed together with the samples being analyzed for comparison, resulted in relative errors of about 1-20% depending on the polarities of the acids in the mixture, the number of acid components in the samples being analyzed and the available instrumentation.

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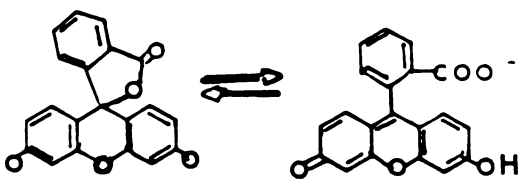
SYNTHESIS AND CHARACTERIZATION OF THE FLUORESCEINS

A. HISTORICAL

Fluorescein was the name given to the product obtained from the fusion of phthalic anhydride and resorcinol.[1] This compound (1) emitted a strong, beautiful, green fluorescence in alkaline solution so that it was used in the qualitative detection of resorcinol and phthalic anhydride. Also it found use in determining the course of flowing water and the mixing of different bodies of water.[2]

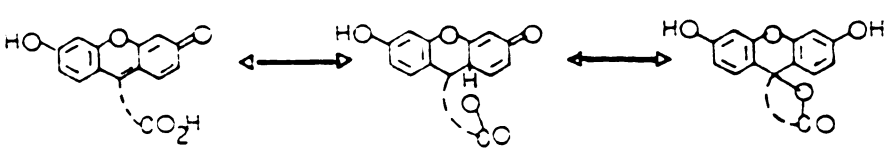


1

In 1881 during an extended investigation on the condensation of phenols with aliphatic acids, Nencki and Sieber[3] described for the first time succinyl fluorescein and one or two of its derivatives. This fluorescein was analogous in nearly all respects to those of the original fluorescein except that it did not form the chloro-derivative with phosphorus pentachloride.

Biggs and Pope[4] in 1923 prepared the succinyl fluorescein and its derivatives using the method used by Nencki and Sieber. Succinic anhydride was boiled under reflux with sulfuric acid for six hours and they reported a yield of 90% using 73% sulfuric acid instead of the concentrated sulfuric acid used by Nencki and Sieber which only gave a yield of 60-70% of the theoretical because of the excessive amount of by-products formed.

In 1924, Dutt and Thorpe[5] doing a study on ring-chain tautomerism, prepared the fluoresceins of succinic anhydride and substituted succinic acids as well as those of glutaric anhydride and substituted glutaric acids using the method of Biggs and Pope. In this study it was established that the change from colorless base to the colored salt was accompanied by a change in structure from the lactone (III) to the quinone (II) form and that the mechanism of the change was similar to that which existed in the case of the mutarotatory sugars, being the case of ring-chain tautomerism between individuals (II) and (III).



II Intermediate III

In 1970, Spassovska and Panayotov[6] used the condensation reaction of resorcinol and polymer anhydrides to synthesize fluorescing polymers. In this study the fluoresceins of acrylic, itaconic, methacrylic and maleic anhydrides were also synthesized to serve as models for comparison as regards structure, properties and behavior. The method used in the synthesis of the fluoresceins of the polymer and those of the monomer was similar to the method adopted for the preparation of the fluorescein of phthalic anhydride[7] which involves heating the phthalic anhydride and resorcinol mixture to 180°C for 45 minutes in the presence of anhydrous zinc chloride as condensing agent. Spassovska heated the mixture of polymer anhydride- or monomer anhydride- and resorcinol to 180°C for 30 minutes, added the anhydrous zinc chloride and then reheated the mass to 200°C for 150 minutes.

The latest work reported on the preparation of fluorescein through resorcinol condensation with the acid or anhydride was that of a USSR patent of 1975[8] which involves condensing phthalic anhydride and resorcinol in the presence of ortho- or polyphosphoric acid as a condensing agent.

No study had been reported in the use of p-toluenesulfonic acid or the resin, Dowex 50W, as catalyst in the preparation of fluorescein. But p-toluenesulfonic acid had been used as catalyst for esterification,

transesterification, ketalization, acetylation, dehydration and cyclodehydration.[9,11] It had been found to be as effective an acid catalyst as sulfuric acid and is generally preferred to sulfuric acid because it is less damaging to reactants. Likewise, Dowex 50W is a strong acid cation exchange resin made by the nuclear sulfonation of styrene-divinylbenzene beads. These resins behave as strong acids and catalyze reactions, which are normally catalyzed by mineral acids, such as esterification, epoxidation and alkylation.[10]

B. EXPERIMENTAL

REAGENTS

All chemicals used were chemically pure or analytical grade reagents and were used without further purification.

Dowex 50W x 12 was dried in an oven at about 60-70°C for a day or two. Then the temperature of the oven was raised to 100° and kept at this temperature for 2 hours.

Inasmuch as commercial polyphosphoric acid (PPA) is very viscous and reaction products obtained when it was used were difficult to dissolve, the PPA used in this study was a 1:1 weight-volume ratio of phosphorus pentoxide dissolved in phosphoric acid (85%). The weighed amount of phosphorus pentoxide was placed in a flask provided with a magnetic bar stirrer and a stopper. The measured volume of phosphoric acid was slowly added (the reaction is exothermic and sufficient heat is generated to dissolve the phosphorus pentoxide). The mixture was stirred and kept at a temperature of 40° for a day or two to equilibrate because equilibration of this mixture is a slow process. This mixture is stable and can be prepared long before the anticipated use. PPA prepared by just mixing the needed components without equilibration will not yield reproducible results.

Preparation of the Fluoresceins

Different methods were tried to produce the fluorescein with the least amount of side product impurities. These methods made use of different condensing catalysts.

1. Use of Sulfuric Acid as Condensing Catalyst (Biggs Method[4])

A 0.1 mole of the acid or anhydride was added with slightly more than 0.2 mole of resorcinol to 53 ml of 73% sulfuric acid. The mixture was boiled under reflux for 6 hours after which the refluxed material was cooled, dissolved in 1 liter of distilled water and carefully neutralized with dilute sodium hydroxide. The precipitate was filtered, washed well with distilled water and crystallized from 5% hydrochloric acid. The crystal was recrystallized several times from hydrochloric acid and finally dissolved in warm sodium carbonate (10%) solution, precipitated by acetic acid, collected, washed well and dried to constant weight at 160°C.

2. Use of Polyphosphoric Acid as Condensing Catalyst

To 30 ml of PPA heated to about 70°C, 0.1 mole of the acid or anhydride was added. The mixture was stirred to dissolve the acid or anhydride. Slightly more than 0.2 mole of resorcinol was added and the stirring and

heating were continued, until the components were well mixed. The temperature was then slowly raised to 110°C and kept at about 110-120°C for about two hours. The red viscous mass was cooled and dissolved with sufficient 0.1 M sodium hydroxide to neutralize most of the PPA. The solution was then carefully neutralized, the precipitate formed was filtered, washed well with distilled water and crystallized from 5% hydrochloric acid. The crystals obtained were recrystallized an additional 2 or 3 times from 5% hydrochloric acid and finally dissolved in warm sodium carbonate solution and precipitated with acetic acid. The precipitate was filtered, washed well, then dried to constant weight at about 160°C.

3. Use of p-Toluenesulfonic Acid as Condensing Agent and Cyclohexane as Solvent

A 0.1 mole of acid or anhydride, slightly more than 0.2 mole of resorcinol and 0.05 mole of p-toluenesulfonic acid were mixed well in 125 ml of cyclohexane and refluxed for 4 hours. The mixture was heated by an oil bath maintained at 125-127°C. The reaction mixture did not dissolve in cyclohexane but upon reflux a red viscous melt was produced. The red viscous mass obtained was cooled, the solvent was decanted and excess solvent was removed by a rota-vapor distillation under vacuum. The solvent-free mixture was dissolved in 5% sodium bicarbonate

and filtered. The filtrate, which was at about pH 8.0 was very slowly adjusted to pH 6.0 with 2.5% sulfuric acid. It was then set aside until effervescence stopped. It was again filtered, and the dark wine-red clear filtrate was again adjusted to pH 6.0 with the 2.5% sulfuric acid. If a precipitate formed, it was filtered and the process repeated until no precipitation occurred at this pH. To the clear, dark wine red filtrate at pH 6.0 was slowly added more 2.5% sulfuric acid until precipitation was complete (pH approximately 4.7). The precipitate was collected, washed well with distilled water, then dissolved in warm sodium carbonate and reprecipitated with acetic acid. The precipitate was collected, washed well and dried to constant weight at about 160°C.

4. Use of p-Toluenesulfonic Acid as Condensing Catalyst and 1,2 Dichloroethane as Solvent

With the same mixture of reactants as in (3) above, 125 ml of 1,2 dichloroethane was used. The mixture was refluxed at an oil bath temperature of 110-120°C for 4 hours. The refluxed material was cooled and transferred to a separatory funnel. To it was added about 600 ml of 1 M sodium hydroxide, shaken and set aside to separate the solvent layer which was then removed. To the solvent-free fluorescein solution dilute hydrochloric acid was slowly added until precipitation was complete. The

precipitate was collected and washed with water. The precipitate was dissolved in 1 M sodium hydroxide and reprecipitated with dilute hydrochloric acid. The precipitate was collected and washed with distilled water. The redissolving and reprecipitating operations were carried out several times more, and finally the precipitate was dissolved in warm sodium carbonate, precipitated with acetic acid, collected, washed and dried.

5. Use of Dowex 50W x 12 as Condensing Catalyst

To the same amounts of acid or anhydride-resorcinol mixtures as used in the previous methods, 10 grams of the dried Dowex and 125 ml of 1,2 dichloroethane was added. The mixture was stirred with a magnetic stirrer and refluxed for 6 hours at an oil bath temperature of about 110-120°C. The refluxed material was cooled, 1 M sodium hydroxide was added and transferred to a separatory funnel. The solvent was separated as in the previous method and the solvent-free fluorescein solution with the resin was poured into a column provided with a sintered glass filter. The solution was filtered at a rate of about 0.3 ml per minute. To the filtrate was added dilute hydrochloric acid until precipitation was completed. Once again the dissolving and reprecipitating operations with sodium hydroxide and hydrochloric acid were performed a few times more and finally the precipitate was dissolved in warm

sodium carbonate, reprecipitated with acetic acid, collected, washed and dried.

Instrumentation

The following instruments were used to characterize the prepared fluoresceins:

Varian Model A 56/60D Spectrometer operated at 60 MHz was used to obtain the proton NMR spectra.

A Varian CFT-20 Fourier Transform NMR Spectrometer, equipped with computer controlled pulse generation and data collection, operated at a field strength of 1.8682T and at a frequency of 20 MHz was used to obtain the carbon-13 NMR spectra. Samples dissolved in dilute sodium hydroxide solution, placed in 10 mm NMR tubes with D_2O for locking the system, were used.

Varian MAT CH 5/DF, a double-focusing, high resolution ($M/\Delta M > 10,000$), Mass Spectrometer, provided with a peak matching unit which permits mass determination with an accuracy of better than 2 ppm by comparing the peak of the sample to the reference peak of known mass, was used to obtain the mass spectra and molecular formulas of the samples. All determinations were taken with the electron impact source operated at an electron energy of 70 ev., ion acceleration voltage of 3 Kv and direct probe

at 225°C. Reference used in mass determination was perfluoroalkane (PFK).

Perkin Elmer Model 457 Grating Infrared Spectrophotometer was used to obtain the infrared absorption spectra.

Unicam Sp 800B UV-Vis Spectrophotometer was used to obtain the absorption spectra of some of the compounds. A pair of 1 cm quartz cells was used.

Thomas Ashley Oil Bath Immersion Apparatus was used to obtain melting point data.

Beckman Expanded Scale pH Meter equipped with a Sargent combination glass-saturated calomel electrode (#S-30070-10) was used for all pH measurements.

In the TLC separation, a sandwich type developing chamber (Eastman Kodak) was used. A dilute solution of known concentration of the fluorescein in methanol was prepared. A few microliters of the solution from a micropipette (Accupette Pipets, DADE) was applied on the TLC sheet (6060 Silica Gel, Eastman Kodak) at predetermined points, about 2 centimeters above the edge of the sheet. The spots were dried and developed with methanol.

A computer-centered spectrophotofluorometer, designed and constructed in this laboratory by Kelly[13] was used to measure the excitation and emission spectra as well as

the quantum efficiencies of the compounds synthesized. These measurements were made with a 200 watt mercury o a 150 watt xenon arc lamp as sources and a 4.0 nm bandpass for both the excitation and emission monochromators. One centimeter quartz cells were used as sample and reference cells. The spectra obtained were background corrected and for the emission spectra were source, photomultiplier tube and primary absorption corrected.[13,14]

To obtain excitation spectra, the solutions were scanned through an excitation range of 300-500 nm with emission fixed at 510 nm.

The emission spectra of the compounds were obtained over the wavelength range of 500-650 nm, with the excitation wavelength of 435.8 nm.

The quantum efficiency measurements were made by the comparative method[15] with quinine sulfate as the reference material.

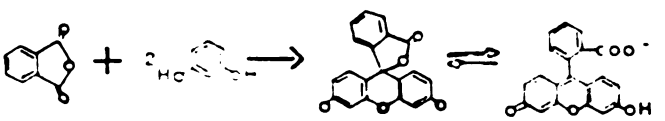
An emission scan was made with a 1.00×10^{-4} M quinine sulfate solution in 1.0 N H_2SO_4 using an excitation wavelength of 365.4 nm and emission wavelength range of 370 to 620 nm to obtain the emission curve. Without changing the instrument settings except the excitation wavelength and the emission wavelength range, the fluorescein solutions were scanned over the emission wavelength range of 500 to 600 nm with the excitation wavelength set at 435.8 nm. The areas under the emission curves are

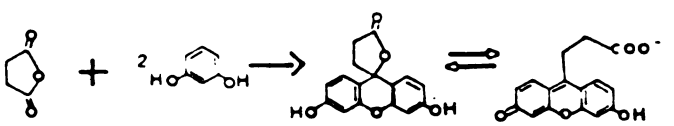
proportional to the number of quanta emitted. The ratio of the areas under the respective curves, corrected for the differences in the absorbances of the two solutions, the quinine sulfate and the fluorescein solutions, is directly proportional to the ratio of the quantum efficiencies of the two compounds. The accepted quantum efficiency of 0.546 for quinine sulfate was used to evaluate the quantum efficiencies of the fluoresceins.

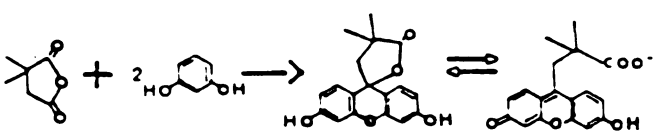
C. RESULTS AND DISCUSSION

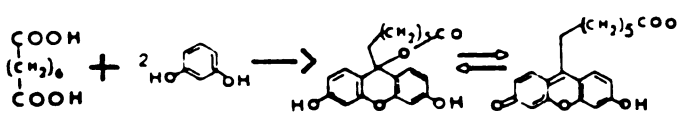
1. Preparation of the Fluoresceins

The synthesis of the fluoresceins involves the condensation of two moles of resorcinol with one mole of the acid or anhydride with the subsequent removal of 3 moles of water in the case of the acid or 2 moles of water in the case of the anhydride.[1,7] This reaction may be represented as follows:









The role of the condensing agent and/or dehydrating agent in such reactions is crucial.

Review of existing methods in the synthesis of the fluoresceins show that the most popular condensing catalysts are anhydrous zinc chloride for a heterogeneous reaction and concentrated sulfuric acid for a homogeneous one. As one would expect, the use of concentrated sulfuric acid, a strong oxidizing and dehydrating agent, would result in side reactions. Biggs and Pope[4] had confirmed that the method used by Nencki,[3] in which concentrated sulfuric acid was the condensing catalyst, resulted in the formation of by-products which accounted for a yield of only 60-70% of the theoretical. They then suggested the use of 73% sulfuric acid as the condensing catalyst.

The use of Biggs' method, gave a good yield. The yield was quite dependent on the amount of 73% sulfuric acid used, the temperature at which the condensation reaction was made to occur, the length of time of heating and the amount of excess resorcinol present. This method resulted in a yield of 44-130% (unisolated) of the theoretical as shown in Table 1. Purification through several recrystallizations lowered the yield to less than 10%. A yield of 100% or more obtained when the condensation reaction occurred at high temperatures, indicates that other species absorbing at 484-486 nm were also produced in addition to the fluoresceins of interest.

Table 1. Influence of Temperature, Heating Time and Amount of Condensing Agent on the Yield of Fluoresceins.

Acid/Anhyd. Sample	73% H_2SO_4 ml	Reaction Temp., °C	Heating Time Hours	% Yield*
Succinic Anhyd.	10	100-105	22	44
Succinic Anhyd.	10	100-105	48	79
Succinic Anhyd.	10	120-125	6	65
Succinic Anhyd.	10	140-150	6	104
Succinic Anhyd.	10	140-150	3	104
Succinic Anhyd.	15	120-125	6	61
Succinic Anhyd.	15	140-150	6	99
Succinic Anhyd.	15	140-150	3	89
2,2 Dimethyl-Succinic Acid	10	100-105	48	70
" "	10	120-125	6	60
" "	10	145-150	6	125
" "	15	100-105	22	63
" "	15	120-125	6	58
" "	15	145-150	6	120

*% Yield was based on concentrations in the reaction mixture determined by spectrophotometry. 0.01 mole of acid/anhydride was used in the reaction mixture.

Modifying the condensing agent through use of a more dilute sulfuric acid lowers the yield as shown in Table 2 without totally eliminating the problem of side reactions and tar formation.

The use of sulfuric acid as condensing agent is also not commendable since the impurities introduced into the product are difficult to isolate from the fluoresceins. Repeated recrystallizations do not seem to eliminate the existence of these impurities. A TLC separation performed on the purified product by using a 6060 silica gel sheet and methanol as developer, yielded 5 spots in the chromatogram which only lessened in intensity upon repeated recrystallizations but none was totally eliminated. The different spots in the chromatogram were eluted with dilute sodium hydroxide. The extracts showed the following emission characteristics:

Spot 1 - a light pink spot moved as fast as the solvent front, emitted no fluorescence when it was irradiated with a 365.4 nm light from a 200 watt mercury arc source.

Spot 2 - an orange pink spot about 0.5 cm below the solvent front, emitted strong fluorescence at 420 nm when it was irradiated with the same exciting light source.

Spot 3 - a pinkish violet spot about 1.2 cm below the solvent front, emitted slight fluorescence at 420 nm when it was irradiated with the same exciting light source.

Table 2. Influence of Condensing Agent on the Yield of Fluoresceins.

Acid/ Anhydride	Condensing Catalyst	Reaction Temperature °C	Heating Time Hours	% Yield
Succinic Anhyd	73% H_2SO_4	100-105	48	79
" "	50% H_2SO_4	100-105	48	76
" "	30% H_2SO_4	100-105	48	19
" "	30% H_2SO_4 + p-TSA	100-105	48	66
" "	50% H_2SO_4 + p-TSA	100-105	48	11
2,2 Dimethyl-succinic Acid	73% H_2SO_4	100-105	48	70
" "	50% H_2SO_4	100-105	48	16
" "	30% H_2SO_4	100-105	48	0.3
" "	30% H_2SO_4 + p-TSA	100-105	48	69
" "	50% H_2SO_4 + p-TSA	100-105	48	14

p-TSA is p-toluene sulfonic acid.

*% Yield was based on concentrations in the reaction mixtures determined by spectrophotometry.

Spot 4 - a dark orange-yellow spot about 2.5 cm below the solvent front.

Spot 5 - a light yellow diffused spot immiediately below spot 4. Both spots 4 and 5 emitted strong fluorescence at 510 nm and slight fluorescence at 420 nm.

Elemental carbon analysis of a purified succinyl fluorescein sample obtained from the sulfuric acid method gave C = 67.52% against a theoretical value of C = 67.60%.

The use of polyphosphoric acid (PPA) as condensing catalyst was explored. This reagent had been used for cyclodehydration[9] and sometimes had been found to be more effective cyclodehydration reagent than sulfuric acid, hydrofluoric acid or sodium aluminum chloride. It has good solvent power and contains anhydride groups which combine with the water formed, preserving its effective acidity. Unlike sulfuric acid, PPA is not an oxidizing agent and has no tendency to enter into aromatic substitutions and therefore less prone to promote rearrangement.

With PPA as the condensing catalyst, a product was obtained identical in all respects to that obtained from the use of sulfuric acid. It had the same melting point, showed identical mass spectra, proton-NMR, carbon-13 NMR, and absorption and emission spectra. Elemental carbon analysis of the succinyl fluorescein obtained when this catalyst was used, gave C = 67.66% against a theoretical value of C = 67.60%.

TLC chromatograms obtained from a recrystallized fluorescein of succinic acid gave the same spots as with the fluorescein of succinic acid from the sulfuric acid method. Except for spot 4 all the other spots were very light with spots 2 and 3 almost invisible even with a spotting solution more concentrated than the solution from the sulfuric acid product.

Yields from the use of sulfuric acid and PPA as condensing agents were comparable except that chromatograms obtained from the sample obtained from the use of PPA showed less impurities.

P-Toluene-sulfonic acid (Tosic acid) is known to be as effective an acid catalyst as sulfuric acid. It is generally preferred to sulfuric acid because it is less damaging to reactants.[9] Its use as the condensing catalyst in the foregoing reaction was also explored. Because it was a solid, an inert solvent was needed. Two solvents were tried, cyclohexane and 1,2-dichloroethane. Inasmuch as both solvents boil at about 81°C, the problem of the formation of products from side reactions due to the high temperature at which condensation reaction was made to take place was eliminated or minimized.

The use of p-toluenesulfonic acid (p-TSA) as condensing agent and cyclohexane as solvent produced a product identical to that of the previous methods. Isolation

of the product from the reaction mass was accomplished by dissolving the mass in 5% $NaHCO_3$, and precipitating with dilute sulfuric acid. The precipitate obtained was partly purified by fractional precipitation at a definite pH.

The product obtained even before recrystallization was found to be much cleaner. Its TLC chromatogram showed only 2 spots, a reddish-violet spot which moved as fast as the solvent front and a yellow spot about 2.5 cm below the solvent front. The impurity responsible for the reddish-violet spot was minimized or eliminated by dissolving the product and reprecipitating it at pH 6. This impurity was precipitated at pH > 6 while the yellow spot of the chromatogram was precipitated at pH < 6. An emission scan of the extract of the yellow spot gave an emission peak at about 510 nm. The product appeared to be the same compound as obtained from the other methods. Its melting point, proton-NMR, carbon-13 NMR, mass spectra, absorption and emission spectra were identical to the corresponding compound obtained from the previous methods.

The p-toluenesulfonic acid in cyclohexane also catalyzed the condensation of resorcinol with the substituted succinic acids, suberic acid, adipic acid and pimelic acid. The yields shown in Table 3, with the exception of 2,2 dimethylsuccinic acid, which was about 40-50%, were only about 20-30%.

Table 3. Fluorescein Yield When p-Toluenesulfonic Acid is Used as the Condensing Catalyst.

Acid/Anhydride	Solvent	% Yield*
Succinic Acid	Cyclohexane	30
2,2 Dimethylsuccinic Acid	Cyclohexane	45
Suberic Acid	Cyclohexane	30
Adipic Acid	Cyclohexane	30
Pimelic Acid	Cyclohexane	20
2,2 Dimethylsuccinic Acid	1,2 Dichloroethane	5
Succinic Acid	1,2 Dichloroethane	5
Methylsuccinic Acid	1,2 Dichloroethane	5
Phthalic Anhydride	1,2 Dichloroethane	44

*% Yield is based on the weight of recovered partly purified fluorescein and therefore is only approximate.

The 1,2 dichloroethane was a good solvent for the condensation of resorcinol and phthalic anhydride but not for the other dicarboxylic acids tried as shown in Table 3. A yield of about 45-50% of the red form fluorescein of phthalic anhydride was obtained. Its IR spectrum, shown in Figure 1, was similar to the IR spectrum of fluorescein published by Sadtler.

Dowex 50W x 12, an acid form ion exchange resin was also tried as a condensing catalyst with 1,2 dichloroethane as solvent. Results shown in Table 4 indicate that it was as effective in the reaction as p-toluenesulfonic acid. The yellow form fluorescein of phthalic anhydride was obtained at a yield of about 50% but fluoresceins of the other dicarboxylic acids were only about 5% or less.

2. Mass and Molecular Formula Determination

The mass spectra of the compounds obtained with the double focusing mass spectrometer are shown in Figures 2, 3 and 4. Data obtained from the mass spectra are shown in Table 5.

With a tolerance of 0.0030 AMU, masses obtained based on the given molecular formula are 284.06848, 312.09979 and 340.13107 for succinyl fluorescein, 2,2 dimethylsuccinyl fluorescein and suberyl fluorescein, respectively. These values differed from the mass determined by peak matching as given in Table 5 by -2.96 ppm, 4.49 ppm and -1.21 ppm, respectively.

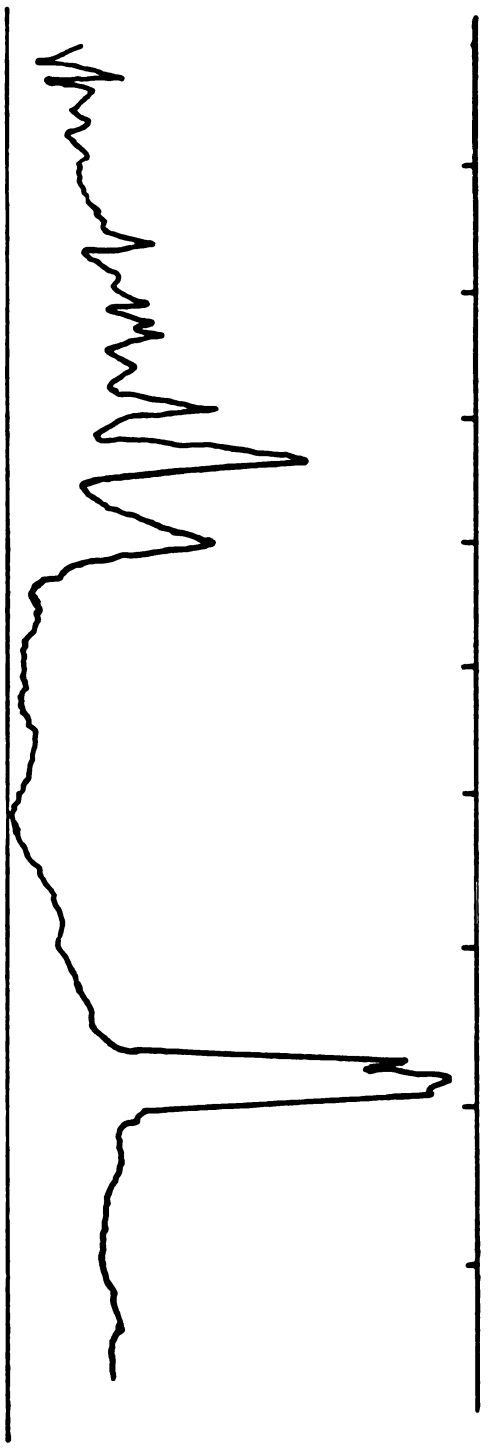


Figure 1. Infrared spectrum of the fluorescein of phthalic anhydride (red form) in Nujol.

Table 4. Fluorescein Yield When Dowex 50W x 12 is Used as the Condensing Catalyst.

Acid/Anhydride	Condensing Temperature °C	Heating Time Hours	% Yield*
Succinic Acid	145-150	20	7
Methylsuccinic Acid	145-150	12	4
2,2 Dimethylsuccinic Acid	145-150	15	7
Phthalic Anhydride	145-150	14	42
Phthalic Anhydride	150-160	5	51
Phthalic Anhydride	108	6	16

*% Yield is based on the weight of recovered partly purified fluorescein and therefore is only approximate. Condensing temperature was the temperature of the oil bath used during the reflux.

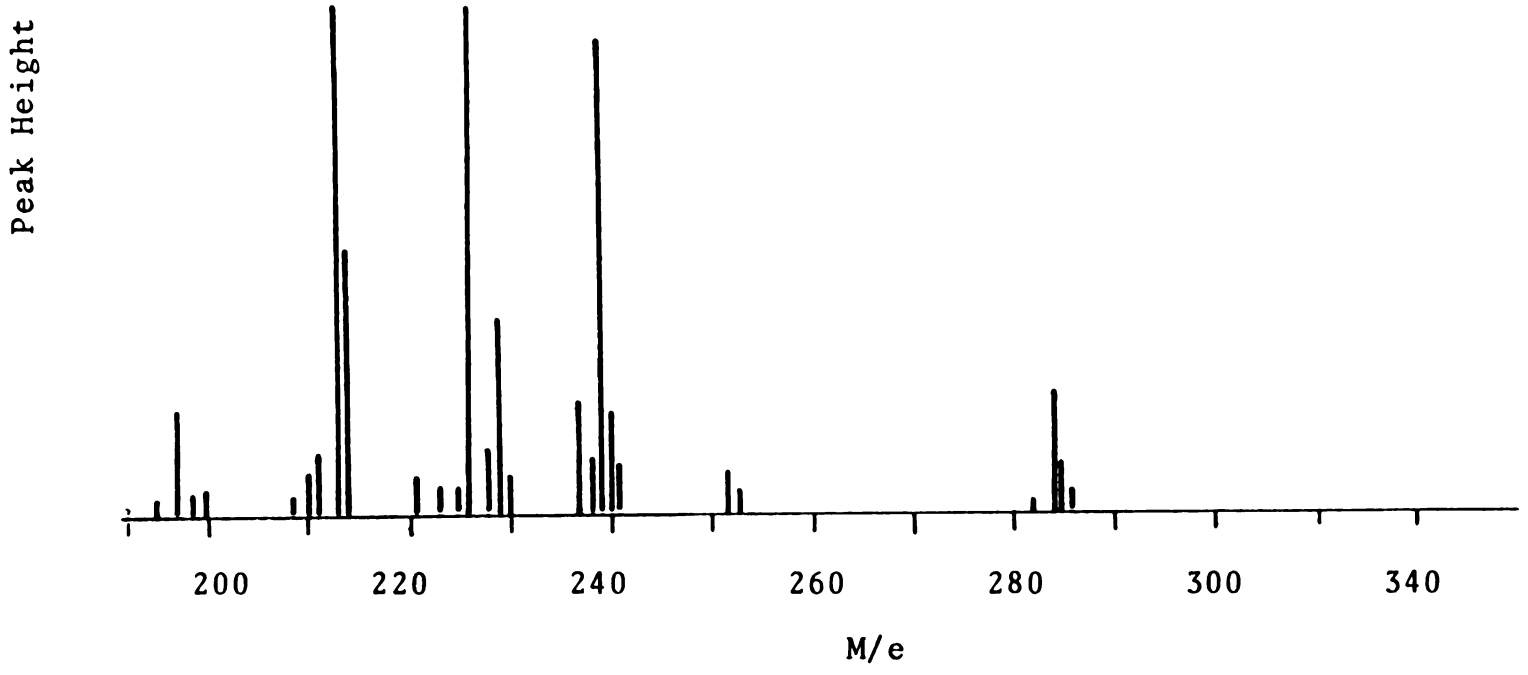


Figure 2. Mass spectrum of the fluorescein of succinic acid.

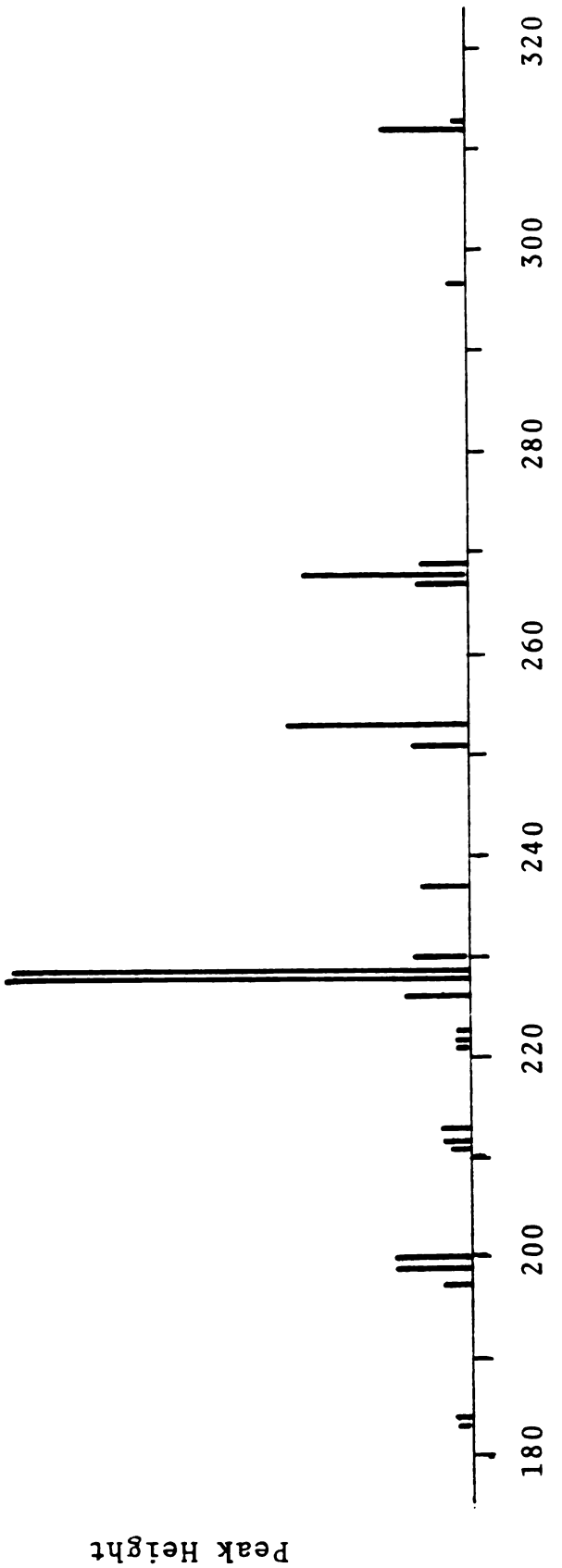


Figure 3. Mass spectrum of the fluorescein of 2,2 dimethylsuccinic acid.

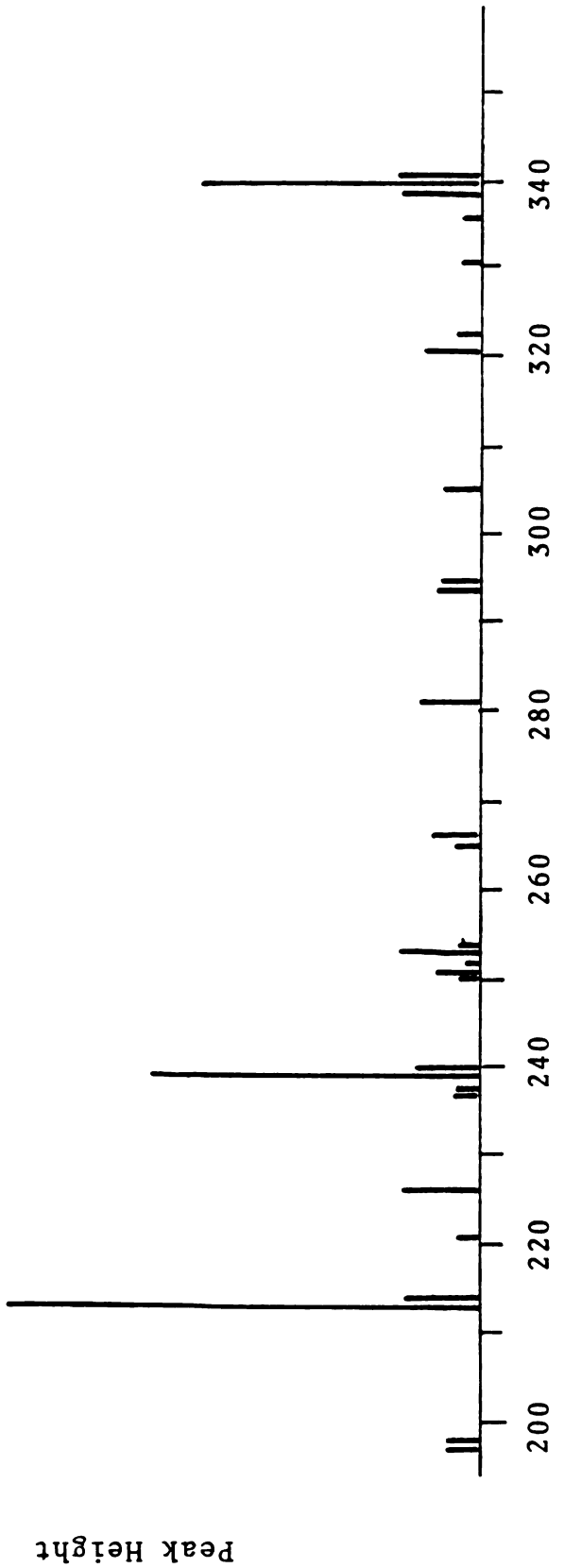


Figure 4. Mass spectrum of the fluorescein of suberic acid.

Table 5. Mass Spectral Data for the Fluoresceins.

Sample	m/e	Mass	Mol. Formula
Succinyl fluorescein	284	284.06763	$C_{16}H_{12}O_5$
2,2 Dimethylsuccinyl fluorescein	312	312.10117	$C_{18}H_{16}O_5$
Suberyl fluorescein	340	340.13965	$C_{20}H_{20}O_5$

3. Proton NMR Spectra

To shed light on the structure of the synthesized fluoresceins, the proton NMR of the compounds were taken. Due to the low solubility of the fluoresceins in the usual deuterated organic solvents the proton NMR spectra were recorded with the compounds dissolved in dilute sodium deuteroxide.

a. Succinyl Fluorescein

If the compound has the structure shown on page 34, it should have 2 sets of non-equivalent aliphatic protons and six aromatic protons which due to the symmetry of the molecule, as a result of the keto-enol tautomerism, should correspond to 3 sets of non-equivalent aromatic protons. The proton NMR of the compound was then expected to show 5 absorption peaks corresponding to the 5 sets of non-equivalent protons - two triplets for the aliphatic methylene protons, two doublets for protons H_c and H_b and a singlet for proton H_a of the aromatic moiety. The -OH proton due to strong intramolecular hydrogen bonding and stabilization through keto-enol conversion was expected to show far downfield and probably would not appear in the spectrum.

As shown in Figure 5, all five absorption peaks are present. The two up-field distorted peaks which should have appeared as triplets, were assigned to the methylene

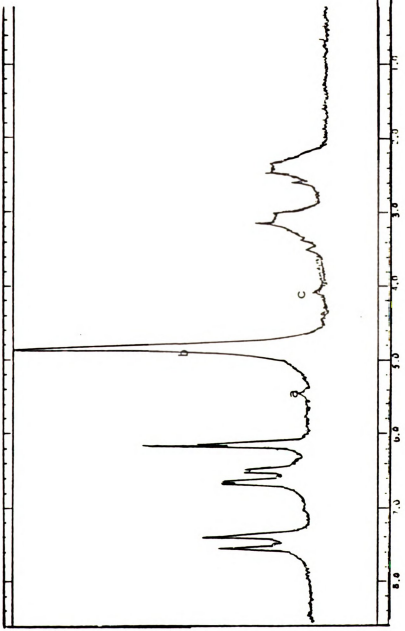


Figure 5. Proton NMR spectrum of the fluorescein of succinic acid (a, b and c are solvent peaks).

protons of the aliphatic side chain based on their position in the spectrum. The down-field absorption peaks, two doublets at δ7.5 and δ6.5 with the same coupling constants, were assigned to protons $H_{c,c'}$ and $H_{b,b'}$, respectively. The doublet at δ6.5 assigned to H_b proton shows also slight coupling with another proton as indicated by the slight splitting of the doublet peaks. The peak at δ6.2 was assigned to the H_a proton. Although a singlet was expected, a slight splitting of the singlet peak was not a complete surprise inasmuch as the aromatic inductive effect can cause slight coupling of this proton with H_c protons. This is shown in the spectrum by the identical splitting of the H_b doublet and the H_a singlet peaks. Integration of the peaks resulted in a 2:2:2:2:2 ratio which accounted for the 4 aliphatic and 6 aromatic protons.

Assignment of peaks, although not unequivocal, are shown in Table 6. The peak marked (b) is due to the residual protons of the solvent, while (a) and (c) are spinning side bands.

b. 2,2 Dimethylsuccinyl Fluorescein

Based on the assumed structure of the compound, (page 37) its proton NMR would show 5 absorption peaks which correspond to the methyl and methylene protons of

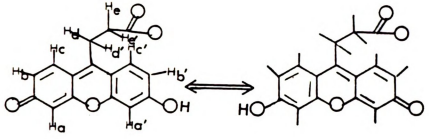


Table 6. Proton NMR Chemical Shifts for the Fluorescein of Succinic Acid.

	Chemical Shifts, (δ, ppm)	Ratio of Integrated Peaks
H_c and $H_{c'}$	7.5	2
H_b and $H_{b'}$	6.5	2
H_a and $H_{a'}$	6.2	2
H_d and $H_{d'}$	3.2	2
H_e and $H_{e'}$	2.4	2

the aliphatic side chain and the three sets of non-equivalent aromatic protons. Figure 6 showed all 5 absorption peaks. From the position in the spectrum, the absorption peaks at δ1.0 was assigned to the methyl protons and δ3.0 to the methylene protons although the methylene proton peak seem to have been shifted somewhat down-field which may be due to solvent effects. Based on the coupling constants and the splitting of the absorption peaks, the doublet at δ7.6 was assigned to $H_{c,c'}$ protons, doublet at δ6.6 to the $H_{b,b'}$ protons, and δ6.2 to the $H_{a,a'}$ protons. Integration of the peaks resulted in a ratio of 2:2:2:2:6 which accounted for the 14 protons in the compound.

Assignments although not unequivocal, are shown in Table 7.

c. Suberyl Fluorescein

The aromatic absorptions were identical to the absorption peaks of the other two fluoresceins except that the long aliphatic side chain seemed to have imposed some geometrical influence on the aromatic moiety. This caused a change in the chemical shift and resulted in a smaller $\Delta\nu/J$ for protons H_a and H_b. The two end methylene proton sets did not show the regular triplet splitting as

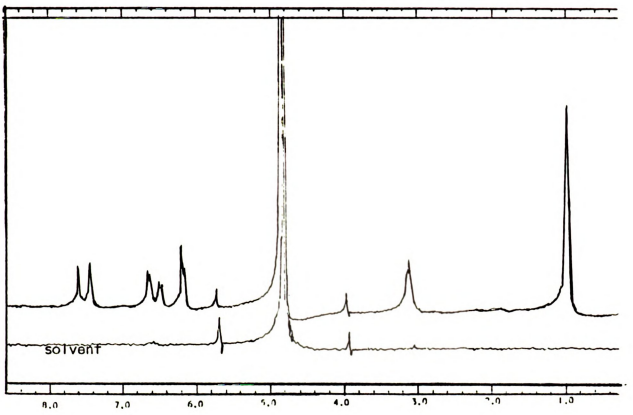


Figure 6. Proton NMR spectrum of the fluorescein of 2,2 dimethylsuccinic acid.

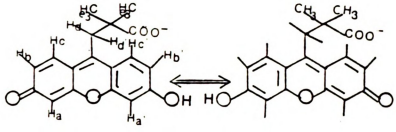


Table 7. Proton NMR Chemical Shifts for the Fluorescein of 2,2 dimethylsuccinic acid.

	Chemical Shifts, (δ, ppm)	Ratio of Integrated Peaks
H_c and $H_{c'}$,	7.6	2
H_b and $H_{b'}$,	6.6	2
H_a and $H_{a'}$,	6.2	2
H_d and $H_{d'}$,	3.1	2
H_e and $H_{e'}$,	1.0	6

expected and the inner methylenes which would probably have very small $\Delta\nu/J$ value appeared as one broad absorption peak. Distortions of the aliphatic proton absorption peaks may be due to the more complex coupling resulting from the almost equivalent protons.

Assignment of the peaks, although not unequivocal are shown in Table 8. Peaks marked a, b and c are due to the residual protons of the solvent.

4. Carbon-13 NMR

In the assumed structures of the three fluoresceins, it is expected that the general pattern of carbon resonance peaks due to the carbons of the aromatic moiety in these compounds would be the same. Due to the symmetry of the molecule imposed by the keto-enol conversion, 7 carbons resonance peaks are expected in the down-field region of the spectra. For the side chain, two resonance peaks are expected for the hydrocarbon carbons of the succinyl fluorescein, three for the 2,2 dimethylsuccinyl fluorescein and six for the suberyl fluorescein and one down-field resonance peak for the carboxyl carbon for each of the fluoresceins.

In the case of succinyl fluorescein all 10 carbon resonance peaks are shown in Figure 8, two up-field due to the aliphatic hydrocarbon carbon peaks and 8 down-field due to the 7 aromatic carbons and 1 carboxyl carbon.

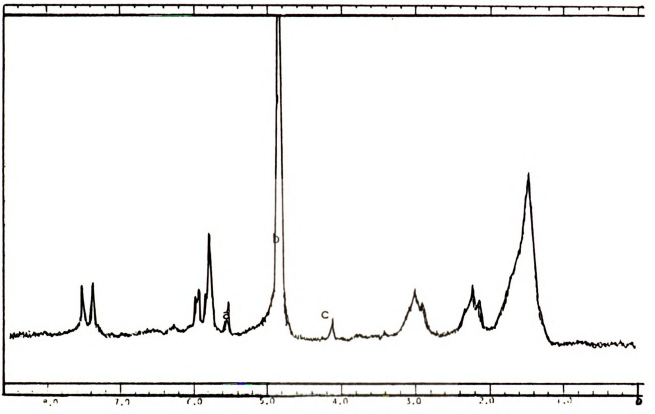


Figure 7. Proton NMR spectrum of the fluorescein of suberic acid (a, b and c are solvent peaks).

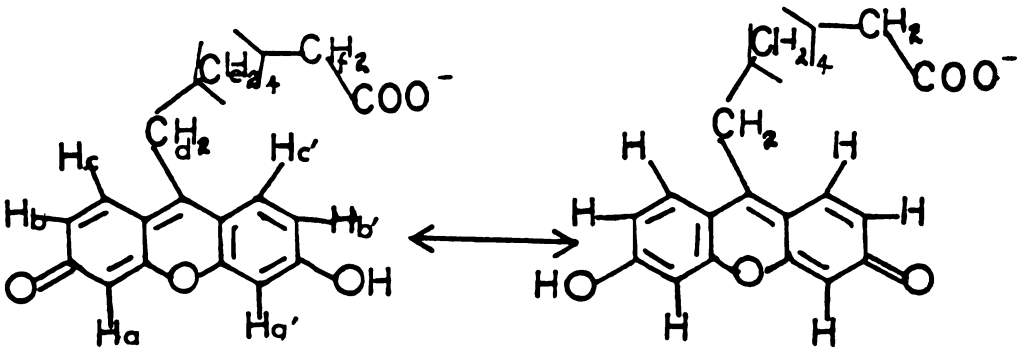


Table 8. Proton NMR Chemical Shifts for the Fluorescein of Suberic Acid.

	Chemical Shifts, ppm	Ratio of Integrated Peaks
H_c and $H_{c'}$	7.4	2
H_b and $H_{b'}$	6.0	2
H_a and $H_{a'}$	5.8	2
H_d	3.0	2
H_e	2.2	2
H_f	1.5	8

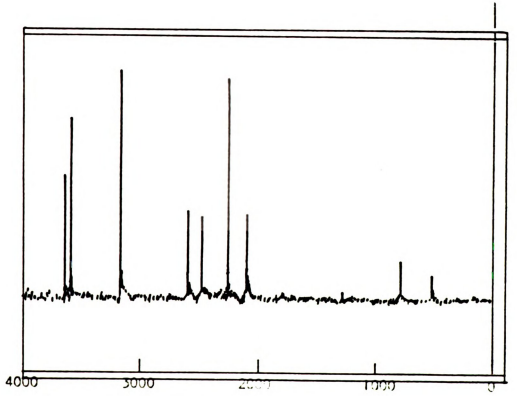


Figure 8. Carbon-13 NMR spectrum of the fluorescein of succinic acid.

All carbon resonance peaks of the 2,2 dimethylsuccinyl fluorescein are also accounted for, as shown in Figure 9. In the spectrum of suberyl fluorescein, Figure 10, the aromatic and carboxyl peaks assigned to the 8 down-field peaks are accounted for but only 3 out of the 6 expected aliphatic hydrocarbon carbon peaks appeared. The 4 inner carbons of the $-CH_2-$ group are all almost equivalent, and therefore highly probable that these 4 carbons do not give rise to the expected four peaks but instead a single peak.

Chemical shifts of the different resonance carbon peaks are given in Table 9.

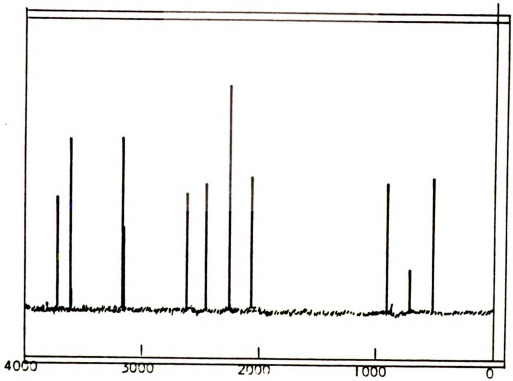


Figure 9. Carbon-13 NMR spectrum of the fluorescein of 2,2 dimethyl succinic acid.

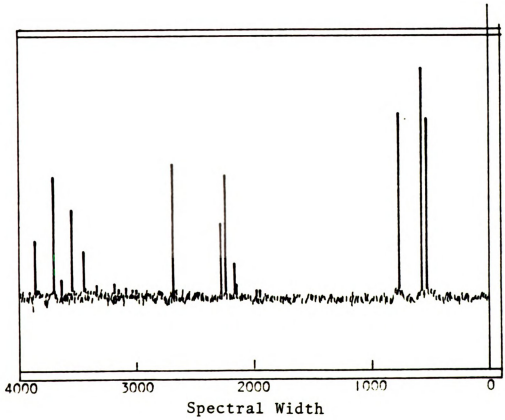


Figure 10. Carbon-13 NMR spectrum of the fluorescein of suberic acid.

Table 9. Carbon-13 Chemical Shifts.

	Chemical Shifts, ppm		
Succinyl fluorescein	181.69	179.33	158.13
	157.43	129.73	123.75
	112.96	104.81	39.69
	25.89		
2,2 dimethylsuccinyl fluorescein	186.66	181.56	158.93
	158.33	131.76	123.79
	114.50	104.73	47.16
	37.20	27.65	
Suberyl fluorescein	193.41	185.64	177.77
	172.58	134.76	114.73
	112.83	108.77	39.16
	30.11	27.48	27.30

II

ABSORPTION AND FLUORESCENCE STUDY

The absorption spectra of the fluoresceins presented in Figure 11 showed major absorption bands with maxima about 484-486 nm and minor absorption bands at shorter wavelengths.

The intensity of absorption at these concentrations, about 10^{-5} M, show that these compounds have large molar absorptivities. Indeed, calculations at these concentrations and pH, gave ϵ_{max} of 6.70×10^4 1. mole^{-1} cm^{-1}, 8.10×10^4 1. mole^{-1} cm^{-1} and 3.56×10^3 1. mole^{-1} cm^{-1} for the fluoresceins of succinic acid, 2,2 dimethylsuccinic acid and suberic acid, respectively. Although the compounds have both non-bonding electrons on the oxygen and π electrons of the aromatic ring system, it is probable that absorption is due to π-π^* rather than n-π^* transitions and so it follows that fluorescence emission is from π^*-π transitions which are strong transitions unless partially forbidden by symmetry factors.

Examination of the excitation and emission spectra presented in Figures 12 and 13, show an almost mirror-image relationship which is usually the case except for very complex molecules or when other competing relaxation processes are taking place. The distribution of vibrational levels in a particular electronic state determines the shape of the absorption or emission band. In general the 0-0 vibrational transitions are the most probable.

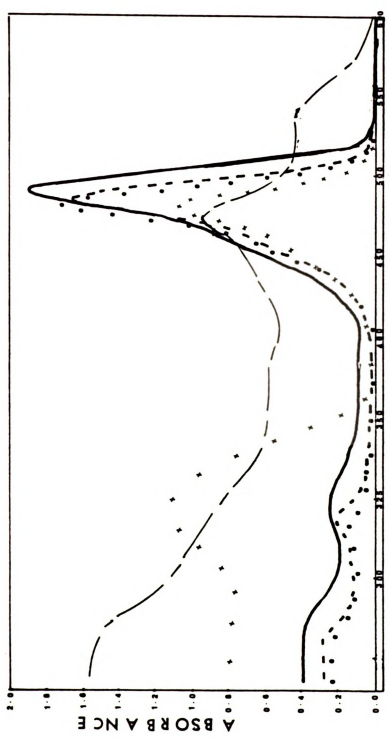


Figure 11. Absorption spectra of 3.0×10^{-5} M fluorescein of phthalic anhydride [——], 2.1×10^{-5} M fluorescein of 2,2 dimethylsuccinic acid [---], 2.8×10^{-5} M fluorescein of succinic acid [eee], 3.7×10^{-4} M fluorescein of suberic acid [xxx], and 3.0 ug/ml resorcinol reaction product [-·-·-].

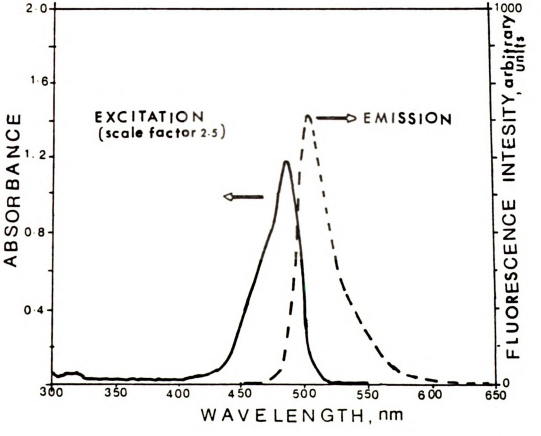


Figure 12. Excitation and emission spectra of 3.6×10^{-6} M succinyl fluorescein.

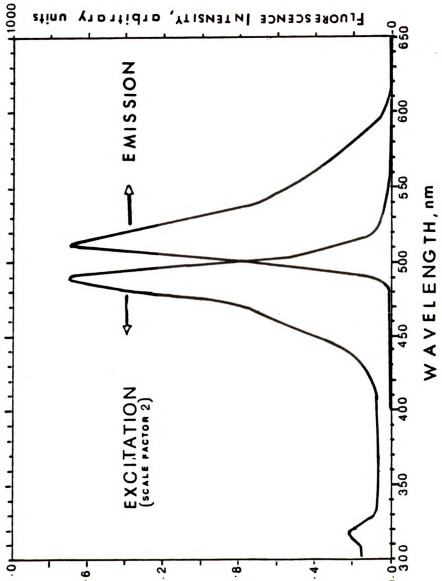


Figure 13. Excitation and emission spectra of 9.0×10^{-6} M 2,2 dimethylsuccinyl fluorescein.

Figures 12 and 13 show that the 0-0 band separation of the fluorescein of succinic acid is about 17 nm while that of 2,2 dimethylsuccinic acid is about 25 nm. Since this energy difference is dependent on the degrees of solvation in the two states, it is therefore, a function of the polarity or polarizability of the molecules of solute and solvent. Deductions can be drawn, that 2,2 dimethylsuccinyl fluorescein is more polar in the excited state than succinyl fluorescein.

The quantum efficiency of fluorescence, which is defined as the ratio of quanta emitted to the quanta absorbed, was determined for the fluoresceins of succinic acid and 2,2 dimethylsuccinic acid in aqueous sodium hydroxide solution at about pH 12 using the comparative technique of Melhuish.[15] The quantum efficiency, QE, values obtained were 0.69 and 0.86 for the above fluoresceins, respectively, relative to the quinine sulfate accepted QE value of 0.546.

In a comparison of the fluorescence yield of fluoresceing (fluorescein of phthalic anhydride) and 6-hydroxy-9-phenyl-fluoron (HPF), Lindqvist[16] concluded that the high fluorescence yield of fluorescein was due to the charge of the carboxyl group in alkaline solution and the ordered structure of water molecules surrounding this charge group which reduces the possibilities of molecular distortion which favors internal conversion. This was

noted with 2,2 dimethylsuccinyl fluorescein which showed larger energy separation of the O-O absorption and emission bands and therefore higher polarity in the excited state than succinyl fluorescein, thus a greater quantum efficiency.

1. pH Influence on Absorption and Fluorescence

The excitation and emission spectra of the fluoresceins of succinic acid and 2,2 dimethylsuccinic acid were studied in aqueous solution at different pH. Samples of the fluoresceins were dissolved in 0.1 M NaOH. Solutions of different pH were prepared by addition of polyphosphoric acid. Solutions with pH greater than 13 were prepared by dissolving the samples in 1 M and 2 M NaOH solution and for low pH in desired concentrations of polyphosphoric acid. Inasmuch as the fluorescence decay was very fast in the very strongly alkaline solutions and samples precipitated at very low pH, absorption and emission studies were carried out within the pH range of 2 to 13.

a. Absorption

An excitation spectrum of the fluorescein of succinic acid is presented in Figure 14. At about pH 13 λ_{max} is at 484 nm. Lowering the pH to 8 did not appreciably change the position of the maximum but caused a slight broadening

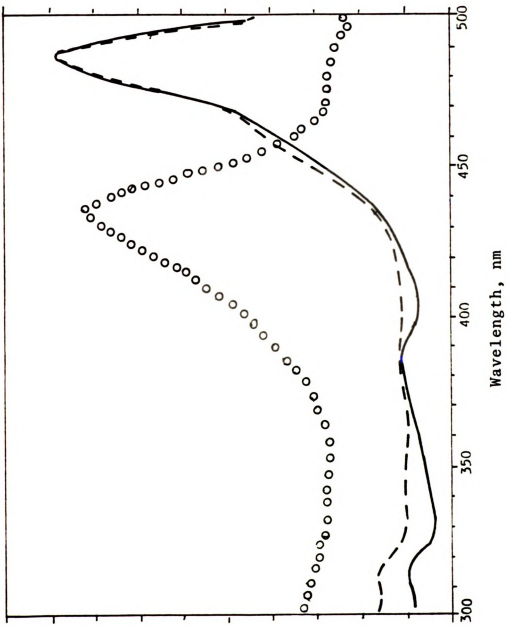


Figure 14. Excitation spectra of succinyl fluorescein at different pH. pH 2.6 [ooo], pH 8.15 [---] and pH 12.9 [——].

of the spectrum on the shorter wavelength side. At a pH of 2.6, this maximum was shifted to about 435 nm, but no appreciable shift towards 435 nm was observed until a pH of 4.

With the fluorescein of 2,2 dimethylsuccinic acid, shown in Figure 15, the same pattern of change was observed. The λ_{max} at 486 nm at pH 13 did not change when the pH was lowered to 8, although a broadening of the band on the short wavelength side occurred. At a pH of 2 the maximum was shifted to 440 nm but no appreciable shift in the maximum was observed until about a pH of 4.0.

b. Fluorescence

At about pH 13 to 8, the fluorescence spectra of succinyl fluorescein, Figure 16, are identical. At pH 2 its fluorescence spectrum has the same λ_{max}, at about 508 nm, but broadened towards longer wavelengths. Shoulders at about 540 nm and 555 nm had developed and emission extended beyond 600 nm.

For 2,2 dimethylsuccinyl fluorescein, the fluorescence spectrum, Figure 17, has λ_{max} at 510 nm at all pH levels studied, except that at pH 2 a broadening towards longer wavelength and development of shoulders at 540 nm and 555 nm were observed. Also the emission had extended beyond 600 nm.

In Zanker and Peter's study[17,18] for fluorescein

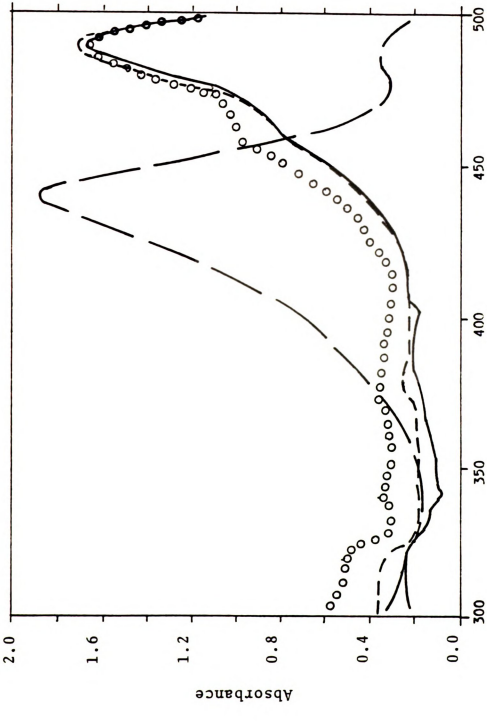


Figure 15. Excitation spectra of 2,2 dimethylsuccinyl fluorescein at different pH. pH 2.15 [— —], pH 6.95 [ooo], pH 8.4 [---] and pH 12.7 [——].

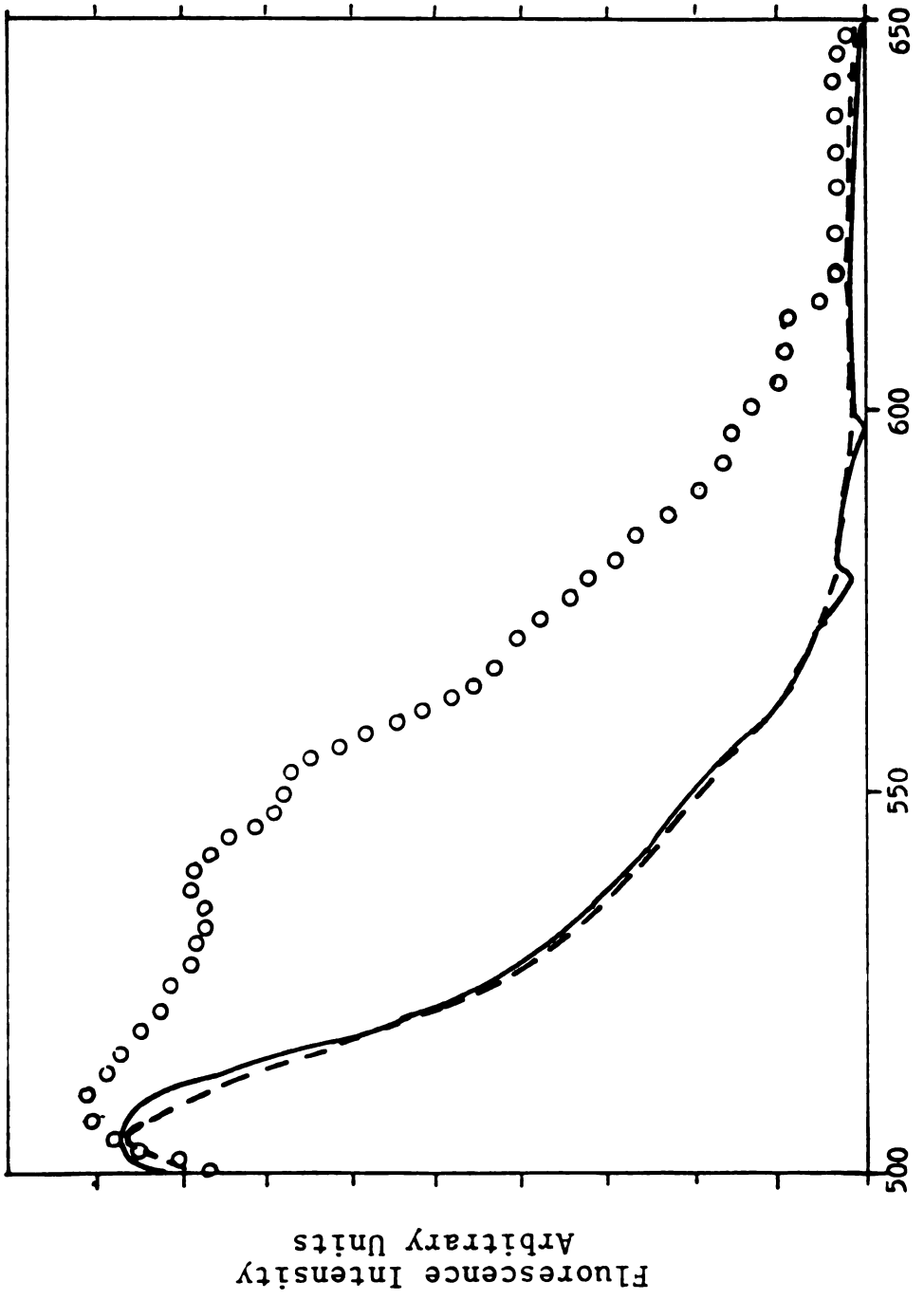


Figure 16. Emission spectra of succinyl fluorescein at different pH. λ_{ex} 435.8 nm, pH 2.2 [ooo]; λ_{ex} 468 nm, pH 8.15 [---] and pH 12.9 [——].

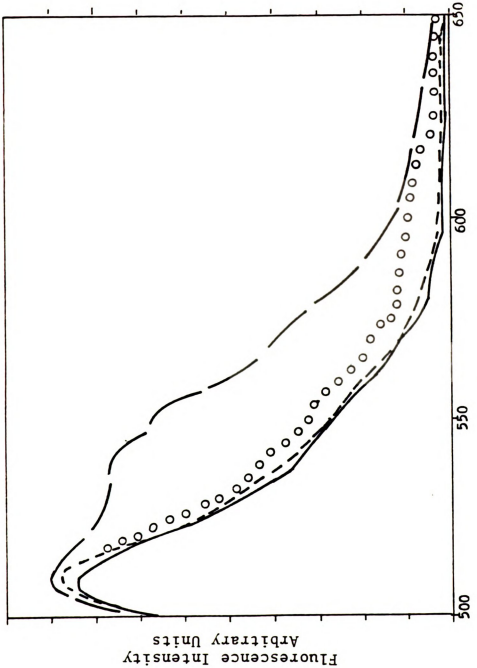


Figure 17. Emission spectra of 2,2 dimethylsuccinyl fluorescein at different pH. λ_{ex} 435.8 nm, pH 2.2 [– – –]; λ_{ex} 468 nm, pH 6.95 [ooo], pH 8.4 [---] and 12.7 [——].

(fluorescein of phthalic anhydride) in dioxane with varying amounts of acid and base present, they concluded that fluorescein existed as the cation, the neutral quinonoid molecule, the neutral lactonic molecule, the monoanion, the dianion, or the ampho-ion depending upon the pH. Martin and Lindqvist[19] studied the absorption spectrum of this compound in aqueous medium and compared it with the spectrum of 6-hydroxy-9-phenyl-fluoron (HPF) in which the carboxyl was replaced by hydrogen. They concluded that at pH 0, 3.3, 5,5 and 12, the cation, the neutral molecule, the monoanion and the dianion forms, respectively, predominate and that at about pH 3, the neutral molecules are found in the proportion of 5/8 in the lactonic form which does not absorb in the visible region, 1/4 as ampho-ion which had the same spectrum as the cation, and 1/8 in the quinonoid form which exhibits the same absorption as the monoanion. Comparison of the absorption spectrum obtained by Lindqvist[16] for fluorescein and the absorption spectra of the fluoresceins of succinic acid and 2,2 dimethylsuccinic acid obtained in this study, revealed a similarity among the spectra. Apparently the two fluoresceins exist in the same forms as the original fluorescein in the pH range of 2 to 13. Figures 18 and 19 show the probable species in which these two fluoresceins exist in aqueous solution at pH 2 to 13.

Fluorescence spectra of these compounds at about pH 2 to 13 using an excitation wavelength of 435.8 nm for pH

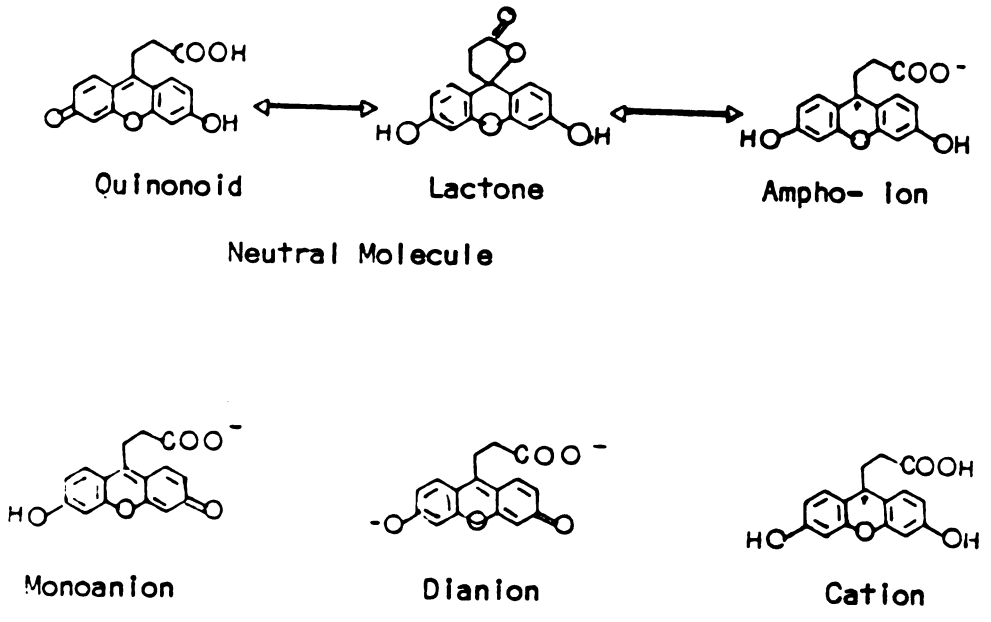


Figure 18. Different species of succinyl fluorescein in aqueous solutions.

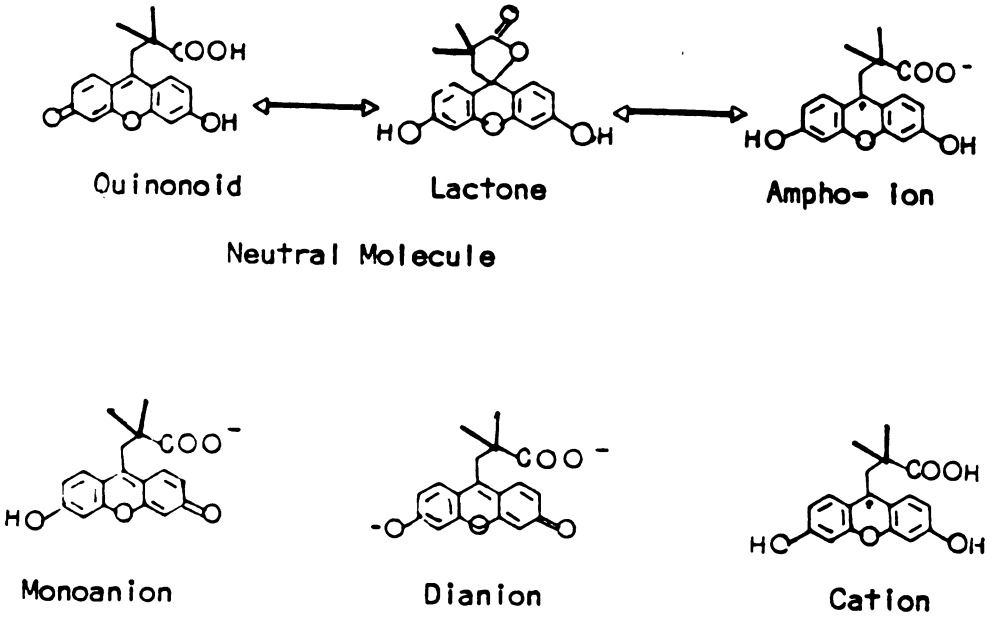


Figure 19. Different species of 2,2 dimethylsuccinyl fluorescein in aqueous solutions.

2 and 468 for pH > 5, presented in Figures 16 and 17 showed only one emission maximum, 508 nm in the case of succinyl fluorescein and 510 nm in the case of 2,2 dimethylsuccinyl fluorescein. Considering that the lactone form does not absorb in the visible, that the cation predominantly exists in very strongly acid medium and the dianion in a strongly basic medium, the emission is then principally due to the quinonoid and the monoanion forms which have basically the same fluorophore inasmuch as the carboxyl is not conjugated to the light-absorbing xanthene moiety.

It would seem probable that for the fluoresceins of succinic and 2,2 dimethylsuccinic acids, the fluorescence yield of the different species would be almost the same as those of the fluorescein. From Lindqvist values[16] of the fluorescence yield, 0.9-1 for the cation, 0.20-0.25 for the neutral molecule, 0.93 for the dianion and an estimated 0.25-0.35 for the monoanion at pH 5.5, it follows that at low pH, fluorescence intensity will increase with decrease in pH due to a larger contribution from the cation. Likewise, at very high pH, the same trend will be observed with increase in pH due to increased contribution of the dianion. At pH 5.5 to 12.0 fluorescence intensity would be almost constant because the predominating species would be the neutral molecule and the monoanion.

An experiment done on the two fluoresceins using a filter fluorometer at constant concentration but varying

pH showed that fluorescence intensity is almost constant at pH 6.0 to 12.0 but at pH lower than 5.0 it increased with decrease in pH as shown in Figure 20. The observed decrease in fluorescence intensity at high pH which should not have happened, may be due to some quenching process or decomposition of the dianion.

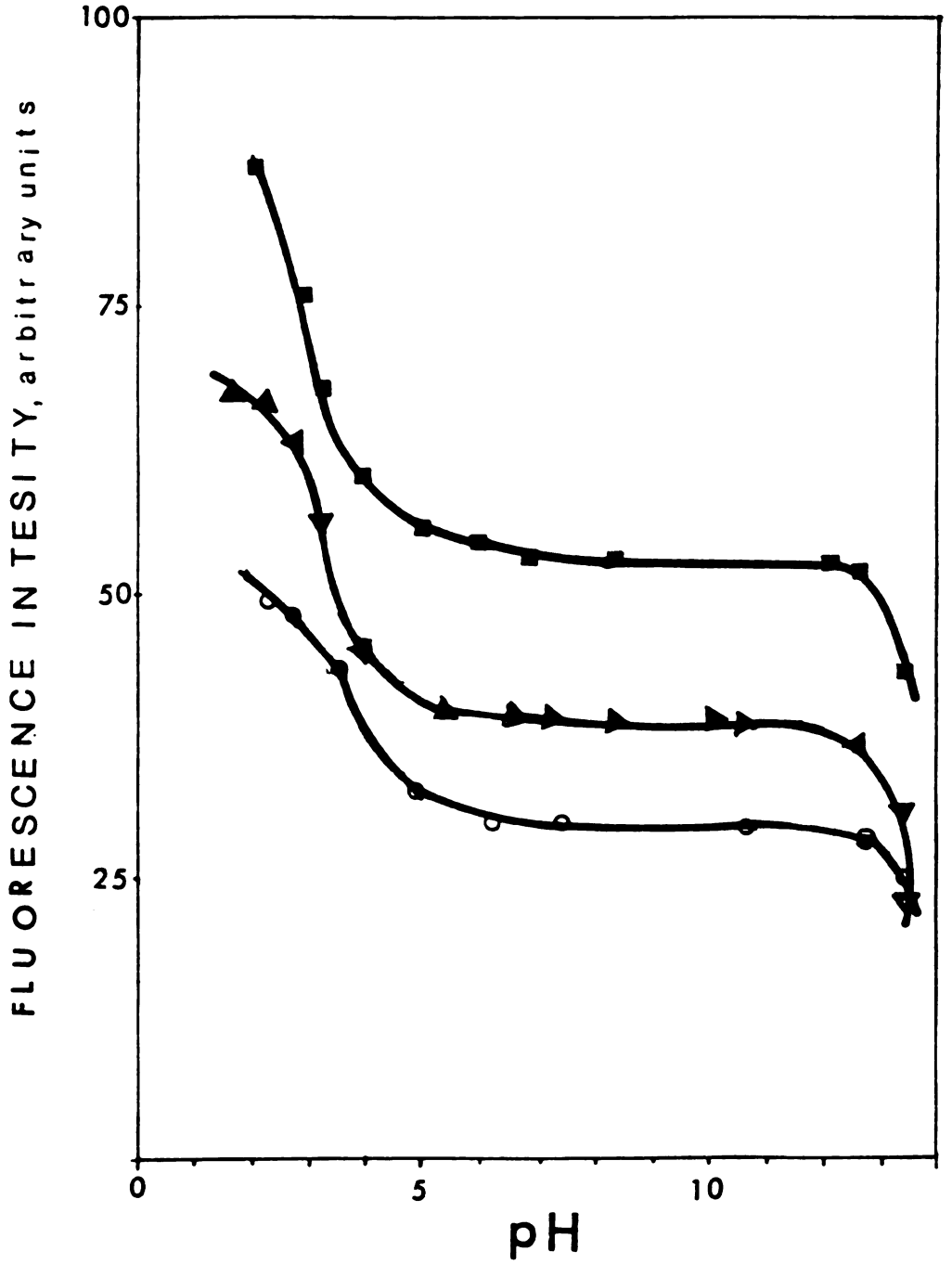


Figure 20. Effect of pH on the fluorescence of 0.16 ug/ml 2,2 dimethylsuccinyl fluorescein [□□], 0.14 ug/ml succinyl fluorescein [▷▷] and 1.60 ug/ml suberyl fluorescein [ooo].

III

ANALYTICAL APPLICATIONS

The use of fluorometric methods for the analysis of organic acids is not new. The dicarboxylic acids, malic, sebacic, succinic, o-phthalic acids and the polycarboxylic acids, fumaric, α-ketoglutaric, oxalacetic, isocitric and citric acids have been determined by fluorometric methods.[20-25] The conversion of the acids to the highly fluorescent fluorescein type compound using resorcinol and sulfuric acid was the basis for the analysis. Unfortunately, the method was not specific. Other dicarboxylic and polycarboxylic acids undergo similar reactions and even in the absence of the acid, resorcinol and sulfuric acid also gave a fluorescing specie which was measurable at the same excitation and emission conditions the fluorescein fluorescence was measured. Thommes,[22] measured this resorcinol blank fluorescence to amount to about 20-25% of the total fluorescence.

Many of the organic acids are found in biological fluids and extracts as metabolic reagent, product or by-product. Qualitative and quantitative information of these compounds may be utilized as indicators in the progress of these biological processes. In foods lactic, succinic and other acids are produced by microbial or enzymic action and can serve as useful indicators of spoilage as in the case of eggs in which the amount of lactic and succinic acids are used as means of detecting spoilage in frozen and dried eggs.[26-29] It is also known that plant organs

accumulate quantities of specific acids especially those which participate in the metabolic citric acid cycle and the characteristic identity and proportions of these accumulations can serve to identify the fruit or vegetable and detect adulteration in these products based on the acid pattern.[30-33] In man, the identity and quantity of organic acids present in blood, urine or other fluids are useful or may be useful indicators of a normal or abnormal condition.

The presence of minute quantities of several organic acids in these biological fluids and extracts and the need of establishing individual acid profiles for different kinds and sources of the biological samples requires a technique of analysis which is amenable to handling large number of samples and capable of determining several components present in very small quantities in a sample.

The high quantum yield of the fluorescein makes fluorometric method amenable to the determination of very small quantities of these acids. The non-specific nature of the acid-resorcinol reaction makes possible the simultaneous conversion of the acids to their respective fluoresceins in a single reaction. The use of thin layer chromatography to separate the different fluoresceins and the scanning fluorometer to measure the fluorescence intensities of the separated fluoresceins should make the resolution of acid mixtures possible and should be capable of handling large

numbers of samples.

Insofar as the investigator can ascertain, no such study had been published although TLC-Fluorescence method of analysis had been applied to other organic compounds.[36-46]

The determination of acids in fruits and fruit products and the lactic and succinic acids in frozen and dried eggs have been performed by gas chromatography. The official method[34-35] for the determination of lactic and succinic acids involves the following steps: (1) the liberation of the organic acids from the other components in the egg with sulfuric acid and phosphotungstic acid, (2) extraction of the lactic and succinic acids with anhydrous ether, (3) the evaporation of the ether from the extract and reflux the residue with BF_3-n propyl alcohol to convert the acids to n-propyl ester, (4) addition of $(NH_4)_2SO_4$ solution and then acetophenone as internal standard, (5) extraction with $CHCl_3$ and drying with anhydrous Na_2SO_4 and, (6) injection of the dried $CHCl_3$ extract into the gas chromatograph. In the case of fruit juices and extracts, the polybasic acids are separated by precipitation as lead salts or by anion exchange. The isolated acids were then converted into their silyl derivatives and determined by gas chromatography.

In this study, a mixture of succinic, methylsuccinic and 2,2 dimethylsuccinic acids was analyzed using a TLC-fluorescence method. The reaction of the acid with

resorcinol was utilized to convert the acid to its fluorescein derivative. Instead of using sulfuric acid as the condensing catalyst and reaction medium, polyphosphoric acid (PPA) was used. PPA has not been used in previous analytical studies but it is described in Russian patent.[8] It was used as the condensing catalyst in the preparation of fluorescein from phthalic anhydride or phthalic acid. Thommes[22] did not find phosphoric acid to be a possible substitute for sulfuric acid in o-phthalic acid analysis. In the present study, PPA was found to convert the acid to fluorescein in the presence of excess of resorcinol to a level of constant yield. It was further observed that very small quantities of the acid as described in the succeeding pages can be analyzed with very little or no interference from the resorcinol blank. Likewise, mixtures of the acids can be quantitatively determined in a single run without undertaking the rigorous and time consuming separations of the acids.

A. EXPERIMENTAL

1. Apparatus and Reagents

A Turner Fluorometer, Model 110, equipped with a mercury-lamp source, was used to measure the fluorescence intensity of the solutions. A combination of Turner filters #110-816 + 110-813 [2A + 47B] which passed the 436 mu radiation was used as primary filter and the sharp cut-off filter #110-818 [2A-12] which passed the 510 mu radiation for secondary filter.

The sensitivity at which measurements were obtained depended upon the intensity of fluorescence but in most cases, the range selector was set at 10X and in addition to the primary filter a 10% transmission neutral density filter was used.

A Turner Fluorometer, Model 111, provided with an automatic TLC-paper strip scanning accessory was used to measure the intensity of fluorescence of the thin layer chromatograms.

A linear chart recorder with an input of one volt and run at a chart speed of 4 cm per minute was used to record the spectra.

A compensating polar planimeter (K & E 620022) was used for measuring the area under the fluorescence peaks for the TLC separated fluoresceins.

Glass micropipets, (Accupette pipets, DADE), disposable,

5 ul, graduated, calibrated T.C. with ± 0.5% accuracy, were used for TLC spotting.

ITLC-SG paper, (Gelman Instrument Company), and Whatman Chromatography paper, SG 81, were cut to fit the scanner and dried for about 24 hours at 100° before use.

TLC developer mixtures were prepared from the following components: Methanol reagent, A.C.S., (Matheson Coleman & Bell Manufacturing Chemists and Fisher Scientific Company) without further purification; Methanol, dried by storing it over molecular sieve; chloroform, analytical reagent, (Mallinckrodt, Inc.) without further purification; ammonium hydroxide, analytical reagent, (Mallinckrodt) was used as received.

For the calibration curve an acetone solution or an aqueous solution of the acid sample was prepared such that the solution contained about 1 mg acid per ml of solution.

For analysis of an unknown acid sample, the solid may be directly weighed into the reaction vessel or a solution may be prepared in the same manner as the solution for the calibration curve.

Resorcinol, certified reagent (Fisher Scientific Company) was used without resubliming. An aqueous solution of resorcinol was used whenever identical replicate amounts of resorcinol were needed, otherwise, the desired amount of resorcinol was weighed and added to the acid

sample.

Polyphosphoric acid (PPA) was prepared by dissolving 1:1 weight-volume ratio of phosphorus pentoxide in phosphoric acid (85%). The weighed amount of phosphorus pentoxide was placed in a flask. The measured volume of phosphoric acid was slowly added (the reaction is exothermic and sufficient heat is generated to dissolve the phosphorus pentoxide). The mixture was stirred by a magnetic stirrer and kept at a temperature of about 40° for a day or two to equilibrate because equilibration of this mixture is a slow process. This mixture is stable and can be prepared long before the anticipated use. PPA prepared by just mixing the needed components without equilibration will not yield reproducible results.

2. Procedure

a. Preparation of the Fluoresceins

Aliquots of 0.1 to 0.4 ml of the acetone or aqueous solutions of acid sample were evaporated to dryness in a 5 ml test tube in a water bath at a temperature of 30-40° for the acetone solution or in an oven at 70-80° for the aqueous solution. The use of a higher temperature is not recommended because spattering of the sample occurs.

To each acid sample, 10 times its weight of resorcinol was added, then 0.2 ml (7 drops) of PPA. The mixtures were warmed in an oven and the test tubes containing the warmed mixture twirled to mix the contents. The test tubes were placed in a sand bath and the sand bath was placed in the oven. The oven temperature was raised and the sand bath temperature maintained at about 130-140° for 2 hours. The test tubes were then removed and cooled to room temperature.

For the TLC-Fluorescence method, the acid samples were mixed with larger excess of resorcinol (greater than 10 times) and the temperature was maintained at 120-130° for about 2 hours.

b. Preparation of the Solutions from Single Acid Samples for Fluorescence Measurements

The cooled reaction mixture was dissolved in 0.1 M NaOH and diluted to 100 ml. A one ml aliquot was diluted to 100 ml with distilled water. This solution, at a pH of about 6, was used for fluorescence measurement.

c. Preparation of Solutions from Multiacid Samples for TLC-Fluorescence Measurements

The cooled reaction mixture was dissolved and diluted with methanol to 50 ml. This solution, at a pH of about 2.0 was used for TLC spotting.

ITLC-SG or SG81 paper strips were spotted about 2 cm above the bottom end with 1 or 2 ul of the above solution from a disposable micropipette. The spots were dried for about 30 minutes at 100-105° in an oven or 5 minutes with an infrared lamp if the relative humidity of the room was less than 30%.

The dried strips were arranged in the sandwich type Eastman Kodak TLC developing chamber and developed with an 80:21.5:3.6 chloroform-methanol-ammonia mixture when ITLC-SG paper was used or 80:20 chloroform-methanol mixture when SG 81 paper was used. The developed spots were rapidly dried at about 105° in an oven and scanned.

3. Measurements

The blank knob of fluorometer was set to zero with a solution prepared by mixing 0.20 ml (7 drops) of PPA in 100 ml of 0.1 M NaOH, withdrawing 1 ml aliquots and diluting each aliquot to 100 ml with distilled water. This mixture duplicates the solvent system of the solution.

Because of the exploratory nature of the work, where the unknowns vary over a wide range of concentrations and information on the reagent blank was required, the 100 reading was not set. Instead, the resorcinol blank and the standards were read on each range in turn, without moving the blank knob.

To obtain fluorescence intensity measurements for

samples containing only a single acid, a correction for the fluorescing species formed from excess resorcinol in the reaction mixture had to be made. This was accomplished by processing an amount of resorcinol equal to the estimated excess (two moles of resorcinol react with one mole of acid) present in a sample mixture in the manner identical to the preparation of the samples. The fluorescence intensity of this "blank" was subtracted from sample intensities to yield corrected fluorescence intensities. To obtain reproducibility, standard acid samples were processed along with each batch of acid samples. From the corrected fluorescence and known concentration of the standard sample, and the corrected fluorescence of the acid sample, the concentration of acid in the sample analyzed was calculated.

For multiacid samples, fluorescence intensity peaks were recorded. Figures 26 and 27 show typical recorded fluorescence intensity peaks for separated fluoresceins formed from the identified acids. Peaks for standard samples, processed in the same manner as the samples analyzed, were also recorded. The areas under these peaks were measured by a planimeter. Concentrations of acids in the analyzed acid samples, were evaluated by comparison of the standard sample and analyzed sample peak areas. No blank correction for excess resorcinol had to be made since the resorcinol product was separated in the TLC separation step.

B. DEVELOPMENT AND EVALUATION OF THE ANALYSIS PROCEDURE

To optimize the experimental conditions to obtain reproducible yields of the fluorescein type products in the reaction between resorcinol and dicarboxylic acids in PPA medium, studies were carried out to determine the optimum resorcinol-acid ratio, reaction temperature, duration of heating the reaction mixture, and the volume of PPA required. Interference due to excess resorcinol was examined, precision in instrumental measurements was evaluated and the procedures for determining single acids or resolution of mixtures of acids were developed and tested.

1. Optimum Ratio of Acid to Resorcinol in the Mixture

A constant amount of acid was added with varying amounts of resorcinol such that the acid-resorcinol ratio in the mixture was about 1:2, 1:4, 1:6, 1:8, 1:10 and 1:12. The optimum amount of PPA was added. The fluorescence intensity of the solutions were measured.

Results presented in Table 10 showed variable results when the ratio of succinic acid to resorcinol in the reaction mixture was less than 1:10. This may be due to incomplete reaction. When the ratio was 1:10 or greater the intensity of fluorescence became consistent. It is apparent that an excess of resorcinol was needed to drive

Table 10. The Effect of Varying the Ratio of Succinic Acid to Resorcinol in the Mixture*.

Resorcinol, ug	Acid/Resorcinol Ratio	Fluorescence Reading
1.528×10^2	1/2.03	6.5
"	"	3.8
3.056×10^2	1/4.06	27.5
"	"	24.5
4.584×10^2	1/6.08	35.8
"	"	36.8
6.112×10^2	1/8.11	43.0
"	"	48.0
7.640×10^2	1/10.14	50.0
"	"	49.5
9.168×10^2	1/12.17	52.0
"	"	52.0

*An 80.8 ug of succinic acid was used in each reaction mixture.

the reaction to completion. The excess resorcinol present in the reaction mixture (reaction needs 2 moles resorcinol for every one mole of acid) also underwent a similar reaction producing species whose fluorescence were measurable at the wavelength the fluorescein fluorescence was measured. It was then necessary that for the determination of a single acid by direct fluorescence measurement on their solutions, that the amount of excess resorcinol be controlled. For analysis by TLC-fluorescence this large excess has no undesirable effects because the product from resorcinol is separated from the desired species during the TLC separation. The use of a 1:10 acid to resorcinol ratio was then adopted.

2. Fluorescence Contribution of Resorcinol Blank

The problem of blank fluorescence was investigated by carrying out the reaction at varying resorcinol concentration in the absence of the acid.

Figure 21 was obtained by plotting the fluorescence reading obtained against the concentration of the resorcinol used to produce the fluorescing species. From this curve it is evident that the blank fluorescence contribution of the excess resorcinol was increased with an increase in the resorcinol concentration in the reaction mixture.

From a reaction of 150 ug of succinic acid with about

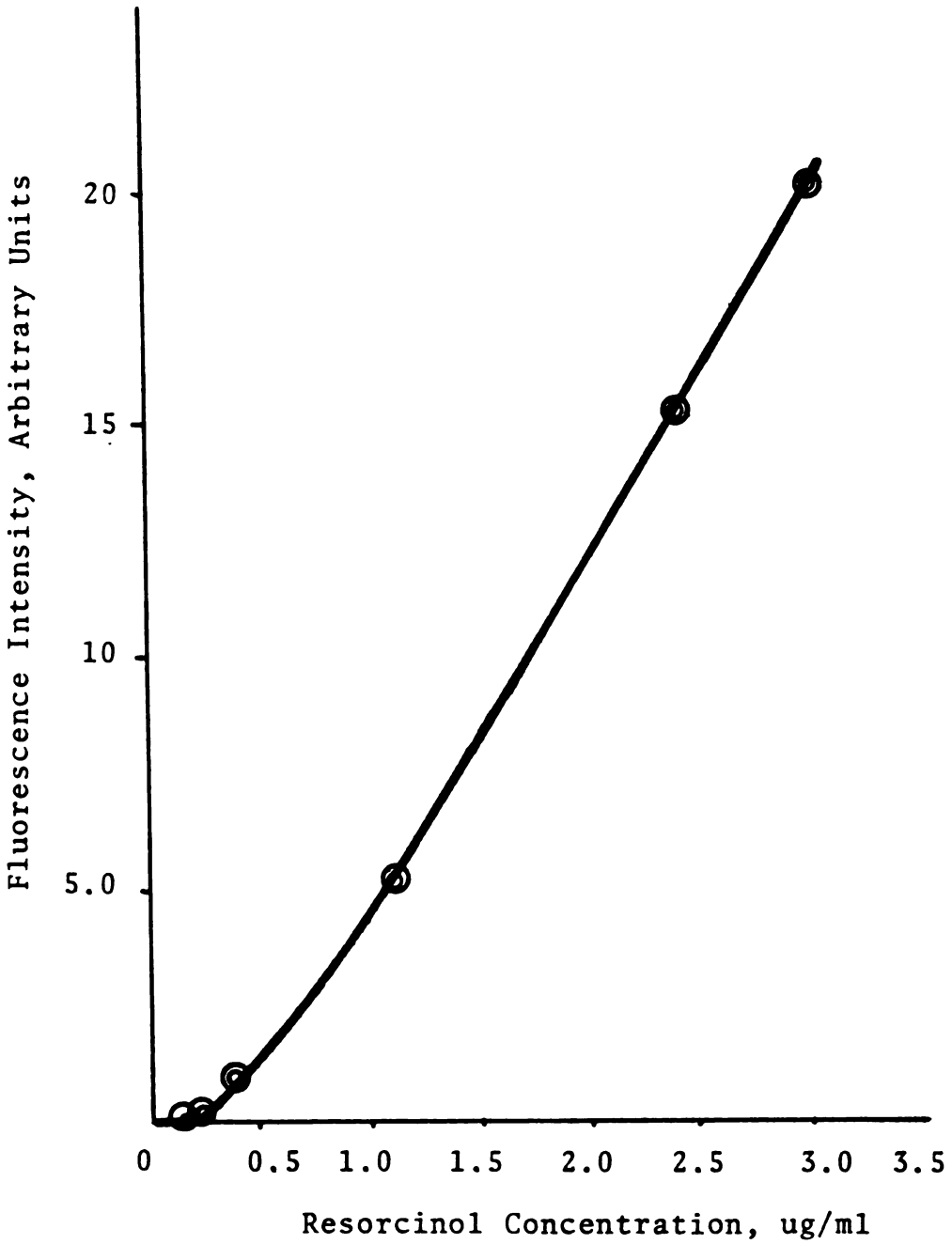


Figure 21. Calibration curve for blank fluorescence [fluorescence of product formed from resorcinol only].

1.5 mg or 1500 ug of resorcinol, the reaction product was diluted so that the acid concentration was 0.015 ug/ml or the resorcinol concentration was 0.15 ug/ml. At this dilution the fluorescence reading was 11.0 for the sample and for a corresponding resorcinol blank it was zero. The use of the same amounts of solvent to dilute the product from the reaction mixture of 776.5 ug succinic acid and 7.8 mg or 7800 ug of resorcinol resulted in a solution having the equivalent of 0.078 ug/ml of succinic acid and a resorcinol concentration of 0.78 ug/ml. The fluorescence reading for this diluted solution was 51.5 and for a resorcinol blank it was 2.4, a resorcinol fluorescence blank contribution of 4.7% of the total fluorescence.

In reactions using 10.4 mg or 10400 ug of resorcinol with varying amounts of succinic acid such that the acid/resorcinol ratios were 1/69.6, 1/34.8, 1/23.2, 1/17.4, 1/13.9 and 1/10.0 fluorescence contributions to the total fluorescence were 32.5%, 18.6%, 12.7%, 9.2%, 7.2% and 4.7%, respectively.

From the figure, it is apparent that if the amount of excess resorcinol in a reaction mixture is less than 2 mg or 2000 ug, no correction on the fluorescence reading obtained is necessary when the reaction mixture is diluted to ten thousandths of its original volume. At higher excess resorcinol, correction must be made on the sample fluorescence reading. The correction is determined by

running simultaneously with the acid sample and the standard, blanks whose resorcinol concentrations are approximately equal to the excess resorcinol present in the acid standard and the sample. The corrected fluorescence of the sample and the standard will be equal to their respective fluorescence readings minus the fluorescence reading of their respective blank.

When analysis is made by TLC-fluorescence, no correction is necessary since the fluorescence spectrum of the blank did not show any fluorescence peaks in the region of the fluorescein peaks even when the resorcinol content of the reaction mixture was more than 69 mg. The fluorescing species from the resorcinol blank moves up the TLC strip as fast as the solvent front and therefore cannot cause any interference.

3. Effect of Reaction Temperature and Duration of Heating

As expected the intensity of fluorescence increased with increasing reaction temperature as shown in Table 11. When the ratio of acid to resorcinol in the reaction mixture was less than 1:10, the temperature and duration of heating were critical factors. Random variation in fluorescence intensity was obtained. With 1:10 ratio of reaction mixture, heating at 120-130° for two or three hours gave reasonable reproducibility but with lower conversion of acid to fluorescein. Heating the reaction

Table 11. Effect of Reaction Temperature and Time of Heating on Product Fluorescence and Yield.

Temperature °C	Heating Time Hrs.	% Conversion of Succinic Acid*
110°-120°	1	61.1 ± 8.0
110°-120°	2	72.8 ± 9.0
120°-130°	1	73.1 ± 3.0
130°-140°	1	94.2 ± 3.0
130°-140°	2	96.5 ± 3.0

* % Conversion was based on the intensity of fluorescence of the succinic acid samples compared to the intensity of fluorescence from a fixed concentration of the purified fluorescein of succinic acid.

mixture to 13 -140° for one to two hours gave reproducible results with a 90-100% conversion (based on a comparison of fluorescence intensity of the sample and the standard prepared from purified samples of the fluorescein of the acid used in the reaction mixture). To insure a high percentage of conversion at the least amount of excess resorcinol in the mixture, the temperature of heating chosen was 130-140° for two hours.

Although the blank fluorescence was high at high reaction temperature and long heating (Figure 22), the fluorescence from the sample was very much more than obtained from the excess resorcinol (Figure 25), the contribution from the blank was small or negligible after the reacted mixture was diluted to the desired volume for fluorescence measurements.

When analysis was made by TLC-fluorescence method a large excess of resorcinol was used. An acid/resorcinol ratio of 1/20, 1/30, 1/40 and 1/50 gave the same area of fluorescence peaks for the same acid concentration in the reaction mixture when the temperature of heating was in the range of 120-140° for two hours.

4. Effect of Varying Quantities of PPA on the Intensity of Fluorescence

The volume of PPA needed for the reaction was determined for a reaction of a fixed quantity of acid with

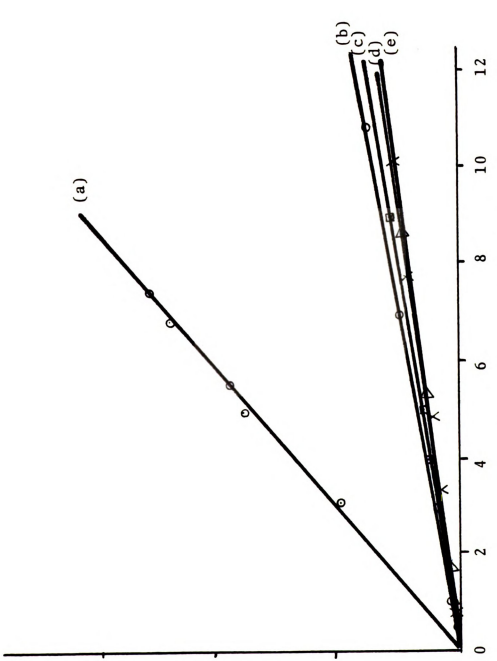


Figure 22. Fluorescence of resorcinol blank as a function of concentration and reaction temperature. (a) 140-150°, 30 minutes; (b) 120-130°, 1 hours; (c) 130-140°, 30 minutes; (d) 110-120°, 2 hours; (e) 110-120°, 1 hour.

10 times its weight of resorcinol.

It was noticed that the use of 0.08 ml (3 drops) of PPA gave a high yield but random variations in the fluorescence intensity among samples of the same acid content occurred. This variation may be due to incomplete reaction because some of the acid was deposited on the reaction vessel wall above the rest of the reaction mixture. Likewise, the use of 0.30 ml (10 drops) lowered the yield, which may be due to dilution of the reagent. The use of 0.15 to 0.20 ml (5 to 7 drops) of PPA was found to give a high yield and consistent results. To insure good mixing and sufficient reaction medium, 0.20 ml of PPA was used throughout this study.

5. Effect of pH on Intensity of Fluorescence

Purified fluorescein of succinic, 2,2 dimethyl-succinic and suberic acids obtained in the first part of this study were used to determine the influence of pH on the intensity of fluorescence. About 2 mg of the sample was dissolved in 1.0 liter of 0.05 M NaOH. A 5 ml aliquot was diluted with 0.1 M NaOH to almost 100 ml in a volumetric flask, adjusted to the desired pH with PPA and filled with distilled water to the mark. Fluorescence measurements were taken three times a day apart from each other to determine the stability of the compounds in solutions of different pH. Table 1 2 gives the

Table 13. Stability of Fluorescing Species in Alkaline, Neutral and Acid Solutions.

Sample	Conc. ug/ml	pH	Fluorescence 1	Fluorescence 2 24 hrs later	Fluorescence 3 48 hrs later
SAR	0.14	13.60	23.2	21.0	18.0
"	"	13.45	30.8	28.5	25.0
"	"	12.70	36.5	35.0	33.0
"	"	10.65	38.5	38.5	38.5
"	"	10.10	39.0	39.0	39.0
"	"	8.40	39.0	39.0	39.0
"	"	7.35	39.0	39.0	39.0
"	"	7.25	39.0	39.0	39.0
"	"	6.64	39.0	39.0	39.0
"	"	5.45	39.5	39.0	39.0
"	"	4.00	44.6	45.0	45.0
"	"	3.27	55.3	55.5	56.0
"	"	2.75	62.7	63.0	63.0
"	"	2.35	66.0	66.0	66.0
"	"	2.24	66.0	66.0	66.0
"	"	2.15	66.0	66.0	66.0
"	"	1.95	66.5	67.0	67.0
"	"	1.72	67.0	67.5	67.5
DSR	0.16	12.70	52.0	52.0	51.0
"	"	12.15	52.5	52.5	52.5
"	"	8.40	52.5	52.5	52.5
"	"	6.95	52.5	52.5	52.5
"	"	2.94	75.5	77.0	78.0
"	"	2.15	86.5	87.0	88.5
SUR	1.60	12.90	28.5	28.0	Violet Solution
"	"	12.75	28.5	28.5	"
"	"	10.70	29.0	29.0	29.0
"	"	7.48	29.5	30.0	30.0
"	"	7.15	30.0	30.0	30.0
"	"	6.45	32.0	32.0	32.0
"	"	6.25	33.0	33.5	33.5
"	"	2.75	48.0	48.5	Precipitation
"	"	2.32	49.0	49.7	"

Range selector setting: x10 + 10% Neutral Filter
SAR - Fluorescein of succinic acid or anhydride
DSR - Fluorescein of 2,2 dimethylsuccinic acid
SUR - Fluorescein of suberic acid
Exposure to light causes a fast decay of fluorescence of about 22-40% in 6 hrs in strongly alkaline solution.

results of the measurements made. A plot of the fluorescence intensity measured with the instrument range set at X10 and 10% neutral filter inserted with the primary filter, against pH is shown in Figure 20. Results show that the fluorescence intensity was constant in the pH region of pH 5-12. At low pH, the fluorescence intensity increased with decreasing pH while at a pH greater than 12 such as in 1.0 M and 2.0 M NaOH, the fluorescence of the sample decreased. A comparison made with resorcinol blank showed the same trend, however, the loss of fluorescence in 1.0 M and 2.0 M NaOH of the blank solution with time, was twice as much as that of the fluorescein sample.

It was also noted that the fluorescein of 2,2 dimethylsuccinic acid was more stable than the fluorescein of succinic acid and suberic acid in both acid and alkaline media. The fluorescein of succinic acid lost its fluorescence with time in basic medium but was quite stable in neutral and acid media. The fluorescein of suberic acid was unstable both in acid and alkaline media.

Because of the stability shown by the three fluoresceins in the pH region of 5 to 12 and the non-dependence of fluorescence on pH in this pH region, analysis of single acid samples by measuring the fluorescence intensity of their solutions, were carried out in this pH region.

6. Relationship of Concentration and Fluorescence Intensity of the Fluorescein Samples

The influence of concentration on the intensity of fluorescence was studied using the purified fluorescein samples of the acids of interest. A calibration curve was obtained as shown in Figures 23 and 24 for the fluorescein of succinic acid and 2,2 dimethylsuccinic acid. In both samples fluorescence intensity was linear with concentration. At all sensitivity settings, the intensity of fluorescence was linear with concentration.

To check the linear relationship on actual reaction product of the acid and resorcinol, known quantities of succinic acid were used. The curve presented in Figure 25 showed the linear concentration-fluorescence relationshp in the actual reaction product of succinic acid and 10 times its weight of resorcinol.

With small amounts of acid either the dilution and/or the range selector setting was changed. But in all cases, linearity was still observed.

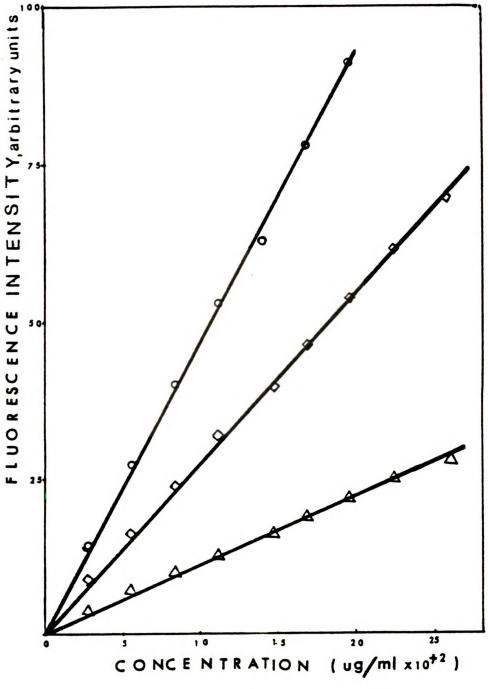


Figure 23. Calibration curve of succinyl fluorescein (purified sample) in the pH region of 6.0-7.5. Sensitivity setting: 10X + 10% ND [ooo], X3 + 10% ND [□□] and X1 [▷▷].

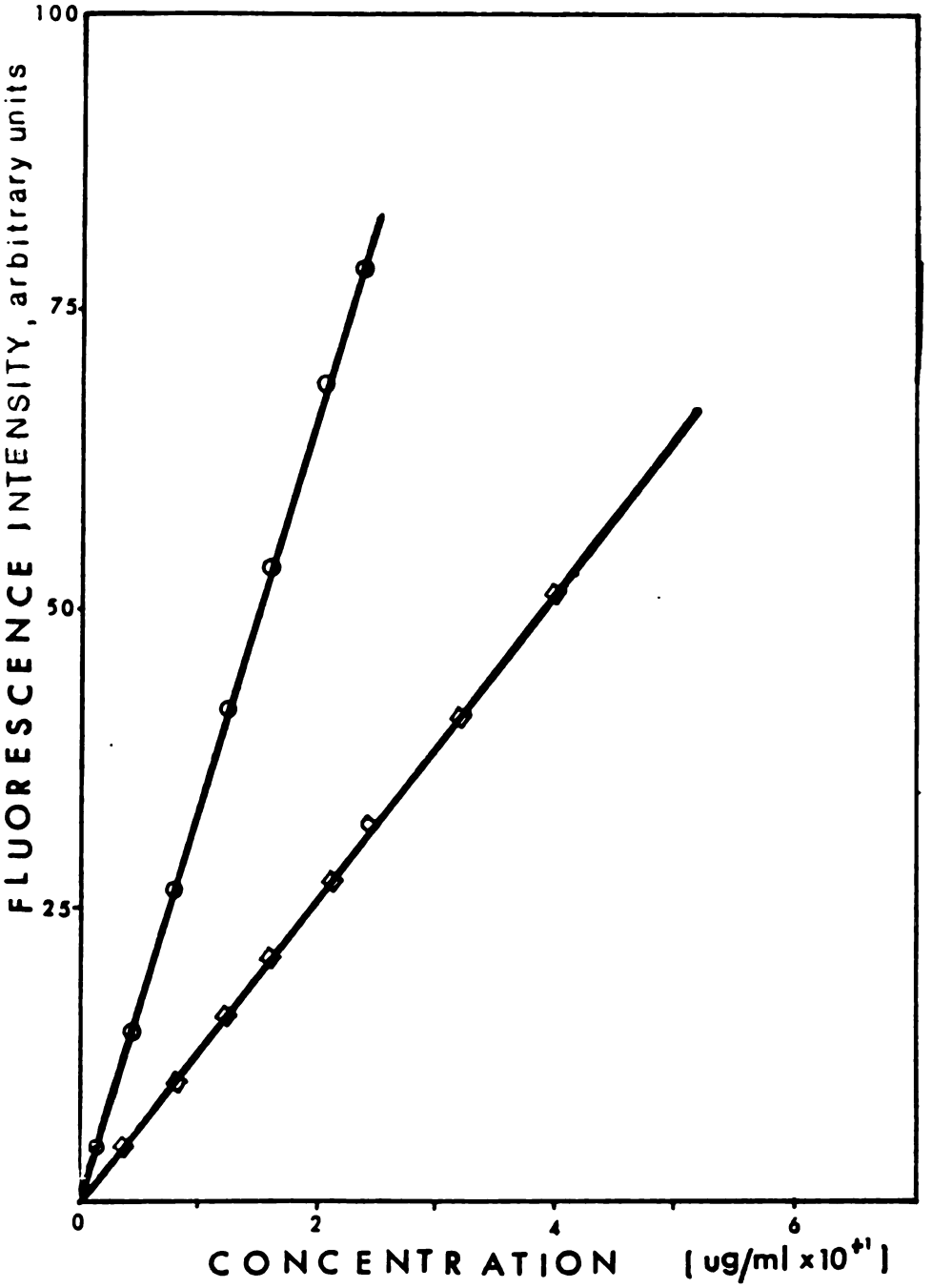


Figure 24. Calibration curve of 2,2 dimethylsuccinyl fluorescein (purified sample) in the pH region of 6.0-7.5. Sensitivity settings: 10X + 10% ND [ooo], 3X + 10% ND [□□].

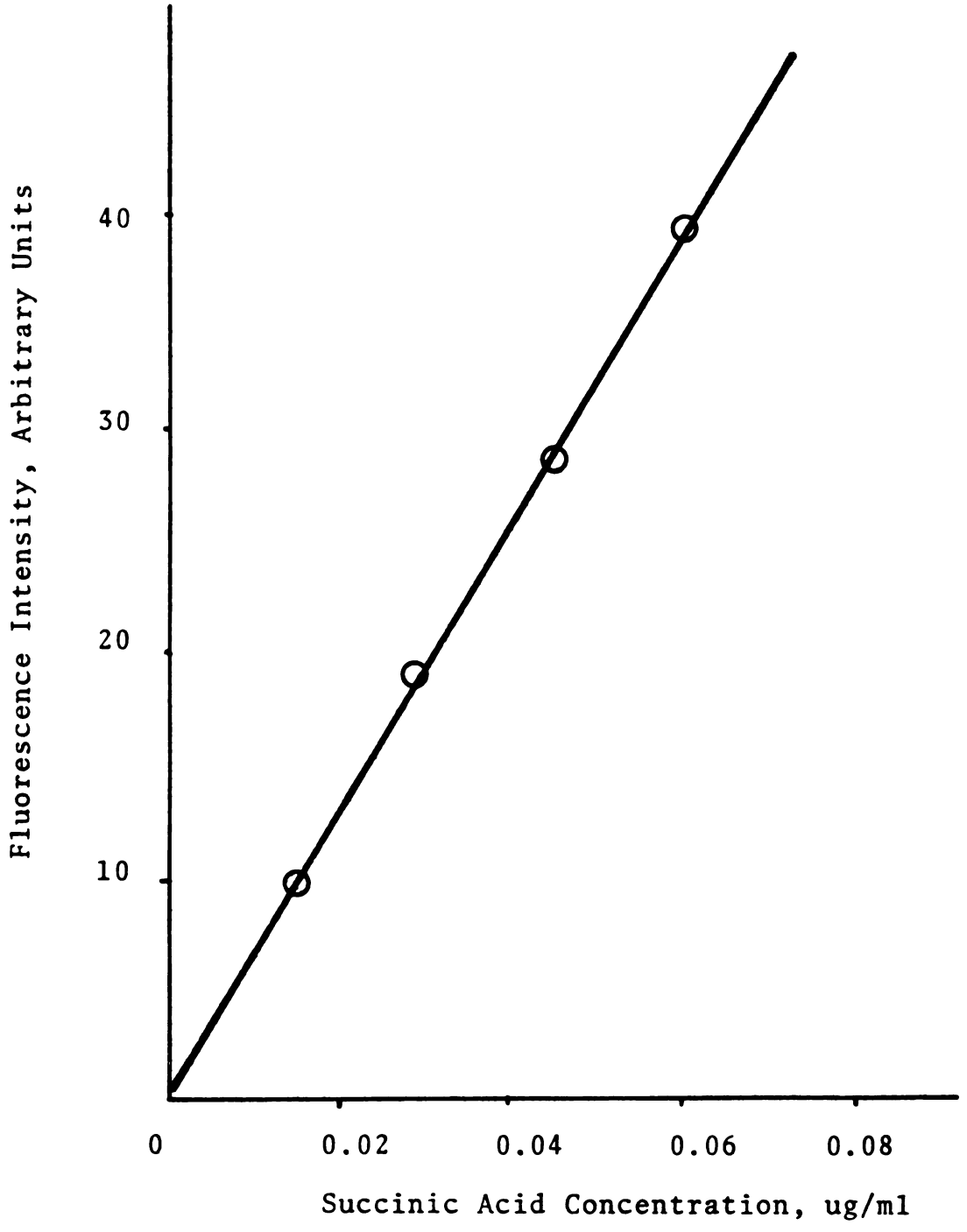


Figure 25. Calibration curve for succinic acid-resorcinol product. Sensitivity 10X + 10% ND.

C. ANALYSIS OF SUCCINIC ACID

If succinic acid, 2,2 dimethylsuccinic acid or methylsuccinic acid is present singly in a sample without any other fluorescein forming compounds as well as interfering substances, each of the acids may be determined quantitatively by direct measurement of the fluorescence intensity of the solution prepared from the reaction mixture of the acid and resorcinol with PPA as condensing catalyst and reaction medium. Analysis may be done using a calibration curve or by comparison with a standard sample run simultaneously with the acid sample.

Calculation of the acid content of the sample using a standard was made as follows:

$$\text{gm acid in sample} = \frac{\text{corrected fluor. of sample}}{\text{corrected fluor. of standard}} \times \text{acid content of standard}$$

Table 13 shows the result of an analysis conducted on succinic acid performed on different days. Heating of the reaction mixture was duplicated closely as possible the heating conditions during which the calibration curve was prepared.

Results obtained using a standard prepared simultaneously with the sample gave a lower relative error

Table 13. Analytical Results for Single Acids.

Succinic Acid Used		Succinic Acid, ug/ml		% Relative Error	
ug	ug/ml	Found[a]	Found[b]	(a)	(b)
First Series					
152.6	0.0076	0.0069±0.02	0.0071±0.02	9.2	6.6
305.0	0.0150	0.0147±0.02	0.0147±0.02	2.0	0.7
381.5*	0.0190	0.0187±0.00	-----	1.6	0·0
534.1	0.0267	0.0256±0.03	0.0260±0.02	4.1	2.6
Second Series					
152.6	0.0076	0.0070±0.03	0.0072±0.01	7.9	5.3
305.0	0.0150	0.0145±0.04	0.0150±0.02	3.3	0·0
381.5**	0.0190	0.0185±0.03	-----	2.6	---
534.1	0.0267	0.0267±0.03	0.0275±0.01	0.0	3.0

* and ** were used as standard for each run.

[a]When a calibration curve was used; [b]when a standard was used.

compared to that obtained when a calibration curve was used. This may be due to variation of the heating conditions for sample reaction from those maintained when standard mixtures were heated to prepare the calibration curve. Heating of the reaction mixture was a critical factor which controlled the conversion of acid to fluorescein except when there was a very large excess of resorcinol present. Preparing the standard reaction products simultaneously with the sample reaction products subjected both sample and standard to identical heating conditions as well as to any other condition which influenced the fluorescence intensity of the sample product.

D. TLC-FLUORESCENCE ANALYSIS OF MIXTURES

1. Separations by ITLC-SG and SG 81 TLC Papers

The formation of fluorescing species by other dicarboxylic and polycarboxic acids as well as by resorcinol alone whose excitations and emissions are in the same spectral region, limits the use of direct fluorescence measurement on complex solutions. Although the quantum yield of fluorescein is high so that analyses of very low quantities of the acid is possible, the applicability is limited by these mutual interferences. The different fluorescing species must be separated.

Thin layer chromatographic (TLC) separation of the fluorescing species formed in the reaction lends itself suitably to handling large quantities of samples with several components.[37,41-53] Thin layer chromatograms of the reaction mixture are prepared and developed. The chromatograms are scanned and the amounts of the different fluorescing species are determined by measuring the area under the fluorescence curve.

Shown in Figure 26 is the array of peaks of a mixture of the fluoresceins of o-phthalic acid, succinic acid, 2,2 dimethylsuccinic acid and suberic acid separated on an ITLC-SG paper and developed with 93:7 chloroform-methanol mixture at a relative humidity of less than 30% and a room temperature of about 24°. Figure 27 shows the

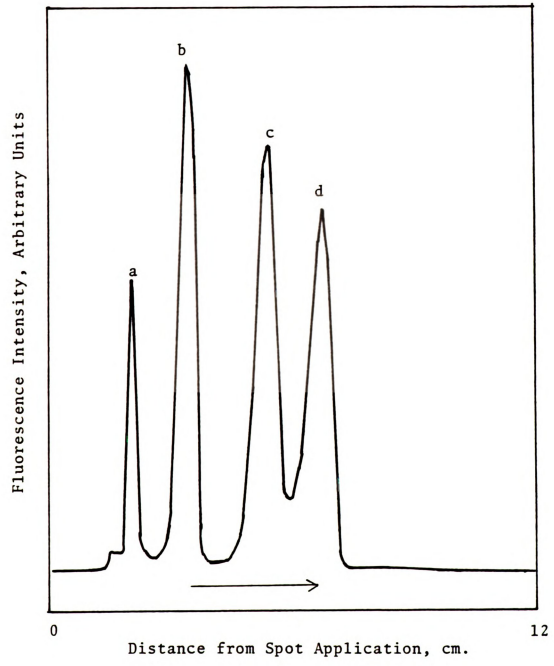


Figure 26. Fluorescence scan of TLC chromatogram of a mixture of fluoresceins (a) succinic; (b) suberic; (c) 2,2 dimethylsuccinic; (d) phthalic acids (ITLC-SG paper; 93.7 chloroform - methanol developer).

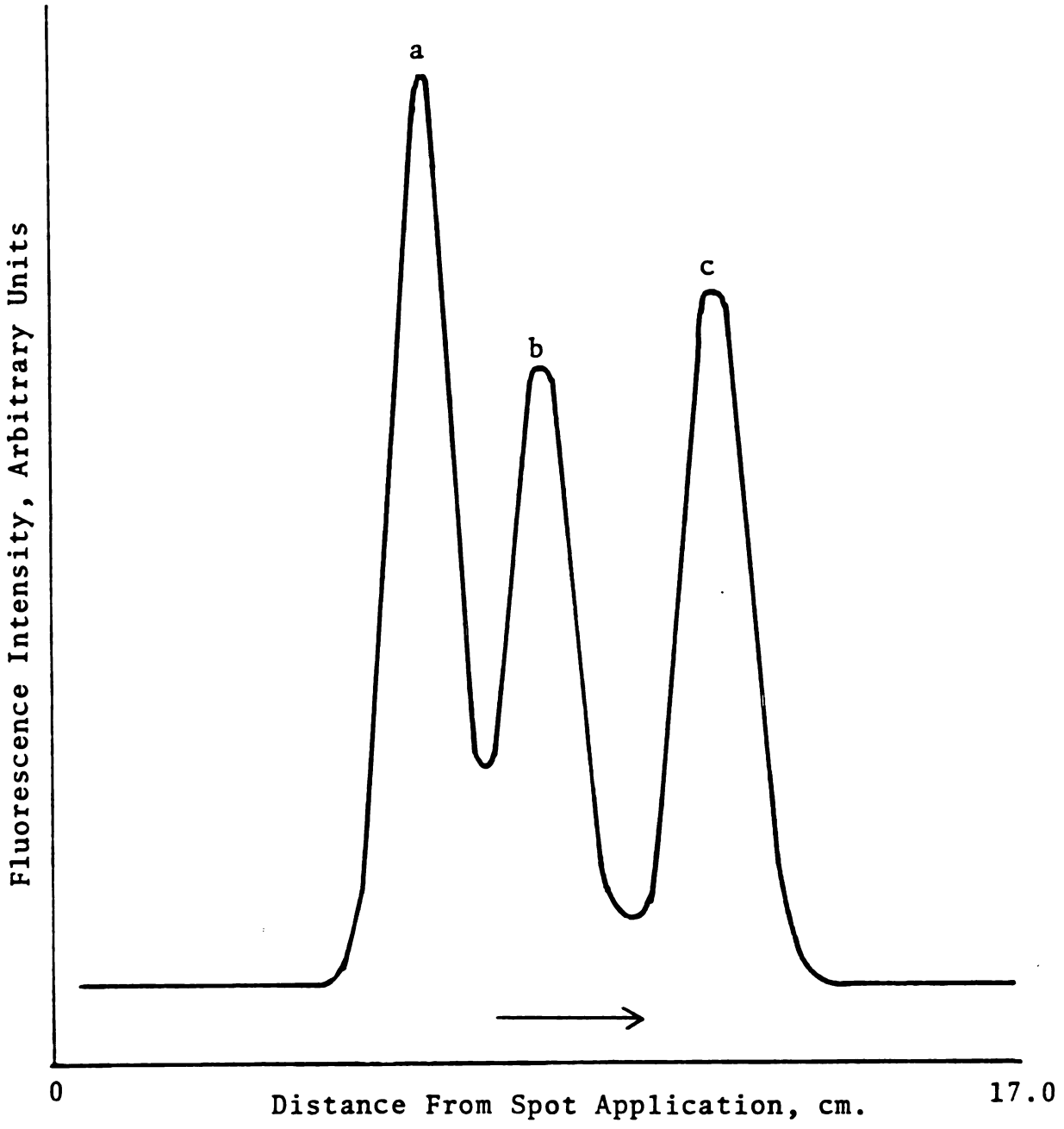


Figure 27. Fluorescence scan of TLC separated reaction product of resorcinol and acid mixture: (a) succinic acid peak; (b) methylsuccinic acid peak; (c) 2,2 dimethylsuccinic acid peak; ITLC-SG paper; 80:21.5:3.6 chloroform-methanol-ammonia developer.

array of peaks of a mixture of the fluoresceins of succinic acid, methylsuccinic acid and 2,2 dimethylsuccinic acid separated on an ITLC-SG paper and developed with 80:21.5:3.6 chloroform-methanol-NH_3 mixture at a relative humidity of 33% and room temperature of 26°. Also presented in Figure 28 is the spectrum of the same mixture as in Figure 27 but separated during a very dry day. At very low % relative humidity such as existed during the dry winter days and the use of a longer strip, a mixture of more than 4 components with slightly varying polarities can be separated with the right mixture of chloroform-methanol and with the right pH of spotting solution. A high relative humidity as is the case during the spring, summer and autumn days (% relative humidity varying from 60-100%), only the fluoresceins with large differences in polarities are separable. In the case of a mixture of the fluoresceins of succinic, methylsuccinic and 2,2 dimethylsuccinic acids only succinic and 2,2 dimethylsuccinic can be distinctly separated with the methylsuccinic appearing as a shoulder on the right side of the succinic peak or on the left of the 2,2 dimethylsuccinic peak depending on the ratio of chloroform to methanol in the developer.

The use of ITLC-SG paper limits the height of development to a maximum of 20 cm since the paper commercially available from Gelman is marketed as 20 cm x 20 cm sheets.

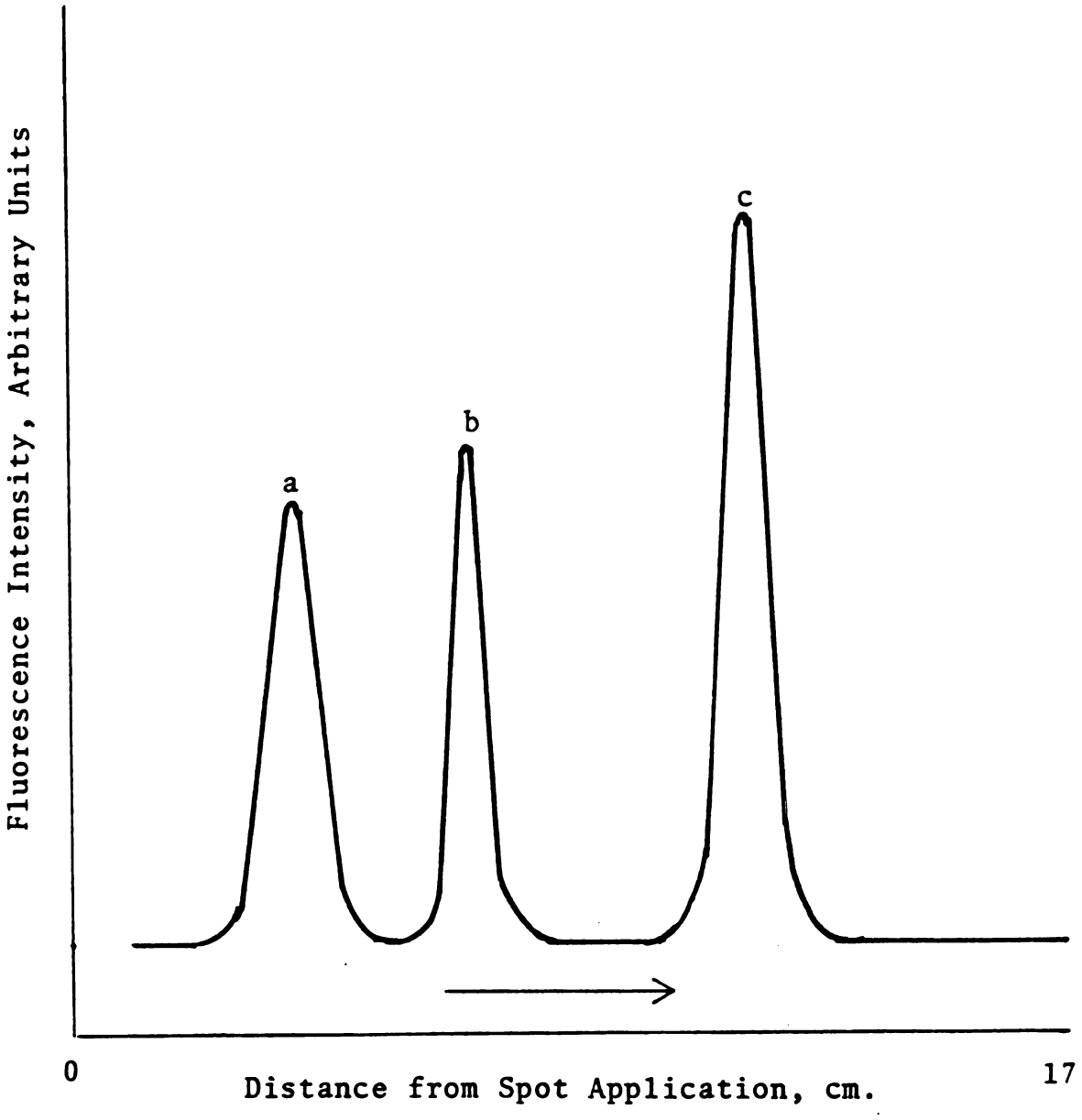


Figure 28. Fluorescence scan of TLC separated aqueous mixture (pH 6.4) of the fluoresceins of succinic acid, methylsuccinic acid and 2,2 dimethylsuccinic acid (a,b,c respectively). [ITLC-SG paper; 93:7 chloroform-methanol developer; relative humidity below 30%].

A technique used to increase the separation on this paper, as if the paper were a long strip, was to open the top end of the sandwich developing column to allow the solvent to escape from the top. The side of the column must be air tight. The length of time the top is opened is limited by the spreading of the spots.

The use of SG 81 paper, available from Whatman in 46 cm x 57 cm sheets, separated the fluoresceins well. It affected the same separation as the ITLC-SG paper for the same mixture of fluoresceins. It has the advantage of giving reproducible results for different batches of development. It was also possible to determine lower fluorescein concentrations than was possible with the ITLC-SG paper. The major disadvantage of the use of the SG 81 for TLC separation was the need of a column so strips can be suspended without wobbling and touching each other. The strips cannot be developed in the sandwhich column because blotting occurs. Furthermore, while 10-15 minutes was all that was needed to develop the 20 cm ITLC-SG strip, it took about an hour to develop the same length of the SG 81 strip.

2. Influence of the Type of Thin Layer Chromatography Papers on the Area of Fluorescence Peak

Table 14 shows the results obtained from the TLC separation of a mixture of the fluoresceins of succinic

Table 14. Influence of Type of TLC Paper on the Area of the Fluorescence Peak.

TLC Layer	Batch #	SAR Conc. ug/ml Spot	DSR Conc. ug/ml Spot	SAR Area cm^2	DSR Area cm^2
ITLC-SG	1	0.0468	0.0460	5.4	13.5
		"	"	3.5	9.7
	2	"	"	2.7	14.0
	2	"	"	2.3	12.7
	3	"	"	5.4	13.0
	3	"	"	5.1	13.4
	3	"	"	5.2	16.1
	3	"	"	5.0	16.2
SG 81	1	0.0149	0.0118	2.4	1.9
	2	"	"	2.4	1.9
	1	0.0298	0.0236	4.9	3.8
	2	"	"	4.9	3.8
	1	0.0372	0.0295	6.6	4.9
	2	"	"	6.5	4.9
	1	0.0595	0.0474	10.1	7.2
	2	"	"	10.0	7.2
	1	0.0744	0.0592	11.8	9.3
	2	"	"	11.8	9.4
	2	"	"	11.8	9.4
	2	"	"	11.5	9.3
	1	0.1190	0.0948	15.1	11.8
	2	"	"	16.0	12.0
	1	0.1488	0.1184	18.2	15.3
	2	"	"	18.2	15.2

Sensitivity setting: X10 for ITLC-SG and X3 for SG 81
Fluorescein of succinic acid (SAR); Fluorescein of 2,2-dimethylsuccinic acid (DSR).

acid (SAR) and 2,2 dimethylsuccinic acid (DSR) on the ITLC-SG and the SG 81 papers when developer mixtures of the composition of 65:21.5:3.6 chloroform-methanol-ammonia and 80:20 chloroform-methanol mixture, respectively, were used at a humidity of about 46% and room temperature of about 26°.

Although reproducibility seems to be poor on the ITLC-SG paper it was observed that at low and controlled humidity and with the use of 80; 21.5; 3.6 chloroform-methanol-NH_3 mixture as developer, the precision among samples simultaneously developed improved as shown in Table 15. When the area of one or two components of the mixture showed very large deviations, examination of the spots under a UV-Visible light indicated that the spots were either shifted away from the straight line center path during development or had spread out irregularly. Either of these departures from ideal development resulted in low fluorescence intensity, therefore smaller peak area. This was due to reduced excitation of only a part of the spot by the incident beam and reduced passage of the emission radiation through the fixed slits through which fluorescence radiation passed to the photomultiplier tube.

As shown in Table 15, an average relative deviation of about 1.8% was obtained for the separation of the fluoresceins on ITLC-SG paper within a single development

Table 15. Precision on Simultaneously Separated Fluorescein in Mixtures on ITLC-SG.

Develop. Group	Sample #**	Area Under the Fluorescence Curve SA, cm^2	MSA, cm^2	DSA, cm^2	% Average Rel. Dev.
1	1	11.47	8.23	[8.83]**	
	2	11.13	8.27	10.30	1.75
2	1	15.40	9.93	12.33	
	2	15.23	9.77	12.27	1.10
3	3	11.80	8.30	10.10	
	4	11.80	8.10	[9.10]**	1.57
4	3	11.30	8.20	10.00	
	4	11.80	[7.90]**	10.00	2.67

*Samples 1, 2, 3 and 4 each contained a reaction mixture of 0.00804 ug succinic acid (SA), 0.00824 ug methylsuccinic acid (MSA) and 0.00753 ug 2,2 dimethylsuccinic acid (DSA) per ul. The acid/resorcinol ratio in samples 1 through 4 were 1/53, 1/23, 1/57 and 1/31 respectively.

Sensitivity setting: X30

**Spot was shifted from center path.

batch and at a relative humidity of 33% and room temperature of 26°. With the SG 81 an average relative deviation of 1.0% was obtained.

The precision obtainable with the instrument was studied by scanning a chromatogram ten times with a rest interval of 15 minutes keeping all the instrument settings the same. To eliminate the error from planimeter reading, the height of the peak was used instead of area since as the chromatogram was left untouched from the first to the last scan. An average relative deviation of 1.38% was obtained.

Since the placement of the chromatogram in the scanner as well as the planimeter reading of the area can affect the reproducibility of a result, its effect on a chromatogram was studied. A chromatogram of a mixture of the fluoresceins of 2,2 dimethylsuccinic acid and succinic acid was scanned, removed, then replaced and rescanned. With 10 scans and 5 planimeter area readings per peak per scan, an average relative deviation of 7.5% was obtained.

This average relative deviation of 7.5% is largely due to the position of the spots in the scanner. The error from the micropipette calibration (about $\pm$0.5%), capillarity effect in spotting, and the "operator" error which Kirchner, Brain and others[54-58] have tried to evaluate in quantitative TLC, do not contribute to this

observed deviation since the same chromatogram was scanned. But this deviation shows that the geometry of the spots relative to the slit location in the instrument used is a critical factor in quantitative TLC-fluorescence. Elimination or minimization of this error through improved instrumentation will greatly improve the precision and accuracy of the method.

3. Influence of Concentration on the Area Under the Fluorescence Peaks of TLC Chromatograms

A 0.0093 gm sample of purified succinyl fluorescein and 0.0074 g of 2,2 dimethylsuccinyl fluorescein were weighed and placed in a 50 ml volumetric flask and filled with methanol to the mark. One and two ul spots of this solution were made on strips of SG 81 paper. The spots were dried, developed with 80:20 chloroform-methanol mixture, dried and scanned. Shown in Table 16 are the results obtained.

The curve shown in Figure 29 obtained from the data presented in Table 16, showed a linear relationship of area to concentration at low fluorescein concentration. The upper part of the curve which showed divergence from linearity corresponded to the area of the fluorescence peaks of the 2 ul spots. Two ul spots of low fluorescein concentration obeyed the linear relationship of area

Table 16. Influence of Concentration on the Area Under the Fluorescence Peaks of the TLC Chromatograms.

Develop. Group	SAR Conc. ug/ml	DSR Conc. ug/ml	SAR Area cm^2	DSR Area cm^2	Spot Vol. ul
1	0.0372	0.0295	6.6	4.9	1
2	"	"	6.5	4.9	"
1	0.0595	0.0474	10.1	7.2	"
2	"	"	10.0	7.2	"
1	0.0744	0.0592	11.8	9.3	"
2	"	"	11.8	9.4	"
1	2(0.00744)	2(0.00592)	2.4	1.9	2
2	"	"	2.4	1.9	"
1	2(0.0149)	2(0.0118)	4.9	3.8	"
2	"	"	4.9	3.8	"
1	2(0.0372)	2(0.0295)	11.8	9.4	"
2	"	"	11.5	9.3	"
1	2(0.0595)	2(0.0474)	15.1	11.8	"
2	"	"	16.0	12.0	"
1	2(0.0744)	2(0.0592)	18.2	15.4	"
2	"	"	18.2	15.2	"

Sensitivity setting: X3

Developer solution: 80:20 chloroform-methanol.

Succinyl fluorescein (SAR); 2,2 dimethylsuccinyl fluorescein (DSR).

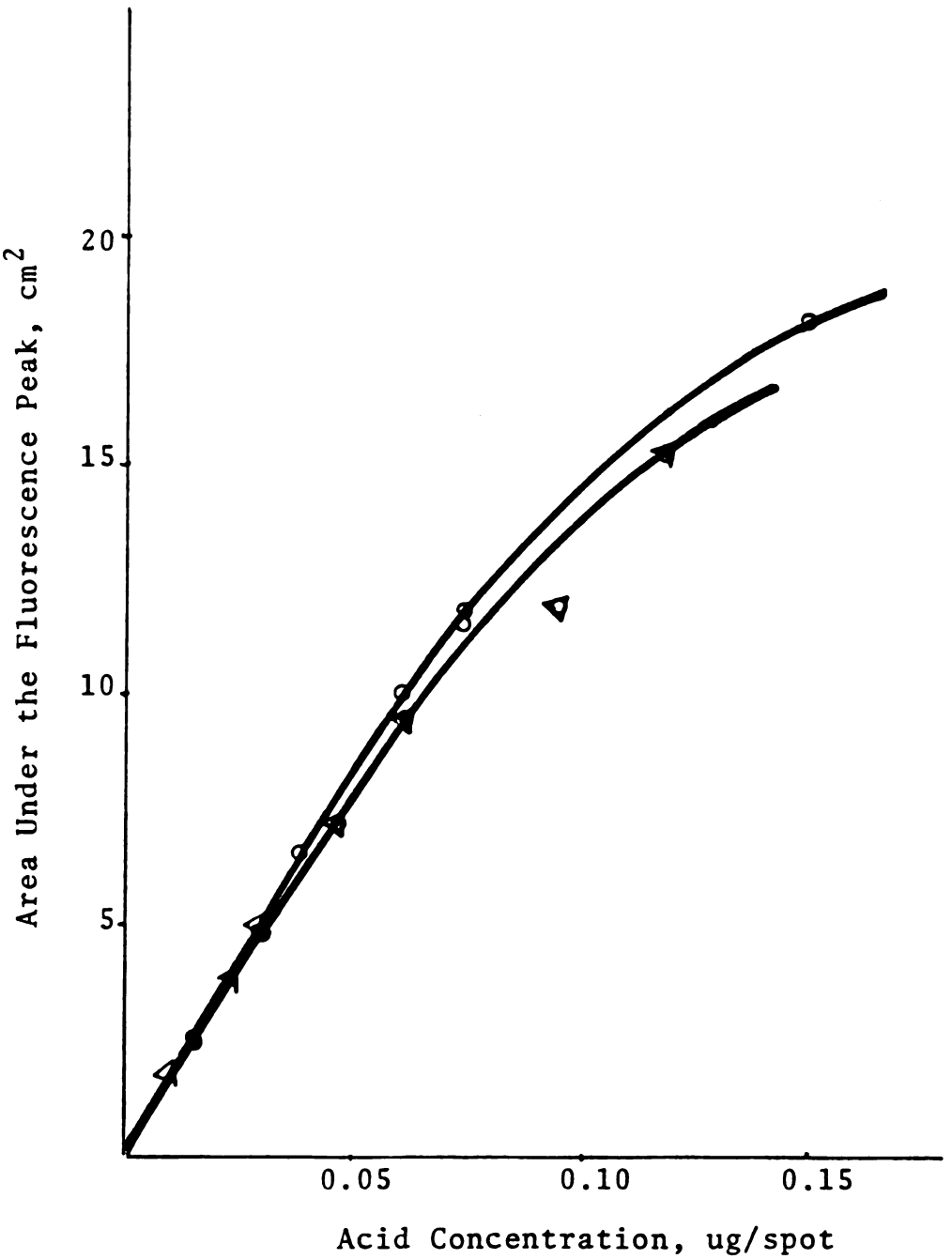


Figure 29. Influence of concentration on the area under the fluorescence peak of TLC chromatograms of fluorescein mixtures (SG 81 paper; 80:20 chloroform-methanol developer).

to concentration. This shows that the size of the spot is a critical factor just as the geometry of the spot is. As explained previously, these losses in fluorescence were caused by illumination of only a part of the spots and detection of only a portion of the emitted fluorescence.

Studies concerning the linear relationship of spot area or peak area or peak height (F) to the amount of substance (M) have resulted in the various functions of F and M. Linear relationships have been found to exist between F and M, F and log M, $\sqrt{F}$ and M, and $\sqrt{F}$ and log M. Kohler,[45] who worked with the non-aminated organic acids, fumaric, glycolic, hippuric, L-lactic, DL-malic, pyrrolidone carboxylic, succinic, and trans-aconitric; spotted 10 ug to 180 ug of the acid on prepared cellulose plates (0.1 mm thickness); developed them two-dimensionally, sprayed them with aniline-xylose reagent, heated the chromatoplates to 140° for 5 minutes and marked the visible spots with pencil and copied them on tracing paper, found a straight line relationship of F and M within the range of 10/30-90/110 ug except for trans-aconitic acid which showed the straight line relationship with F and log M. Nybom,[46] who worked with malic and citric acid at different thicknesses of the cellulose layers and Bondivenne, et al.,[59] who worked with different organic substances, concluded that different relationships exist for different substances.

The accidental error caused by the fact that the spot formed is influenced by the attendant substances chromatographed and the difficulties in outlining the spots makes quantitative determination of substances by direct measurement of spot size hardly possible.[59]

The use of photometric measurement of spot areas in quantitative thin layer chromatography by direct scanning procedure for the TLC plates, was critically evaluated by Klaus,[60] and apparently direct fluorimetry is most likely to give correct results. Purdy and Trutter,[61] suggested on the basis of results obtained by other investigators that a linear relation between $\sqrt{F}$ and log M was most correct. Rasmussen,[41] who used photometric scanning of photoprints of TLC plates (silica gel about 0.2 mm thick) with different exposures for the quantitative determination of dinitrophenylhydrazones of α-ketoglutaric acid and oxaloacetic acid (10 ul spots) obtained curves for standards of the compounds which showed linear relationship of F and M within a certain concentration range.

Smith,[42] worked with cholesterol, n-propionate, oleic acid, xanthanoic acid (9-xanthene-carboxylic acid), androstane-3,17-dione, triolein, D-glucose and N-methylphenylalanine. He spotted 5 ul of the solutions on prepared silica gel G plates (250 u thickness) impregnated with ammonium sulfate; developed the chromatoplates, dried them,

formed the fluorescent derivative by exposure of the dried chromatoplates to vapors of tert-butyl hypochlorite and heated the plates to 150-180° for 15 minutes, found that within the range of 0-10 ug, the amount of substance (M) was linearly related to peak height as well as peak area (F).

Segura and Gotto[43] used plates prepared from the adsorbent, silica gel, alumina and cellulose by spreading the adsorbent on glass to a thickness of 250 um. Spots of 20 and 25 ul were developed and fluorescent derivative of the compounds were formed by placing the chromatoplates in a tank with ammonium hydrogen carbonate, placing the tank in the oven and heating it to 110-150° for 2 to 12 hours depending on the type of adsorbent used. A Farrand Optical TLC scanning accessory attached to an MK-1 spectrofluorimeter was used and the peak areas were determined by planimetry and/or triangulation. For the lipids, phosphatidylcholine, oleic acid, triolein, cholesterol, cholesteryl linoleate; the steroids, progesterone, cortisone and tetrahydrocortisone; and the catecholamines, epinephrine and norepinephrine; developed on silica gel. They stated that a linear response was obtained between the square root of peak area ($\sqrt{F}$) and log of concentration (log M) within 0.5 and 80-100 ug.

Madsen and Latz[36] performed a TLC separation of 5-geranoxy-7-methoxycoumarin and 5,7 dimethoxycoumarin in

expressed citrus oil. They used 0.2 to 2.0 ul spots of the expressed oil sample on precoated analytical chromatoplates of Silica Gel F-254 and developed the chromatoplates in a Brinkmann sandwich developing chamber. Both peak area and peak height (F) obtained from the fluorimetric scanning of the chromatograms were linear with concentration (M). Accurate results were obtained for the quantitative determination of these compounds when care was taken that the maximum emission intensity was measured.

In this study instant thin layer chromatographic papers, the ITLC-SG and the SG 81, were used. As in the SG 81 (Figure 30), a linear relationship between the peak area and the concentration of the corresponding fluorescein was obtained when ITLC-SG paper was used, although linearity only occurred within a single batch of development. Reproducibility among samples developed in different batches were quite poor which may be due to the more porous nature of the sheet and the high content of silica gel which makes it more susceptible to variations in environmental conditions, especially humidity. The lateral diffusion of the spots varied among different batches of chromatograms and affected the peak area, probably because in some cases only a part of the spot was excited and detected. Although the addition of ammonia to the chloroform-methanol developer minimized the spreading

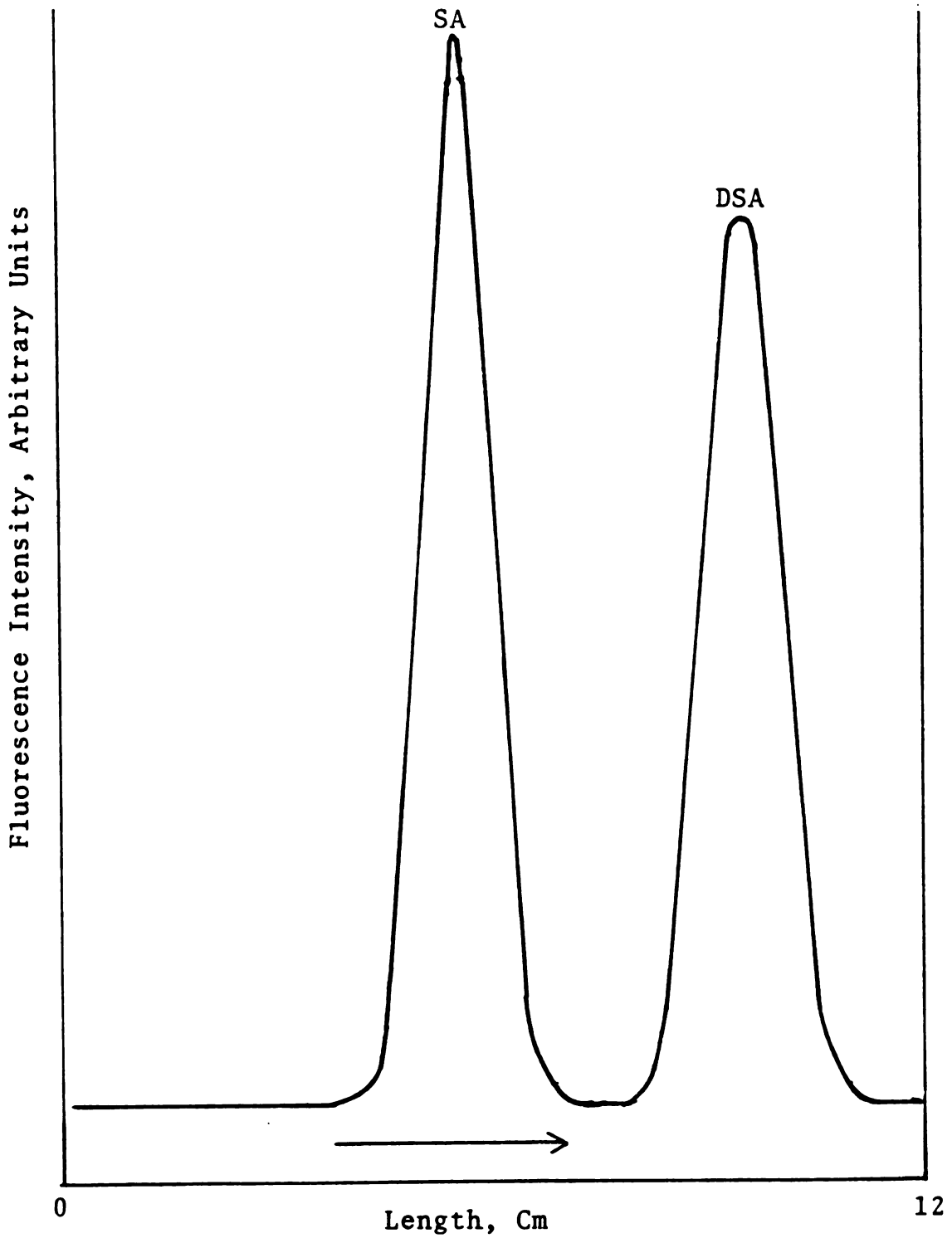


Figure 30. Fluorescence scan of TLC separated fluorescein product of succinic acid (SA) and 2,2 dimethyl-succinic acid (DSA). [ITLC-SG paper; 80:21.5: 3.6 chloroform-methanol-ammonia developer.]

of the spot during development and improved greatly the precision in the area measurements for a single batch of chromatographic peaks; it did not improve the precision in the area measurements when measurements were made on peaks obtained on identical samples but developed in different batches.

The applicability of the separation on mixtures of acid samples for the quantitative determination of the acids was tried on mixtures of succinic acid and 2,2-dimethylsuccinic acid; methylsuccinic acid and succinic acid; and 2,2 dimethylsuccinic acid and methylsuccinic acid. One to two ul spots of the prepared solution of the reaction mixture product were used. The array of peaks from the above mixtures is shown in Figures 30, 31, and 32, respectively. A calibration curve of peak area vs. concentration prepared from the above mixture samples yielded curves shown in Figures 33 and 34. It is apparent that peak area is linearly related to concentration within a certain concentration range.

Although the calibration curves show good linearity, quantitative analysis was best done using a standard, a mixture containing known quantities of the acids being analyzed, prepared and developed simultaneously with the unknown sample. This is so, because as was noted in the previous sections, the resorcinol-acid reaction was influenced by temperature and time of heating. Likewise,

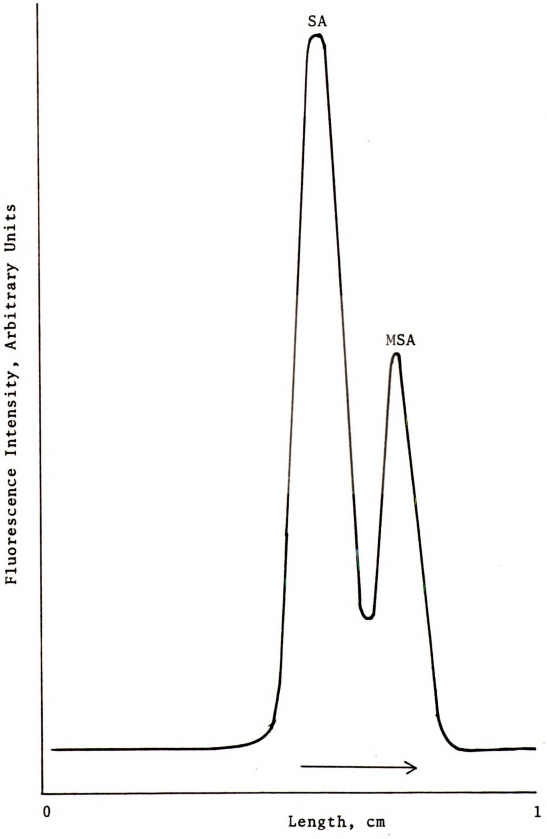


Figure 31. Fluorescence scan of TLC separated fluorescein product of succinic acid and methylsuccinic acid. [ITLC-SG paper; 80:21.5:3.6 chloroform-methanol-ammonia.]

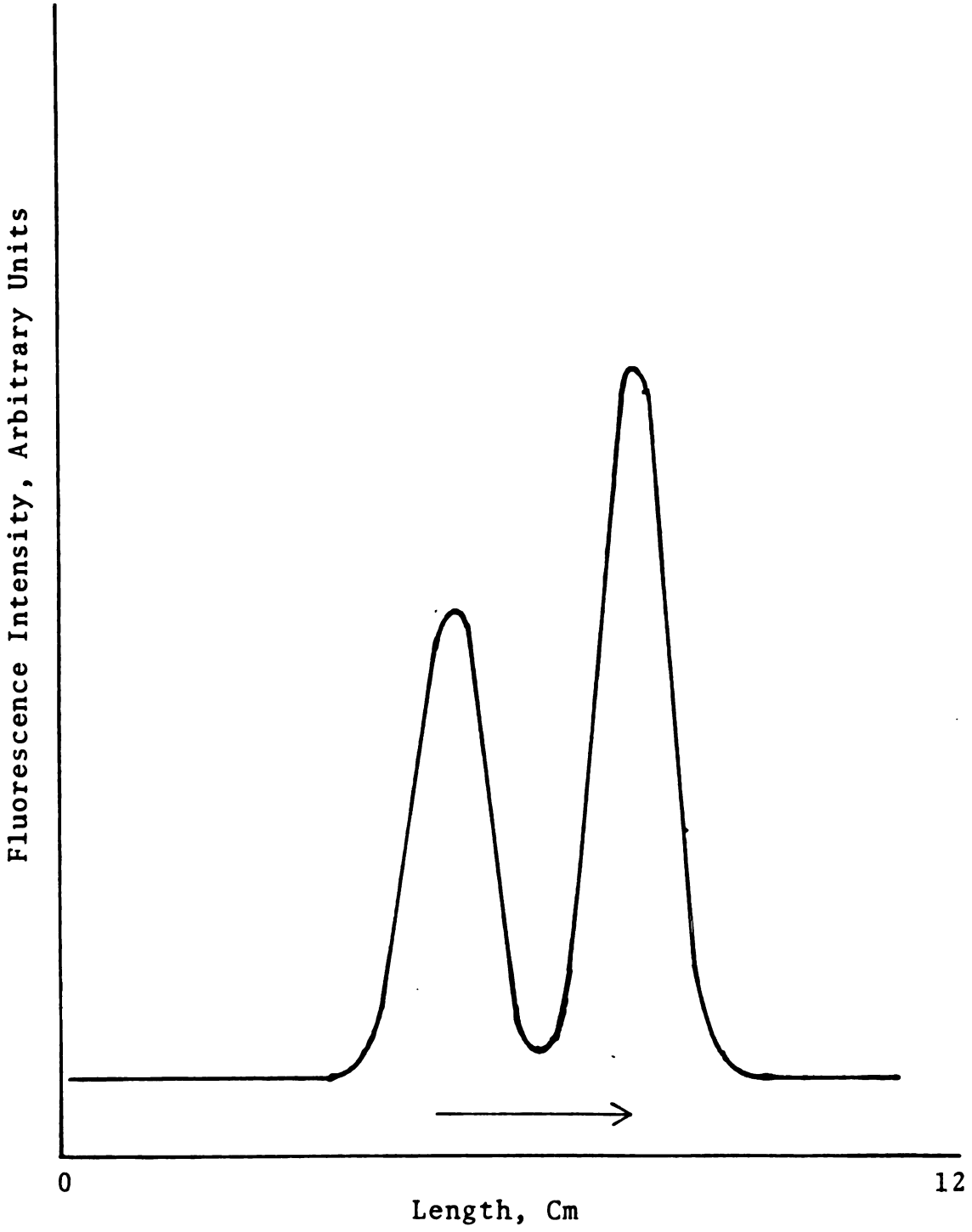


Figure 32. Fluorescence scan of TLC separated fluorescein product of a mixture of methylsuccinic acid and 2,2 dimethylsuccinic acid. [ITLC-SG paper; 80:21.5:3.6 chloroform-methanol-ammonia developer.]

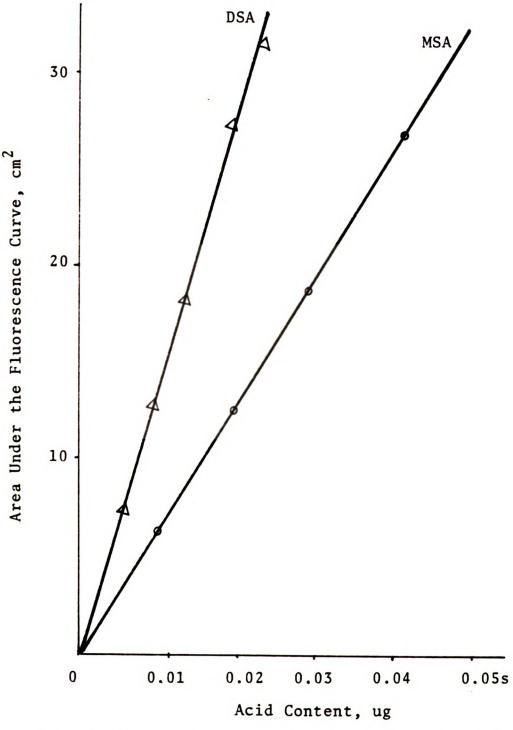


Figure 33. Calibration curve of a reaction product of a mixture of methylsuccinic acid and 2,2 dimethylsuccinic acid with resorcinol.

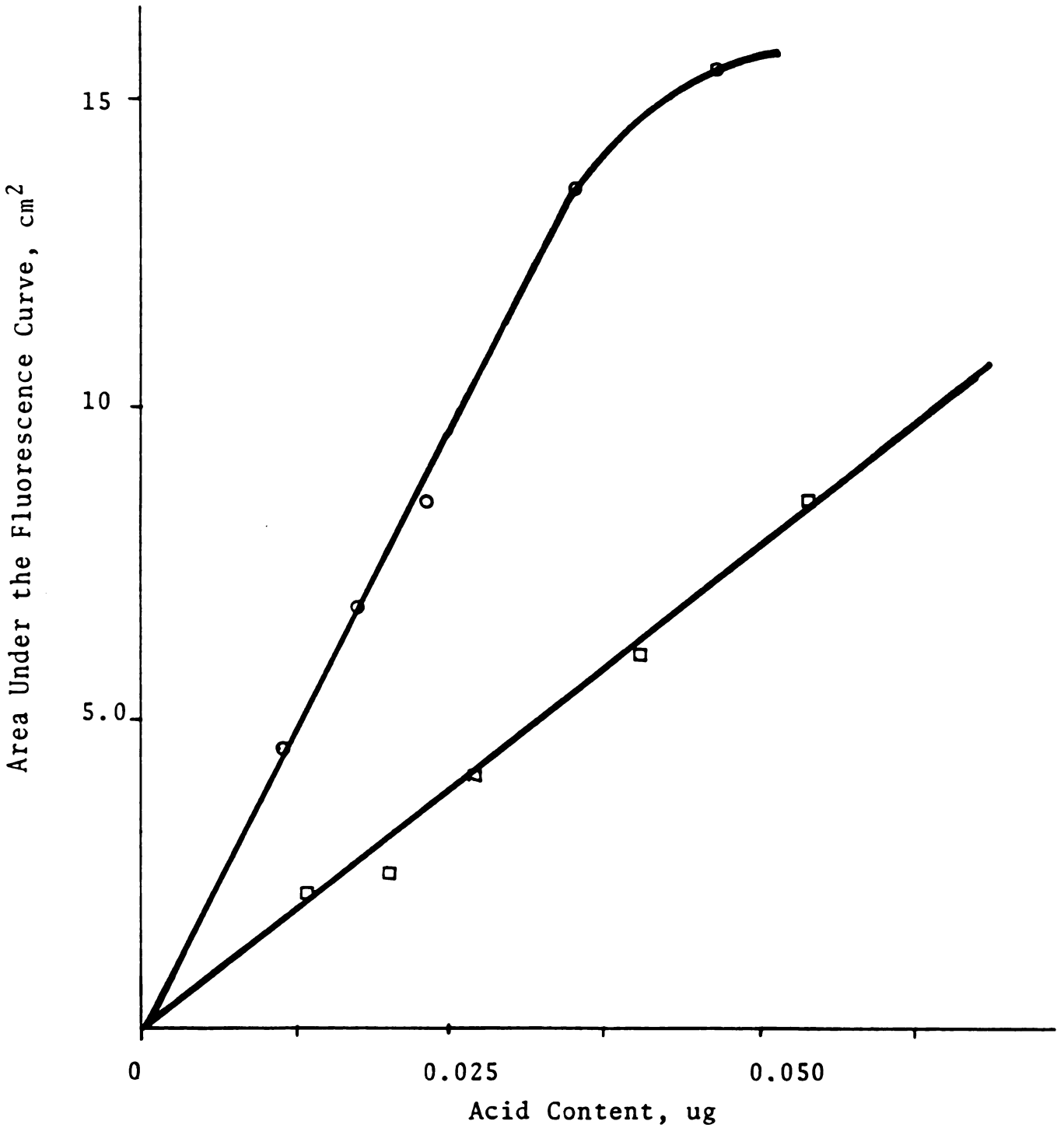


Figure 34. Calibration curve for the reaction products of succinic acid and methylsuccinic acid mixtures and resorcinol.

the preparation of the chromatograms for measurement are also greatly influenced by humidity. Duplicating the conditions used in the preparation of the calibration curve is difficult or impossible. Using a standard prepared along with the samples, subjected both samples and standards to the same conditions.

4. Analysis of a Mixture of Succinic Acid (SA), Methylsuccinic Acid (MSA) and 2,2 Dimethylsuccinic Acid (DSA)

A mixture, containing 0.00804 ug of succinic acid, 0.00824 ug of methylsuccinic acid and 0.007528 ug of 2,2-dimethylsuccinic acid per ul of spotting solution, was used as the standard. Samples of mixtures of the above acids were prepared. The standard samples and sample mixtures were subjected to identical reaction and separation conditions. Areas of the fluorescence peaks for the separated components in standard and sample mixtures were measured. The concentration of each acid in a sample mixture was calculated with the aid of the following simple expression,

$$\text{Acid content of sample, ug/ul/spot} = \frac{\text{area of acid peak of sample}}{\text{area of corresponding peak of standard}} \times \text{acid content of standard, ug/ul/spot}$$

Results obtained from the analyses of several mixtures when the above standard was used are given in Table 17.

It is observed that the percent average relative error, when a standard was used that contained the same components as the unknown sample, was very much less than when the unknown contained less components. It was also noted that this percent relative error was even larger when the components have large polarity differences so that spots were separated from one another unlike those in the standard. From examination of the chromatograms, with UV-Visible light, the large relative error occurred when the spots were not aligned at the center of the strip and when the spots were large.

Table 17. Analytical Results for Acid Mixtures.

Sample Mixture		Acid Used ug/ul	Acid Found, ug/ul*				% Average Rel. Error
			Batch 1	Batch 2	Batch 3	Batch 4	
A	SA	0.01087	0.01288	0.01106	0.01363	0.01218	14.4
	DSA	0.01112	0.01389	0.01122	0.01363	0.01438	19.4
B	SA	0.01162	0.01143	0.00895	0.01213	0.00989	8.8
	MSA	0.00890	0.00899	0.00831	0.00904	0.01024	2.8
C	SA	0.01208	0.01295	0.01224	0.01274	0.01100	1.3
	MSA	0.01066	0.01009	0.01121	0.01035	0.00850	5.8
	DSA	0.01034	0.01155	0.00967	0.01242	0.00903	3.2
D	SA	0.01208	0.01219	0.01141	0.01165	0.01169	2.9
	MSA	0.01066	0.00999	0.01018	0.01055	0.00983	4.9
	DSA	0.01034	-------	0.01055	0.01054	0.01031	1.2
E	MSA	0.00874	0.00952	0.01079	0.00945	0.00788	7.7
	DSA	0.01003	-------	0.00875	0.00979	0.00903	8.4

*Batches 1 and 2 were obtained from the same reaction mixture but spots were developed separately, likewise, batches 3 and 4 were from the same reaction mixture. Reaction temperature for the mixture in batches 1 and 2 was 130-140° for 2 hrs, while for batches 3 and 4, it was 125-130° for 2 hrs.

E. SUMMARY AND CONCLUSION

The reactions of resorcinol with succinic acid, methylsuccinic acid, 2,2 dimethylsuccinic acid, suberic acid, adipic acid, pimelic acid and o-phthalic acid to form their fluoresceins were investigated. The condensing catalysts were polyphosphoric acid, p-toluenesulfonic acid and Dowex 50W x 12 in addition to the most commonly used condensing catalyst, sulfuric acid. From a comparison of the physical properties, mass spectra, proton and carbon-13 NMR, absorption and emission behavior and elemental carbon analysis of the fluorescein product of succinic acid, 2,2 dimethylsuccinic acid and suberic acid obtained from the use of the different condensing catalysts, it was concluded that all catalysts were effective in the resorcinol-acid reaction to produce similar products.

When polyphosphoric acid was used for a reaction mixture of 1:10 or greater, acid to resorcinol, it catalyzed the reaction to a level of constant yield or to completion with about 100% yield when the reaction temperature was maintained in the range of 120 to 140° for about 2 hours. It was also observed that this catalyst was adequate for the formation of fluoresceins in reactions between resorcinol and succinic acid, methylsuccinic acid, 2,2-dimethylsuccinic acid, suberic acid and o-phthalic acid

for use in the determination of these acids by fluorimetry, when these acids are present singly or as mixtures in samples. The blank fluorescence which resulted from the reaction of the excess or the unreacted resorcinol, was negligible when the reaction mixture was appropriately diluted. When a large excess of resorcinol was used, a resorcinol blank fluorescence correction could be evaluated and applied to the sample fluorescence. The blank fluorescence did not constitute an interference when the analysis was carried out by the TLC-fluorescence method because during the process of separation by TLC, the fluorescing species was washed up the TLC paper with the solvent and did not give any fluorescence peaks in the spectral region of the fluoresceins.

The analyses by direct measurement of the fluorescence of the fluorescein solutions prepared from the reaction mixture, was feasible only when other dicarboxylic acids were not present since their fluorescing species all absorbed and emitted in the same spectral region $\lambda_{ex,max}$ = 484-486 nm and $\lambda_{em,max}$ = 508-510 nm. When present singly in a sample, analysis by this method was found to work well even for very low amounts of succinic and the substituted succinic acids down to less than 5 ug. A standard sample of the acid was processed along with the regular sample for comparison of fluorescence intensities.

When mixtures of dicarboxylic acids are present in a

sample, the TLC-fluorescence method of analysis can be applied. The fluoresceins were separated on TLC paper with chloroform-methanol mixtures as developer in an environment of low relative humidity. The fluorescing species formed in the reaction of the excess or unreacted resorcinol, was washed up the TLC paper with the solvent front. For mixtures of succinic acid, methylsuccinic c acid and 2,2 dimethylsuccinic acid fluoresceins, the areas under the fluorescence peaks obtained from the fluorescence scans of their TLC chromatograms, were linear with concentration. Likewise, mixtures of the fluoresceins of succinic acid, 2,2 dimethylsuccinic acid, suberic acid and o-phthalic acid gave areas under their fluorescence peaks obtained from the fluorescence scans of their chromatograms, which were linear with concentration. This method is simple, inexpensive and capable of handling large numbers of samples containing several components.

The method is not without flaws. In the case of the analyses of succinic acid, 2,2 dimethylsuccinic acid and methylsuccinic acid, the TLC paper tested, developer and humidity affect the separation of their fluoresceins. Furthermore, the position of the spots relative to the slit of the TLC scanning fluorimeter used during the fluorescence scan, and the size of the spot were critical factors which could lead to erroneous results.

Further work in the following suggested areas would

improve precision and accuracy of the method and minimize some of the difficulties encountered in the use of the method as well as expand the applicability of the method to analysis of real biological samples.

1. Further study on the stoichiometry of the resorcinol-acid reaction concerning other dicarboxylic acids, polycarboxylic acids and polyfunctional dicarboxylic acids, especially those present in real biological samples, using PPA as the condensing catalyst.

2. Establish a list of compounds which interfere with the test when performed on real biological samples without prior separation of the other components.

3. Evaluate different TLC papers and developing solvents for possible use in TLC-fluorescence analyses.

4. Improve the instrumentation of the TLC scanning fluorimeter so that the variation in peak areas caused by the geometry variation of the spot relative to the excitation and detection slits is minimized or eliminated so that the area under the fluorescence peaks truly represent the concentration of the acid analyzed, regardless of whether the developed spot is exactly at the center of the line of development or at the center of the strip or shifted somewhat from the center.

5. Incorporation into the TLC scanning fluorimeter of an automatic integrator to eliminate the error and the tedium determining the peak areas with a planimeter.

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