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Chelating derivatives of dimethyldihydroxyfluorans

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CHELATING DERIVATIVES OF DIMETHYLDIHYDROXYFLUORANS

by

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Head of Major Department

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Dean of Graduate College

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I. INTRODUCTION

A metallochromic or metallofluorochromic indicator is an organic compound which changes color or fluorescence upon reaction with a metal ion. The first of these indicators was Eriochrome Black T, an azo compound, used by Biedermann and Schwarzenbach (4) as indicator in the titration of calcium plus magnesium with disodium ethylenediaminetetraacetate (EDTA). The unique feature of this indicator is that magnesium, one of the elements being titrated, participates with the indicator in the color change at the end point. Eriochrome Black T itself is colored blue at pH 10 but unites with magnesium at this pH to form a red, slightly-dissociated compound. At the end-point, the first excess of EDTA, the titrating agent, pulls the magnesium away from the indicator, causing the color to change from red to blue and clearly marking the end-point of the titration.

The necessary and sufficient conditions for an azo compound to unite with magnesium and with calcium were established by Diehl and Ellingboe (6). Other dihydroxyazo compounds have been proposed as metallochromic indicators, in particular one designated Calmagite synthesized and popularized by Diehl and Lindstrom (7) as a stable replacement for Eriochrome Black T.

The second big advance in the field of metallochromic indicators was also made by Schwarzenbach who introduced into phenolphthalein type indicators the chelating group methyleneiminodiacetic acid (Schwarzenbach and coworkers) (13, 14, 1). This principle and the same chelating group was used by Diehl and Ellingboe (5) in the

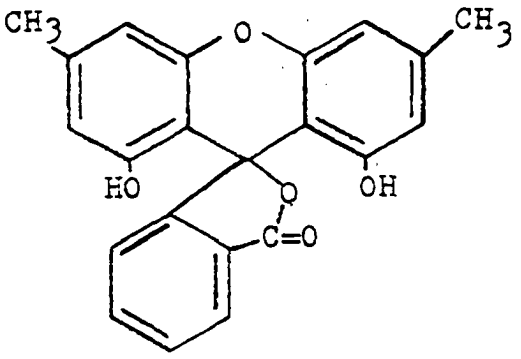
synthesis of a metallochromic indicator which also exhibited fluorescence. The methyleneiminodiacetic acid group was introduced into the fluorescent molecule fluorescein and the latter, itself an acid-base indicator, converted into a chelating molecule responsive to calcium and magnesium. This indicator, fluorescein bismethyleneiminodiacetic acid, the first metallofluorochromic indicator, was given the common name Calcein. It has found extensive use in the determination of calcium in the presence of magnesium in all manner of natural and manufactured products. The determination of calcium is carried out by titration at high pH, 12.5 to 13, with EDTA; at this pH magnesium is precipitated as magnesium hydroxide and does not interfere. The calcium derivative of Calcein exhibites a yellowish green fluorescence which disappears at the end-point in the EDTA titration.

The composition, structure and properties of Calcein were the subject of the Ph. D. thesis of A. J. Hefley (8) who showed that two methyleneiminodiacetic groups are present in the molecule and that one and then a second atom of calcium entered into combination with the molecule. Nuclear magnetic resonance studies of Calcein by Hefley made it likely that the two methyleneiminodiacetic acid groups occupy positions 4' and 5' in the molecule of fluorescein but the evidence was not completely unequivocal.

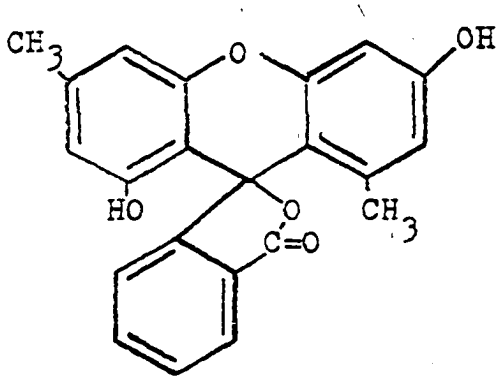
The present investigation is concerned with analogs of Calcein derived from the three possible dimethylfluoresceins and was undertaken to assist in establishing the structure of Calcein, to determine the

effect of substituent groups on the union of such chelating reagents with calcium and magnesium, and to confirm and extend some chemistry in the field of the oxygen heterocyclic, the fluorans.

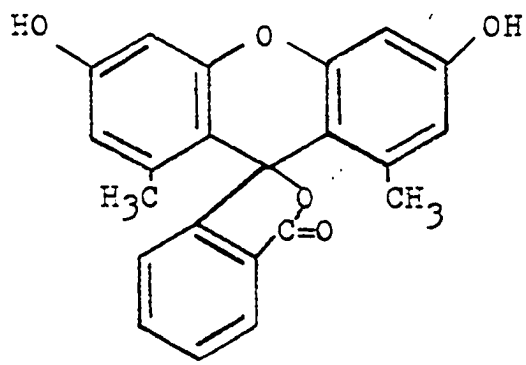
Many other fluorescent acid-base indicators exist. Orndorff and Allen (12) reported the synthesis of three dimethyldihydroxyfluorans. The three isomers (alpha-, beta- and gamma-dimethyldihydroxyfluoran) were synthesized by the condensation of orcinol and phthalic anhydride.



alpha-dimethyldihydroxy-fluoran



beta-dimethyldihydroxy-fluoran

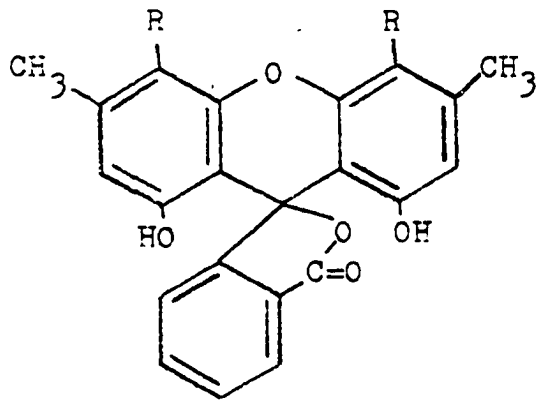


gamma-dimethyldihydroxy-fluoran

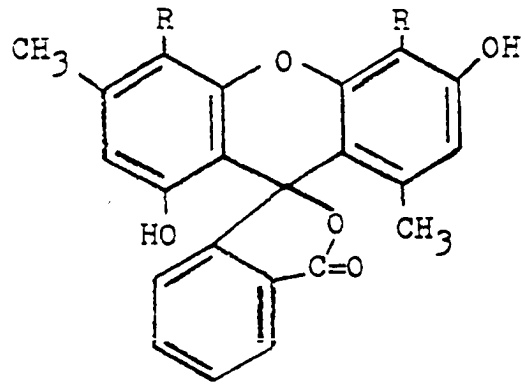
In the present work, the three dimethyldihydroxyfluorans were prepared, separated and purified by a new method, and characterized. The purity, composition, structure and properties of each of these isomers have been established.

The methyleneiminodiacetic acid derivatives of each of the three dimethyldihydroxyfluorans have been prepared, purified and studied as metallofluorochromic indicators. The methyleneiminodiacetic acid groups have been introduced into the parent fluoran by condensation with formaldehyde and iminodiacetic acid. The purity, composition, structure and properties of each of the derivatives have been established.

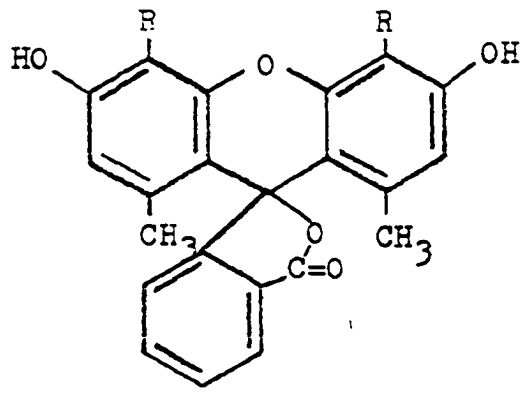
The methyleneiminodiacetic acid group has been introduced into the parent fluoran at positions 4' and 5' in the orcinol ring.



alpha-dimethyldihydroxy-fluoranmethyleneimino-diacetic acid



beta-dimethyldihydroxy-fluoranmethyleneimino-diacetic acid



gamma-dimethyldihydroxy-fluoranmethyleneimino-diacetic acid

Thus the three methyleneiminodiacetic acid derivatives are 1', 8'-dihydroxy-3', 6'-dimethyl-4' 5'-bis /N, N'-di(carboxymethyl)-aminomethyl/fluoran, 3', 8'-dihydroxy-1', 6'-dimethyl-4', 5'-bis /N, N'-di(carboxymethyl)-aminomethyl/fluoran, and 3', 6'-dihydroxy-1', 8'-dimethyl-4', 5'-bis /N, N'-di(carboxymethyl)-aminomethyl/fluoran.

II. THE DIMETHYLDIHYDROXYFLUORANS

A. Experimental Work

1. Synthesis

a. Apparatus and reagents Infrared spectra were obtained with a Perkin-Elmer Model 21 Infra-Red Spectrophotometer.

Nuclear magnetic resonance spectra were obtained with a Varian Associates A-60 Nuclear Magnetic Resonance Spectrometer.

Mass spectra were obtained with an Atlas CH4 Mass Spectrometer.

Clear, precision ground glass NMR tubes of 0.5 cm. o.d. were used for all NMR work.

Fuming sulfuric acid (20-23 per cent), reagent grade, was obtained from the Baker Chemical Company.

Phthalic anhydride, analytical reagent grade, was obtained from the Mallinckrodt Chemical Works.

Orcinol monohydrate (1, 3-dihydroxy-5-methylbenzene monohydrate), an off-white powder, was obtained from the Eastern Chemical Corporation, Pequannock, New Jersey.

All water used was distilled and deionized by passage through Amberlite MB-1 ion exchange resin.

All reagent chemicals were of reagent grade quality.

b. Analysis of orcinol. The analysis used to ascertain purity and composition of the commercial orcinol were:

1. Loss on heating
2. Melting point
3. Infrared spectroscopy

4. Nuclear magnetic resonance

5. Mass spectroscopy

1. Loss on heating A known weight of orcinol hydrate was heated for 12 hours at 50° C.

2. Melting point The commercial material melted sharply at 54° C. Orcinol that had been heated for two hours at 90-100° C. gave a sharp melting point of 113° C.

3. Infrared spectroscopy Infrared spectra were obtained on potassium bromide pellets of orcinol. Spectra were run on three samples. The first spectra was of the commercial material as obtained. The second spectra was of material previously heated for two hours at 100° C. The third spectra was of this anhydrous material after manual addition of water to the pellet.

4. Nuclear magnetic resonance A solution of orcinol in chloroform with a trace of DMSO, to enhance solubility, was used to obtain the spectra. TMS was added as the reference standard. The anhydrous orcinol was used.

5. Mass spectroscopy Mass spectra were obtained at an electron beam energy of 70 electron volts. The instrument scanned to 190 mass units, but found 124 the highest recorded peak.

c. Condensation of orcinol and phthalic anhydride To 0.135 mole (20.0 grams) of phthalic anhydride was added 0.270 mole (38.3 grams) of orcinol monohydrate with stirring. The mixture was fused for thirty minutes at 100°, whereupon the white solid mass became a light yellow liquid. This was followed by the dropwise addition of 20 ml. of fuming sulfuric acid with continued stirring.

Within a few minutes the reaction became very exothermic and a dark red almost black viscous liquid was formed. The reaction was allowed to proceed at 90^{o} to 100^{o} for two hours. The viscous liquid was cautiously poured into 1 M sodium hydroxide solution. The material adhering to the reaction flask was dissolved by several additions of sodium hydroxide solution. The basic solution was dark red almost black. The reaction products were precipitated by the dropwise addition of 1:1 hydrochloric acid with vigorous stirring. Acid was added until the solution measured pH 1. The solution and precipitate were bright yellow-orange. The mixture was allowed to stand for twenty-four hours, then filtered and the solid air dried for twenty-four hours. The solid was then dried under vacuum at 60^{o} for twenty-four hours and subsequently stored in a desiccator over magnesium perchlorate.

2. Separation and purification

a. Method of Orndorff and Allen The first method attempted was that of Orndorff and Allen (12). This method, carried out in pre-pH times, was apparently based on the acidic behavior of the isomers. The isomers were extracted from diethyl ether solutions into 1 M sodium carbonate solutions.

The semi-dried mixture of isomers was extracted into diethyl ether. Material that did not extract into the ether was discarded. The ether solution of the three isomers was extracted with 1 M aqueous sodium carbonate solution. All of gamma-dimethyldihydroxyfluoran and some of the beta-dimethyldihydroxyfluoran were extracted into the

aqueous layer. All of alpha-dimethyldihydroxyfluoran and most of beta-dimethyldihydroxyfluoran remained in the ether layer. The ether layer containing the two isomers was then extracted with 1 M sodium carbonate solution. The beta-dimethyldihydroxyfluoran was extracted into the aqueous layer, the alpha-dimethyldihydroxyfluoran remained in the ether layer. The crude extractions were further purified.

b. Solvent for extraction The reaction mixture containing the three dimethyldihydroxyfluorans as described on p. 8-9 was dissolved in an organic phase. Each of the three dimethyldihydroxy-fluorans was then selectively extracted from the organic phase into an aqueous phase.

The following characteristics of each possible organic solvent were noted:

1. solubility of the dimethyldihydroxyfluorans
2. miscibility with water
3. density
4. acid-base properties
5. toxicity, stability and ease of handling
6. boiling point
7. commercial availability and purity

The following possible solvents were investigated: methyl, ethyl, n-propyl, n-butyl and isopentyl alcohol; ethyl acetate; acetone, di-n-propyl and dimethyl ketone, cyclohexanone; di-ethyl and di-n-propyl ether, furan, tetrahydrofuran and 1, 4-dioxane; dimethylformamide; pyridine; acetonitrile, carbon disulfide, ethylene dichloride, dimethyl sulfoxide, carbon tetrachloride and chloroform; benzene, toluene,

m-xylene and chlorobenzene.

c. Separation as a function of pH

1) Apparatus and reagents A Beckman pH meter was used for pH measurements. A glass electrode was used as the indicating electrode and a saturated calomel electrode as the reference electrode.

Buffers in increments of 0.5 pH units were prepared covering the pH range seven to fourteen.

2) Procedure: determination of optimum pH The crude mixture of isomers was extracted into the organic phase, diethyl ether or methyl isobutyl ketone. The first extraction was from the organic phase with 100 ml. water. All subsequent extractions were made using the prepared buffers, 100 ml. buffer solution per extraction. The pH of each aqueous extraction was measured, adjusted if necessary with aqueous sodium hydroxide and the same aqueous extract recombined with the ether layer, the mixture shaken and the pH again measured. The process was repeated until the desired pH was reached. This required up to five additions of base per extraction. The pH was varied in 1 pH unit increments from pH 3 to pH 7 and in 0.3 to 0.5 pH unit increments from pH 7 to pH 14. The response of the glass electrode in the aqueous solution containing small amounts of dissolved organic solvent was very slow. Two minutes were allowed for stabilization prior to each pH reading. Each set of extractions was then acidified with dilute hydrochloric acid, diluted with water and allowed to stand overnight to assure complete precipitation. The material was filtered and then dried under a vacuum over magnesium.

perchlorate. The dried material was weighed and an NMR spectrum in D_2O-NaOD plus Tier's Salt run. The isomer extracted was determined by NMR analysis, color of the basic solution, fluorescence of the basic solution, and color of the acid precipitate. See Figure 1 .

3) Procedure: selective extraction of alpha-, beta- and gamma-dimethyldihydroxyfluoran Extractions from the organic phase were made with buffers at pH 7, 9, 11, and 13. Each set of extractions was heated to just below boiling for one hour to volatilize any organic solvent. Dilute (3 M) hydrochloric acid was added dropwise with stirring until the pH of each solution was 2. The solutions were digested for another hour at below boiling. The solution and precipitate were allowed to cool overnight, filtered and the solid vacuum dried for 24 hours. The solid was stored under vacuum over magnesium perchlorate.

3. Properties and structure

a. Apparatus and reagents Mass spectra were obtained using an Atlas CH4 Mass Spectrometer.

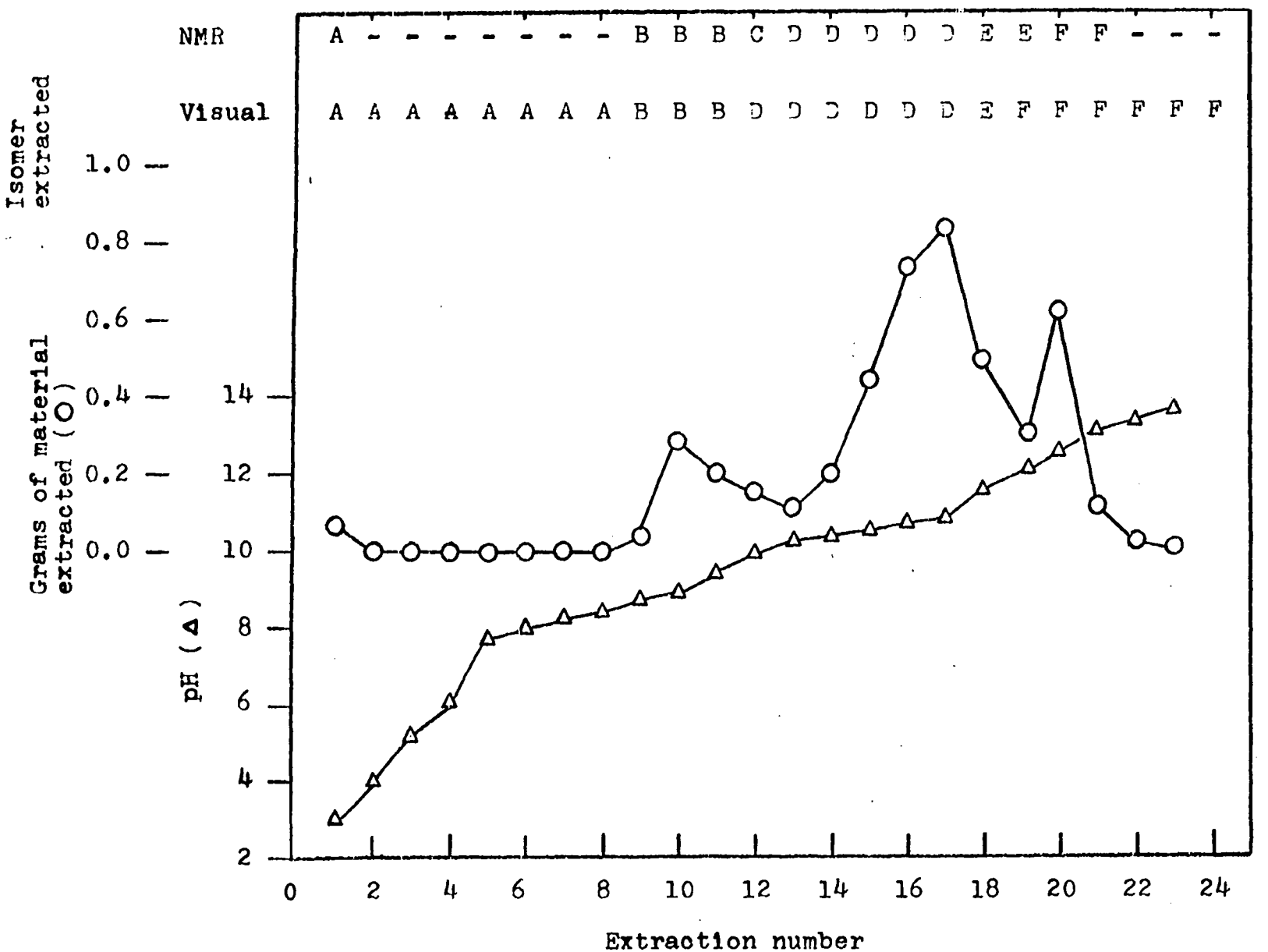
Infrared spectra were obtained using a Perkin-Elmer Model 21 Infrared Spectrophotometer.

Nuclear magnetic resonance spectra were obtained using a Varian Associates A-60 Nuclear Magnetic Resonance Spectrometer. Clear, precision ground glass NMR tubes of 0.5 cm. o.d. were used for all NMR work.

A Leeds and Northrup pH meter was used to monitor the non-aqueous and aqueous potentiometric titrations. The glass

Figure 1. Extraction number as a function of pH, weight of material extracted and isomer extracted as determined visually and by NMR analysis

A. Mixture, not identified
B. Pure gamma-dimethyldihydroxyfluoran
C. Gamma-dimethyldihydroxyfluoran and beta-dimethyldihydroxyfluoran
D. Pure beta-dimethyldihydroxylfuoran
E. Beta-dimethyldihydroxyfluoran and alpha-dimethyldihydroxyfluoran
F. Pure alpha-dimethyldihydroxyfluoran


NMR
A - - - - - - - - B B B C D D D D D E E F F - - -
Visual
A A A A A A A A B B B D D D D D D E F F F F F F
Isomer extracted
Grams of material extracted (O)
1.0
0.8
0.6
0.4
0.2
0.0
pH (Δ)
14
12
10
8
6
4
2
0 2 4 6 8 10 12 14 16 18 20 22 24
Extraction number

indicating electrode was a Corning silver/silver chloride internal reference, high alkalinity electrode, catalog #475024. A modified sleeve-type saturated calomel electrode was used as the reference electrode in the non-aqueous potentiometric titrations. A saturated solution of potassium chloride in methanol was used as the internal salt solution. A saturated calomel sleeve-type electrode was used as the reference electrode in the aqueous potentiometric titrations.

Ultraviolet and visible spectra were obtained using a Carey 14 Spectrophotometer. Perkin-Elmer quartz cells of 10.0 mm. internal path length were used.

b. Mass spectroscopy Mass spectra were obtained at an electron beam energy of 70 electron volts. The mass scale up to 450 mass units was scanned. The highest recorded peak for each isomer was 360 mass units.

c. Infrared spectroscopy Infrared spectra were obtained on potassium bromide pellets of alpha-, beta- and gamma-dimethyl-dihydroxyfluoran.

d. Nuclear magnetic resonance spectroscopy A solution of the isomer in either D_2O-NaOD plus Tier's Salt or in dimethyl sulfoxide plus TMS was used to obtain the spectra.

e. Melting point data Melting point determinations were made with a mercury thermometer by placing the sample between two cover glasses on the stage of a polarizing microscrope which was heated electrically at a constant rate.

f. Fluorescence The presence of fluorescence was qualitatively detected using radiation from a General Electric 15 watt

fluorescent lamp.

g. Elemental analysis Alpha-, beta- and gamma-dimethyldihydroxyfluoran were analyzed directly for carbon and hydrogen. Oxygen was determined by difference.

h. Residue on ignition The non-volatile metal content of alpha-, beta- and gamma-dimethyldihydroxyfluoran was determined by ignition. Samples of approximately 0.5 grams of each isomer were ignited in platinum crucibles at red heat with ample access to air and the weight of the residue determined.

i. Solubility in non-aqueous solvents The solubility of alpha-, beta- and gamma-dimethyldihydroxyfluoran was qualitatively determined in a number of solvents. The solubility was determined by adding a semimicro spatula tip of the isomer (approximately 0.1 grams) to 2 ml. of the solvent and shaking the mixture in a test tube. The mixture was allowed to stand for four hours and then the extent of dissolution was noted.

j. Titrations in non-aqueous solvents A known amount of isomer was dissolved in the appropriate organic solvent.

Alpha-, beta- and gamma-dimethyldihydroxyfluoran were dissolved in ethanol; alpha-dimethyldihydroxyfluoran was also dissolved in pyridine. The respective mixture was titrated potentiometrically. The titrant, sodium hydroxide, was in the same solvent as the isomer being titrated. Response of the pH meter was slow in the non-aqueous media. A thirty second time delay per addition of titrant was observed.

k. Titrations in water A saturated solution of alpha-, beta- and gamma-dimethyldihydroxy-fluoran was titrated potentiometrically

with standard sodium hydroxide. The titration was carried out on a micro scale.

l. Acid dissociation constants by solubility measurements The solubility of beta-dimethyldihydroxyfluoran over the pH range 6.08 to 8.00 was determined by buffering solutions containing an excess of compound at specific pH values and shaking for twelve hours to ensure complete equilibration. Appropriate volumes of the filtrates containing beta-dimethyldihydroxyfluoran were adjusted to pH 10.0, the pH of maximum absorbance, diluted to 100 ml. in volumetric flasks with 0.10 M potassium chloride, and shaken for twelve hours to ensure complete equilibration. The amount of compound in each solution was determined spectrophotometrically on a Cary 14 Spectrophotometer, the absorbance being measured at 430 nm. The absorbance of these solutions was related to concentration by using a calibration curve prepared by making absorbance measurements on standard solutions. The results are shown in Table 1 . A graph of solubility versus $1/[H^+]$ shown in Figure 2 , yielded the value for the intrinsic solubility of beta-dimethyldihydroxyfluoran. From the values of solubility at known pH and from the intrinsic solubility, the first acid dissociation constant of the phenolic group proton was determined for beta-dimethyldihydroxyfluoran.

m. Acid dissociation constants by spectrophotometric measurements The ultraviolet and visible absorbance spectra of alpha- and gamma-dimethyldihydroxyfluoran were obtained at intervals of 0.5 pH units over the pH range 4.0 to 10.0. Solutions on which the spectra were run were prepared by mixing 10.00 ml. of the isomer

Table 1. Solubility of beta-dimethyldihydroxyfluoran as a function of pH

Compound	pH	Solubility (mmoles/ml.)
beta-dimethyldihydroxyfluoran	8.00	14.90×10^{-6}
	7.50	7.53×10^{-6}
	7.00	5.64×10^{-6}
	6.08	4.63×10^{-6}

Figure 2. Change in solubility of beta-dimethyldihydroxyfluoran with pH

Solubility mg./100 ml.
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0
1
2
3
4
5
6
7
8
9
1/ [H+] x 10+2

stock solution, 50.0 ml. of the appropriate buffer solution, and diluting to 100.0 ml. with 0.10 M potassium chloride solution. The pH of each solution was measured after the spectra were obtained. The absorption spectra of alpha- and gamma-dimethyldihydroxyfluoran in the ultraviolet and in the visible wavelengths of light are shown in Figures 3, 4, 5 and 6. The absorbance of each solution was plotted versus the pH of that solution for both compounds. The graphs are shown in Figures 7 and 8a.

The first acid dissociation constant, that of the phenolic proton, of alpha- and gamma-dimethyldihydroxyfluoran was determined in the following manner. The absorbance of a solution containing the acid form of the compound, H_2A, of another solution containing the anion form of the compound, HA^-, and of six additional solutions having pH values numerically equal to the estimated pKa value 0.0, +0.2, +0.4, +0.5, -0.2, and -0.5 and thus containing both forms of the compound were measured on a Carey 14 Spectrophotometer. The wavelength range of 600 to 300 nm. was scanned. The log-ratio method was then used to determine the values of pKa.

B. Results and Discussion

1. Synthesis

a. Analysis of orcinol The orcinol used was a non-stock item and was therefore subjected to careful investigation to establish the purity.

1) Loss on heating A known weight of orcinol hydrate was heated at 50° for twelve hours and resulted in a loss of

Figure 3. Absorption spectra of 2.36×10^{-5} M alpha-dimethyldihydroxyfluoran

A. At pH 7.06
B. At pH 11.80

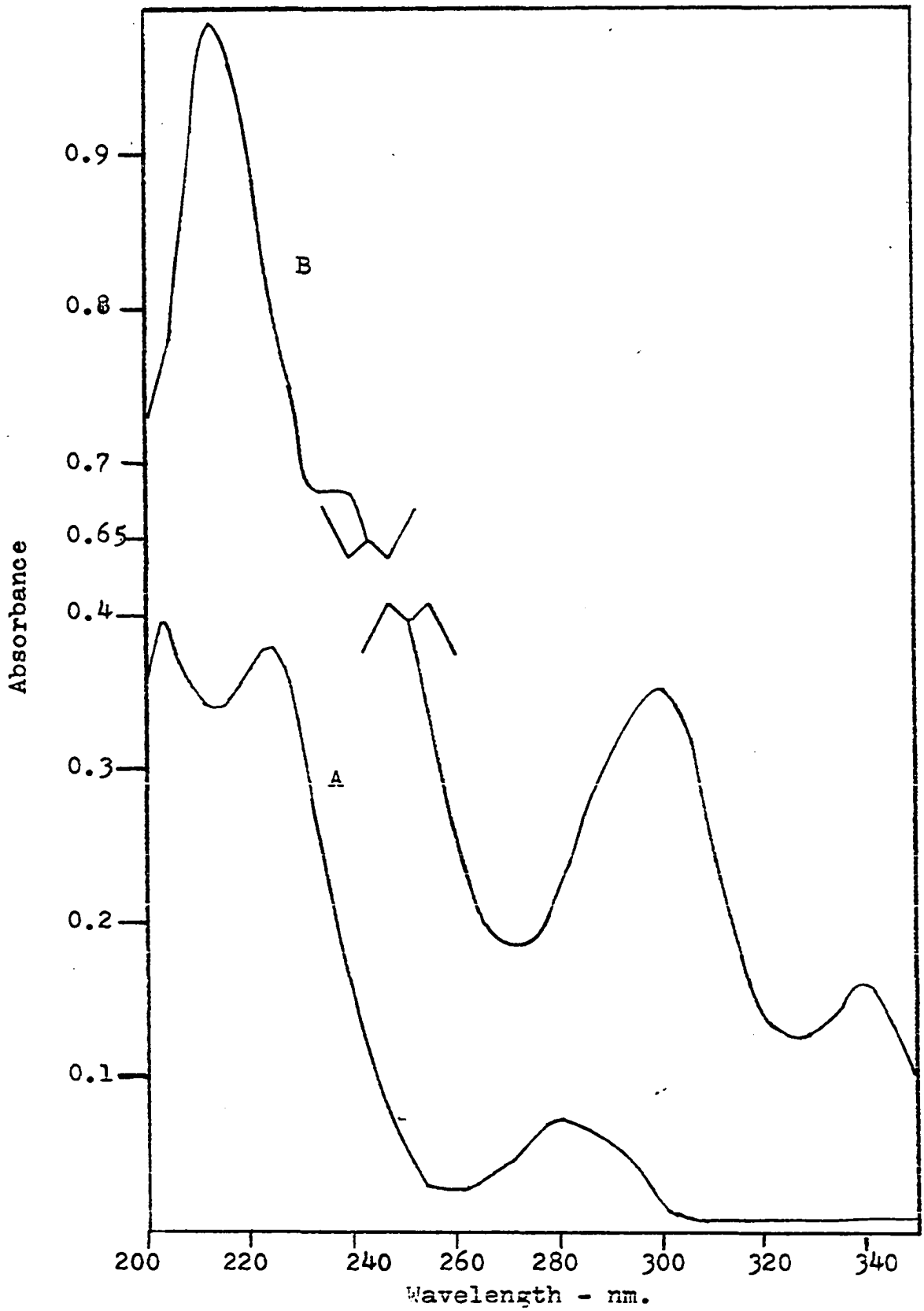

0.9
0.8
0.7
0.65
0.4
0.3
0.2
0.1
Absorbance
B
A
200
220
240
260
280
300
320
340
Wavelength - nm.

Figure 4. Absorption spectrum of 2.36×10^{-6} M alpha-dimethyldihydroxyfluoran at pH 11.39

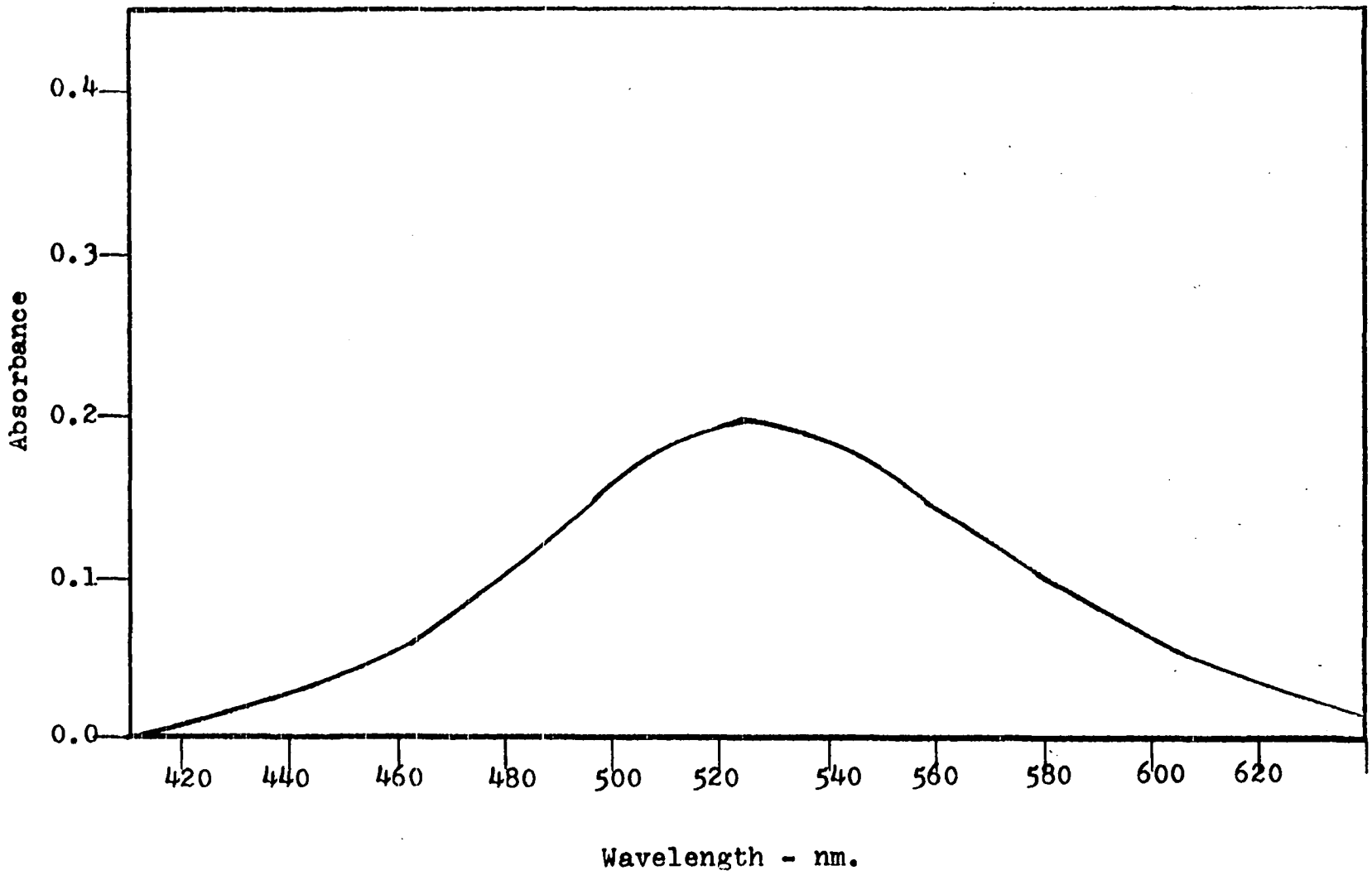

0.4
0.3
0.2
0.1
0.0
Absorbance
420
440
460
480
500
520
540
560
580
600
620
Wavelength - nm.

Figure 5. Absorption spectra of 2.20×10^{-5} M gamma-dimethyldihydroxyfluoran

A. At pH 10.00
B. At pH 6.10

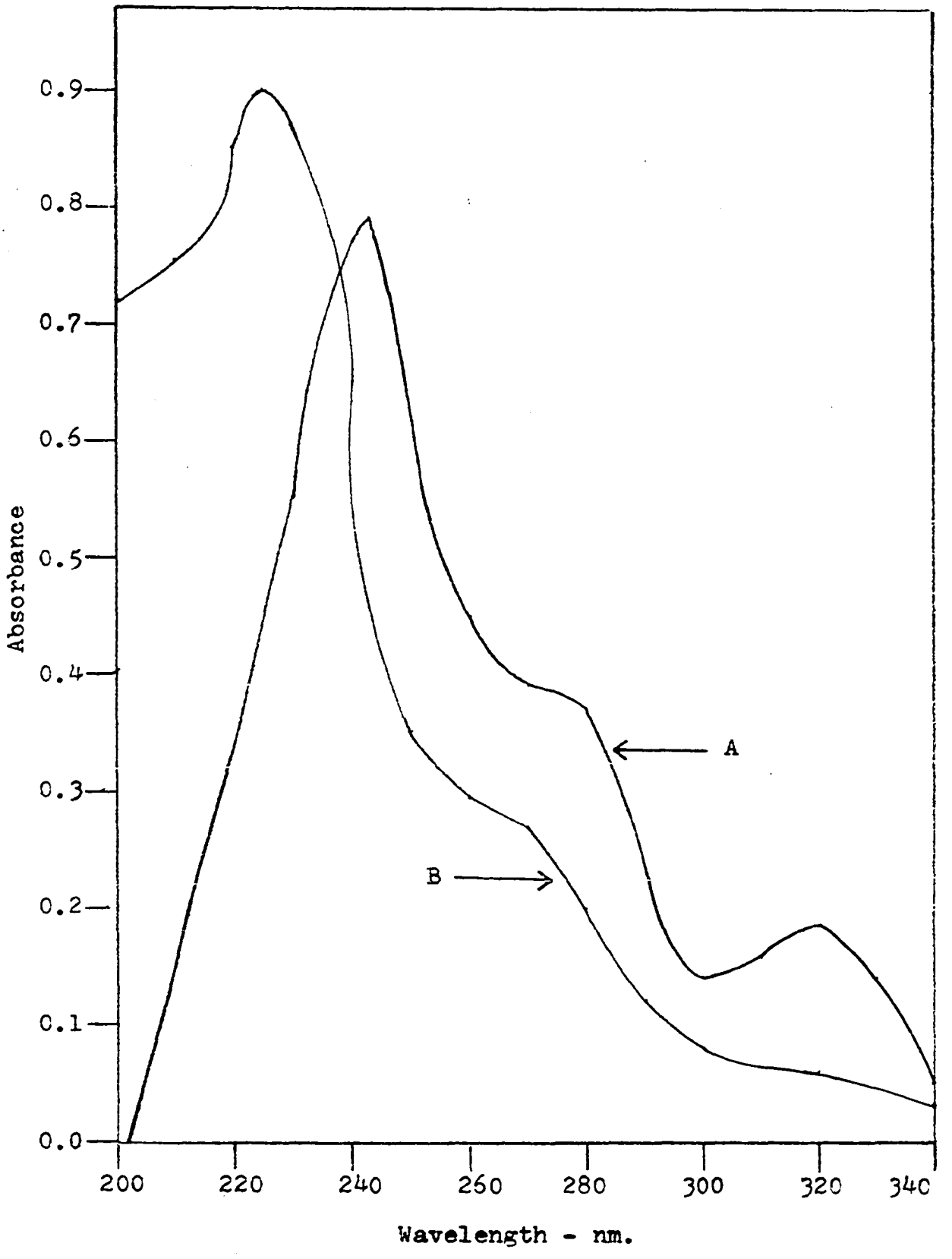

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
A
B
200
220
240
260
280
300
320
340
Wavelength - nm.

Figure 6. Absorption spectra of 2.20×10^{-5} M gamma-dimethyldihydroxyfluoran

A. At pH 10.00
B. At pH 6.10

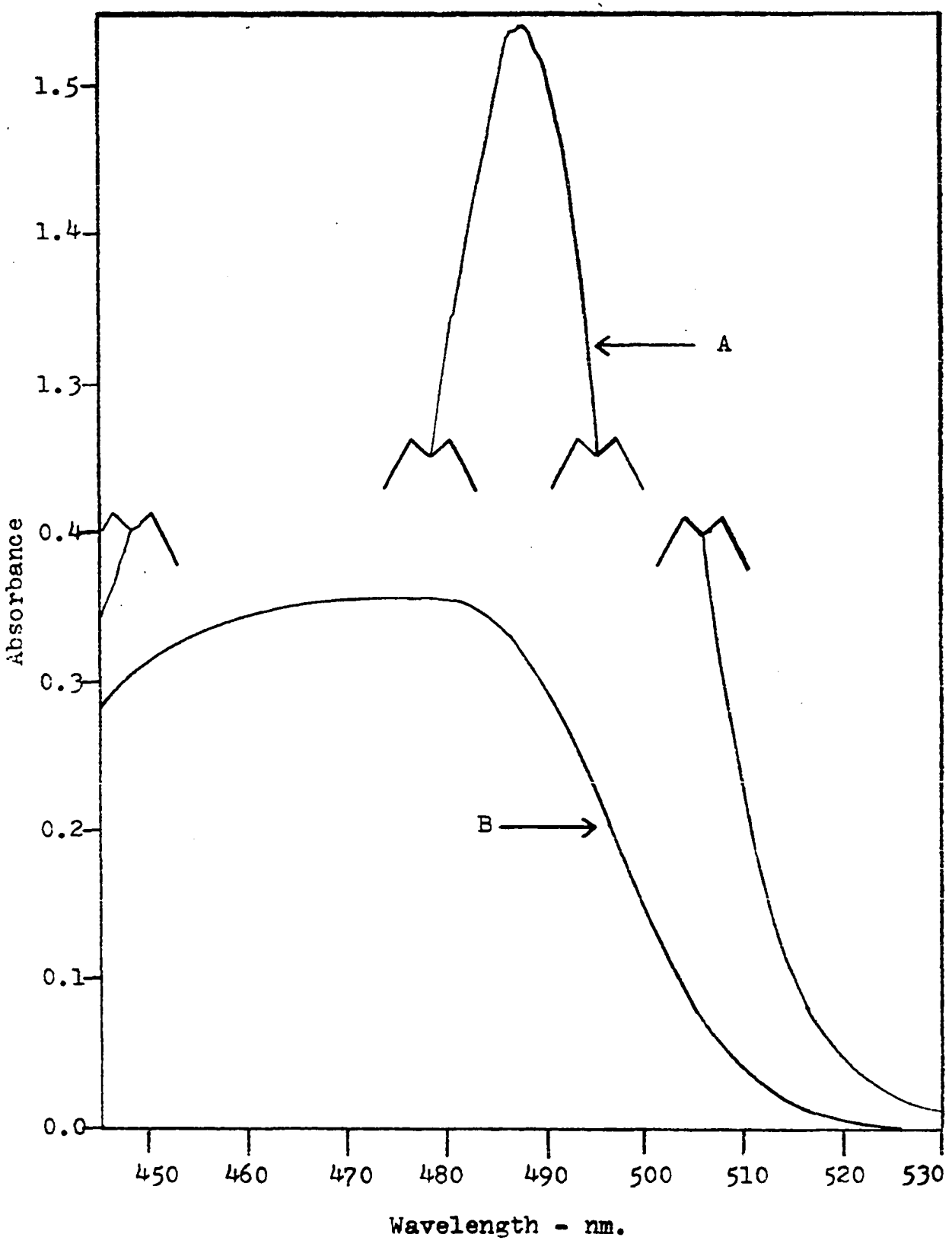

1.5
1.4
1.3
0.4
0.3
0.2
0.1
0.0
Absorbance
A
B
450
460
470
480
490
500
510
520
530
Wavelength - nm.

Figure 7. Change in absorbance of alpha-dimethyldihydroxyfluoran at 530 nm with pH

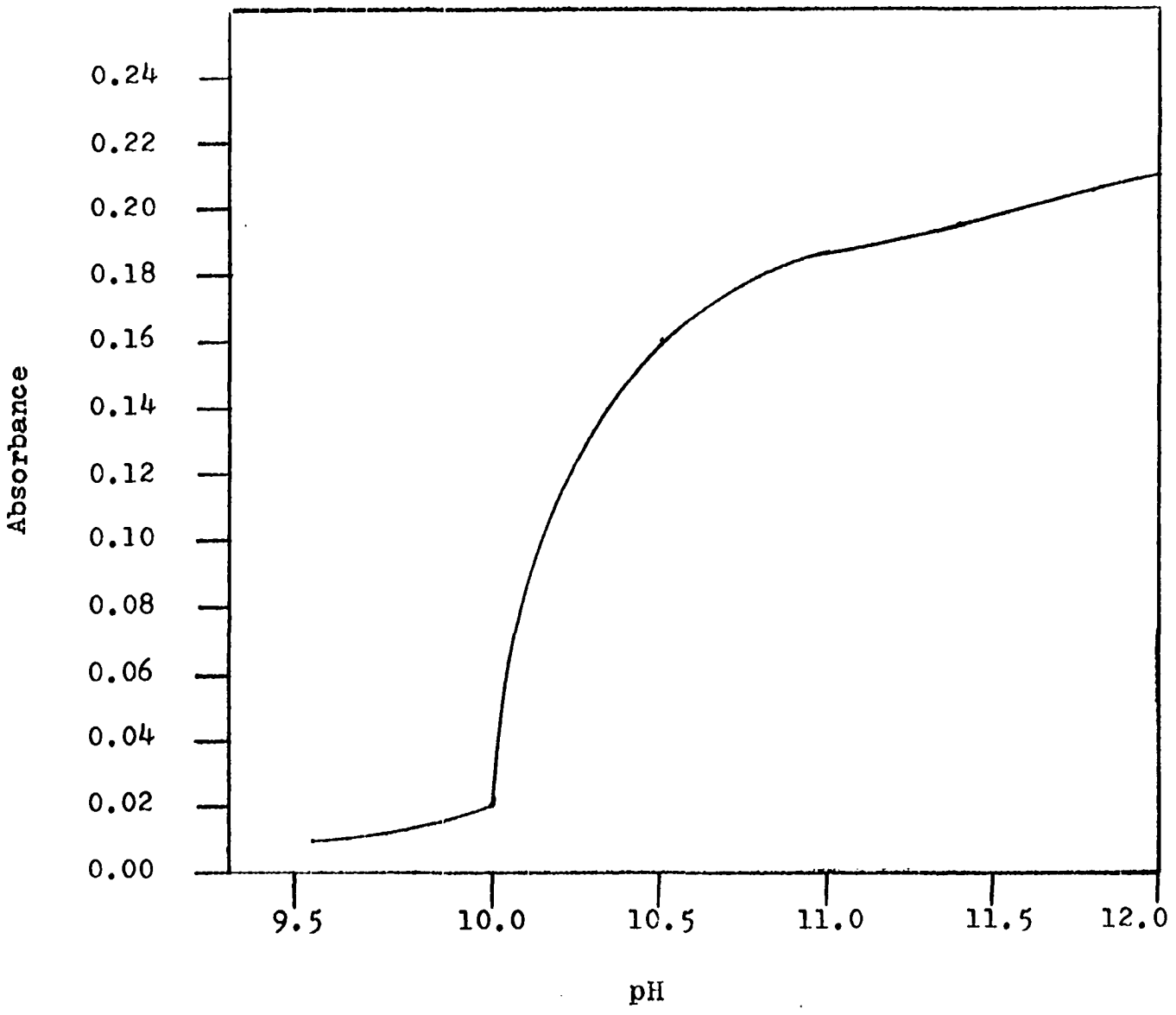
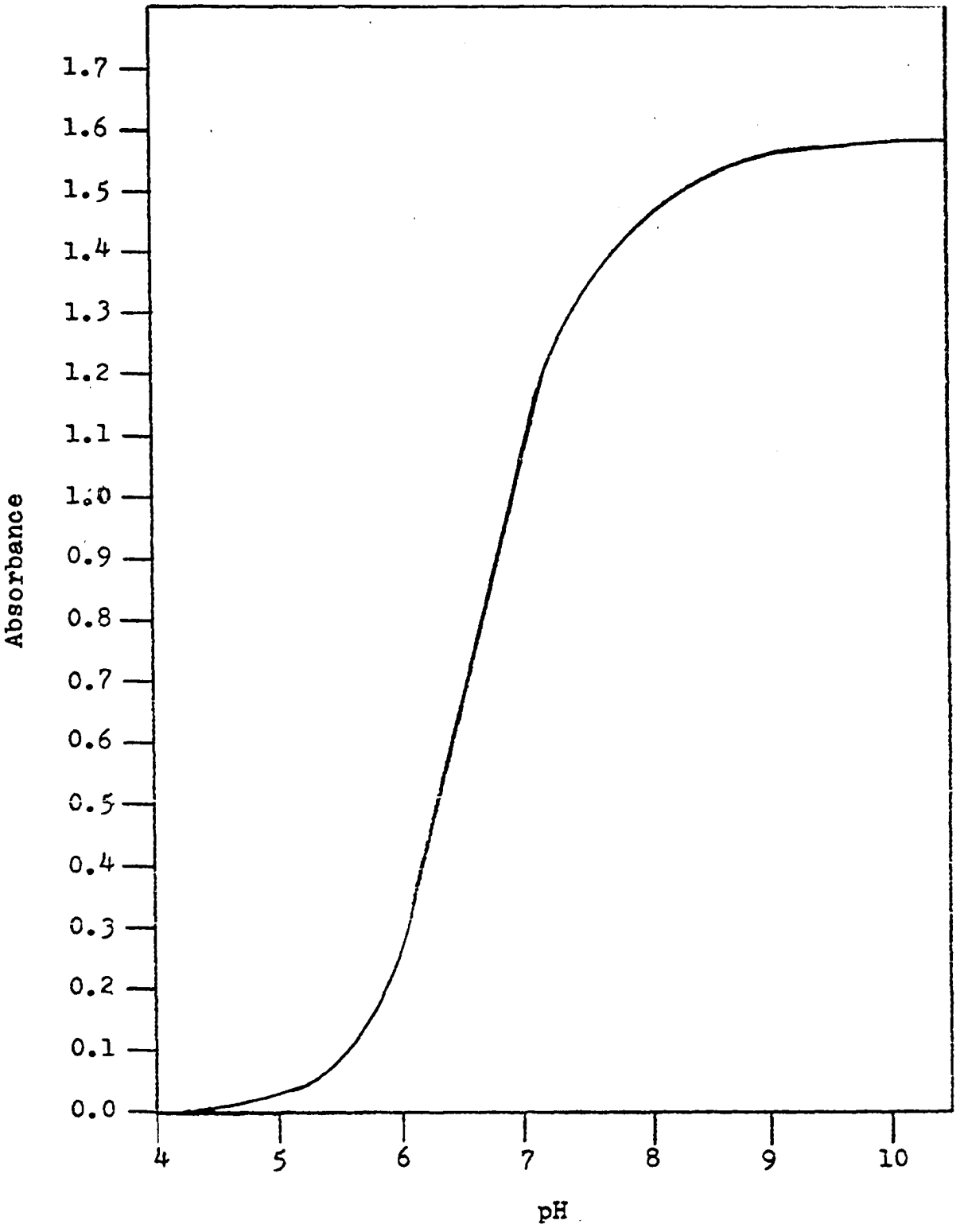

Absorbance
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
9.5
10.0
10.5
11.0
11.5
12.0
pH

Figure 8a. Change in absorbance of gamma-dimethyldihydroxyfluoran at 486 nm with pH


Absorbance
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
4
5
6
7
8
9
10
pH

1.0003 moles of water per mole of compound. No further weight loss was observed even after heating for several days. It was concluded that the commercial orcinol was a monohydrate.

2) Melting point The commercial material melted sharply at 54°; Orndorff and Allen (12) reported the melting point to be 58°. Orcinol that had been heated for two hours at 100° melted sharply at 113°. This latter material was considered anhydrous. Orndorff and Allen reported that the anhydrous material melted at 107°. It appears that the orcinol available to Orndorff and Allen was of lower purity than the material being used in this study.

3) Infrared spectroscopy All three infrared spectra were essentially identical. The broad peak at 2.8 to 3.2 microns was attributed to the hydroxide in orcinol and to any water present. The absorption band in the 6-7 and 11-15 micron region was indicative of 1, 3, 5-aromatic substitution. The hydroxide peak of the anhydrous material was not as broad as that observed of the hydrated material, as first obtained. On the addition of water to the anhydrous material, the hydroxide band was broadened.

The spectra obtained were in accord with the predicted spectra of orcinol. The spectra were indicative of a small amount of water of hydration in the commercial material, a negligible amount in the anhydrous material. The spectra contained sharp peaks consistent with high purity of sample.

4) Nuclear magnetic resonance

a) Operation The sweep generator swept the main magnetic field in the vicinity of 14,092 gauss, at which

energy the proton flipped from its low energy state to its high energy state. The sweep range was 1000 cycles per second. The methyl-silyl protons of either TMS (tetramethylsilane) or Tier's Salt (3-(timethylsily)-propane sulfonic acid sodium salt) set arbitrarily at 0.00 delta were used as an internal reference. The diffuse pattern of the CH_2 peaks of Tier's Salt barely showed on the base line. TMS could be used in any solvent but water in which it decomposes. Tier's Salt was used in aqueous media.

b) Preparation of sample Ideally, samples should be at least 1 M in concentration. This ideal situation rarely presented itself in the current work. Common NMR solvents included carbon tetrachloride, chloroform, acetone, benzene, dimethyl sulfoxide, acetic acid, acetonitrile and water. Unless the desired peaks are known to fall quite far (at least $\pm$ 1.5 ppm) from the solvent peaks, a deuterated solvent is necessary. The above solvents are obtainable in deuterated form. A minimum solvent depth of 30 mm. in the NMR tube must be used. About two milliliters of TMS vapor or about 10 mg. of Tier's Salt was added to each sample in a solvent, and the tube well shaken. The methyl-silyl peak was arbitrarily set at zero parts per million and all subsequent measurements were made in reference to this peak in delta values (ppm downfield from the reference).

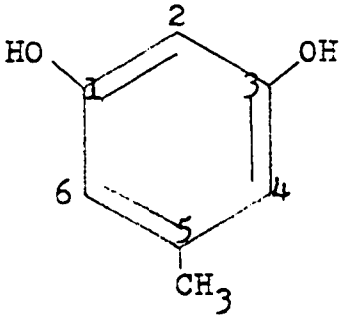
The following solvent systems were used in the current work:

1. D_2O-NaOD with Tier's Salt

2. d_6-Dimethyl sulfoxide with TMS

3. $CHCl_3$ with a trace of dimethyl sulfoxide with TMS

The following peaks were observed in the spectrum of orcinol in the $CHCl_3$ - DMSO - TMS solvent system. (Figure 8b.)



orcinol

The singlet at 2.19 delta integrating to three protons was attributed to the $-CH_3$ group protons. By comparison, the $-CH_3$ group protons of toluene neat appear at 2.32 delta (3).

The singlet at 2.62 was attributed to the $-CH_3$ group protons of dimethyl sulfoxide. By comparison, the $-CH_3$ group protons of dimethyl sulfoxide neat appear at 2.62 delta (3).

The singlet at 6.22 delta integrating to three protons was attributed to the 2, 4, 6 protons of the aromatic ring. The 6 - 8 delta region is characteristic of aromatic protons.

The singlet at 7.28 delta was attributed to the proton of $CHCl_3$.

Figure 8b. A proton magnetic resonance spectrum of orcinol in chloroform-dimethyl sulfoxide

A. Methyl hydrogen atoms (3)
B. Methyl hydrogen atoms of dimethyl sulfoxide

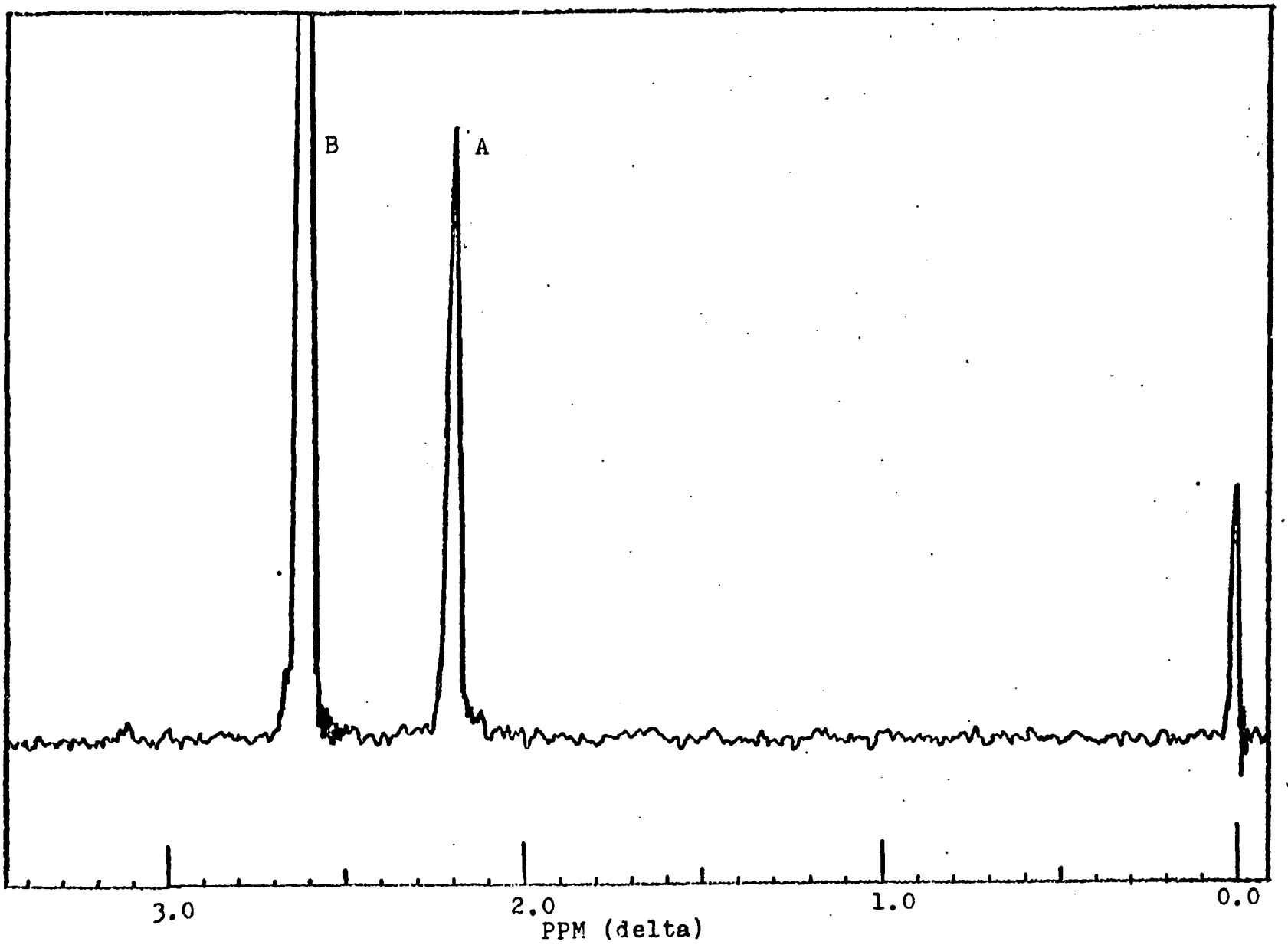
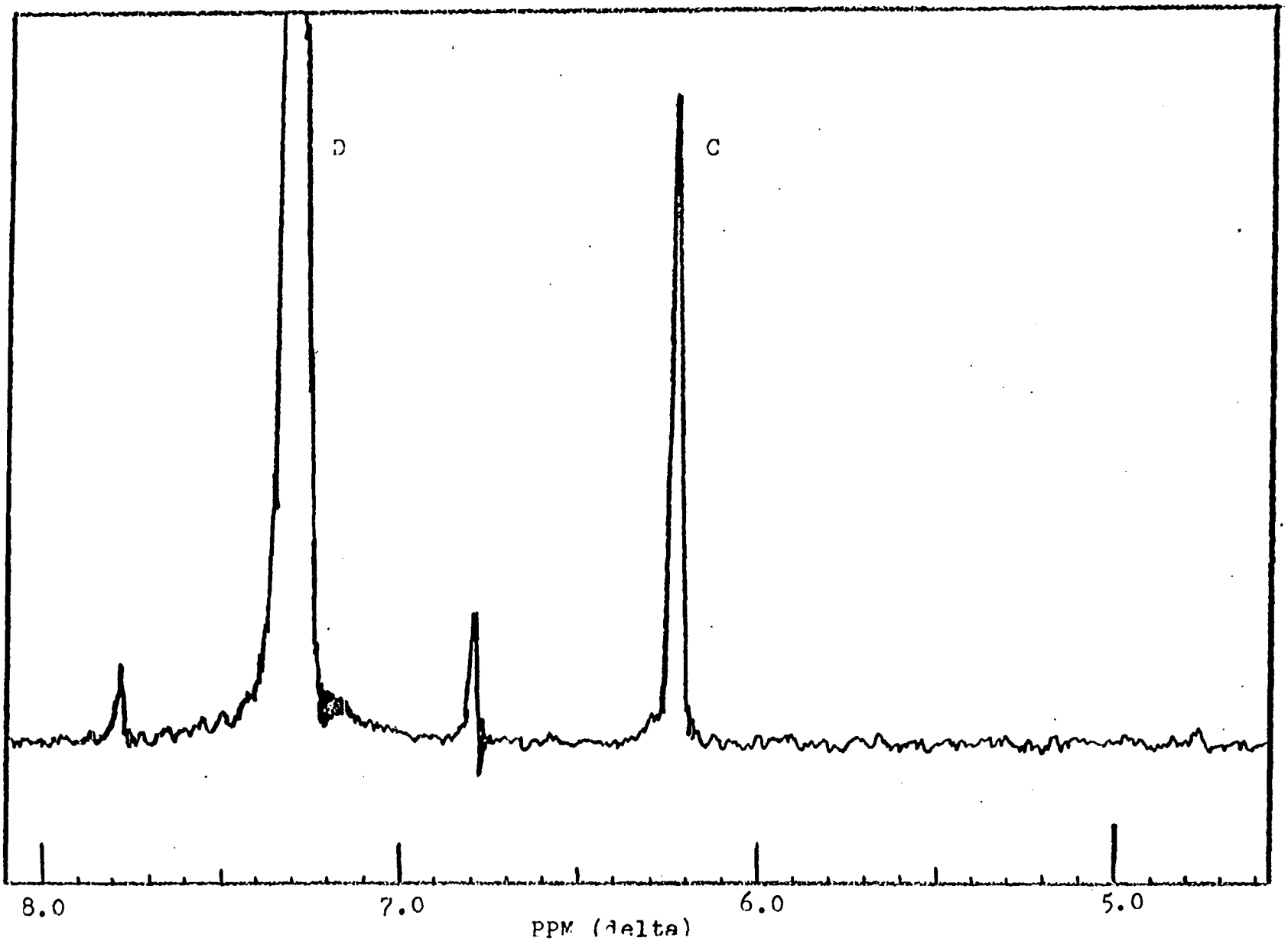

B
A
3.0
2.0
1.0
0.0
PPM (delta)

Figure 8b. (Continued)

C. Ring hydrogen atoms (3)
D. Hydrogen atoms of chloroform


D
C
8.0
7.0
6.0
5.0
PPM (delta)

By comparison, the proton of $CHCl_3$ neat appears at 7.27 delta (3).

The singlet at 9.03 delta integrating to two protons was attributed to the two -OH group protons. The -OH group proton appears in the range 7 - 16 delta.

No water peak was observed. Water usually appears at 4.6 delta, although the exact shift is solvent and pH dependent.

The NMR spectra was in accord with the structure of orcinol.

5. Mass spectroscopy

a. Operation The mass spectrometer bombarded the sample with an electron beam and quantitatively recorded the result as a spectrum of positive ion fragments. Separation of the positive ion fragments was on the basis of mass/charge; the majority of ions being singly charged.

Up to 190 mass units were scanned, but the 124 peak was the highest recorded peak. The 124 peak was both the molecular ion and the parent peak. The 124 peak corresponded to the theoretical molecular weight of the anhydrous material. Any water of hydration had been pumped away by the time the high vacuum vapor phase spectra of the material being examined was recorded. The orcinol gave a complex spectra. Present were peaks at 108, 107, 106, and 105 indicative of the benzene hydroxy plus one methyl group, minus one hydrogen, minus two hydrogens and minus three hydrogens. Phenol at 55 mass units and tolyl ($C_6H_5CH_2$) at 91 mass units were also present. The phenyl and benzene peaks were found at 77 and 78 mass units. The C_5H_5 peak at 65, characteristic of benzene ring rearrangements, was also prominent.

The molecular weight was verified as 124 for the anhydrous orcinol and was in accord with the predicted molecular weight. The spectrum obtained was characteristic of orcinol.

As a result of the loss on heating, melting point, infrared spectroscopy, nuclear magnetic spectroscopy and mass spectroscopy data it was concluded that the commercial orcinol was of high purity and a monohydrate. The commercial orcinol was used as received without further purification.

b. Condensation of orcinol and phthalic anhydride

The reaction products were extremely heat sensitive. Temperatures in excess of 100° caused drastically reduced yields and also considerable tar formation.

To ascertain the conditions best suited for the synthesis, the reaction was allowed to proceed for one hour, two hours and three hours respectively. Reduced yields were noted for the one hour and three hour reaction times. The one hour reaction time was insufficient kinetically and the three hour decomposed the product once formed. Product yield for the two hour reaction time was 96.7 per cent. The two hour reaction time with careful temperature control showed no evidence of tar formation.

2. Separation and purification

a. Method of Orndorff and Allen The purification of the crude extraction of the three isomer mixture by the method of Orndorff (12) was involved and tedius. Large amounts of solvent were required, up to four liters solvent per 30 grams crude product. The alpha- and

beta- isomers were isolated by this method, but the gamma- isomer was lost along the way.

The products (that is, the alpha- and beta- isomers) isolated by the method of Orndorff and Allen were subjected to NMR analysis. Analysis of the NMR spectra showed that the final alpha- isomer fraction and final beta- isomer fraction were not pure but each fraction was contaminated with small amounts of the other isomers. The loss of the gamma- isomer was attributed to its solubility in the large amounts of solvent necessitated by this procedure.

b. Solvent for extraction The organic solvent for the extraction of the dimethyldihydroxyfluorans must have the following properties:

1. Must be highly polar so that the dimethyldihydroxyfluorans are appreciably soluble.
2. Must be immiscible with water such that subsequent extractions with aqueous solutions are feasible.
3. Should have a density much different from one to accelerate the rate of separation of the organic phase from the aqueous phase.
4. Must have no acid-base properties such that at high pH the isomers can be extracted into the aqueous layer without competition from any organic bases formed.
5. Should not be toxic, should be relatively stable and easily handled.
6. Should be commercially available at relatively low cost and high purity.

The mixture of dimethyldihydroxyfluorans as described on p. 8-9 were extracted from the alcoholic organic phase into the aqueous phase only at pH less than 10.

Ethyl acetate was hydrolyzed slowly under the very basic and slow steps in the extraction. At high pH, the dimethyldihydroxyfluorans remained in the organic phase.

The lower ketones (acetone and di-n-propyl ketone) are miscible with water and therefore could not be used. Appreciable amounts of sample were soluble in cyclohexanone; this solvent is slightly soluble in water (50 g./l at 30°) and has a density (0.9421) very close to water. The separation of cyclohexanone and the aqueous layer was slow. Appreciable amounts of sample were soluble in methyl iso-butyl ketone. Methyl iso-butyl ketone is only slightly soluble in water (2 g./100 g. water at 20°), readily dissolved the dimethyldihydroxyfluorans, has a density of 0.801, has a boiling point of 117°/760 mm., is relatively safe to work with and is commercially available at high purity and relatively low cost. Methyl iso-butyl ketone was subsequently used as an extraction solvent.

The ethers (diethyl ether, di-n-propyl ether, furan, tetrahydrofuran and 1,4-dioxane) showed excellent properties with respect to solubility of the dimethyldihydroxyfluorans, immiscibility with water, solvent action on the dimethyldihydroxyfluorans, low boiling point and commercial availability at high purity. Ethers do form peroxides which are explosive on heating. Diethyl ether has a low boiling point (35°/760 mm.) and is commercially available at high purity. Diethyl

ether was also used as an extraction solvent.

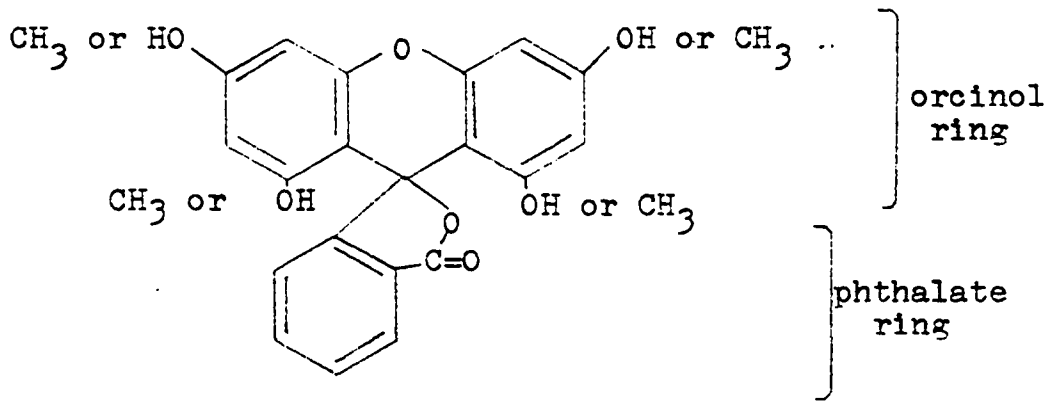
Amides, such as dimethylformamide, did dissolve the dimethyldihydroxyfluorans, but were miscible with water.

Amines, such as pyridine, dissolved the dimethyldihydroxyfluorans but are miscible with water.

The non-aromatic solvents (acetonitrile, carbon disulfide, ethylene dichloride, dimethyl sulfoxide, carbon tetrachloride, chloroform, and ethylene dichloride) and aromatic hydrocarbons (benzene, toluene, m-xylene, and chlorobenzene) did not dissolve appreciable amount of the dimethyldihydroxyfluorans.

Both diethyl ether and methyl iso-butyl ketone were used as solvents for extraction of the dimethyldihydroxyfluorans. Methyl iso-butyl ketone was superior to the diethyl ether in safe handling at boiling point temperatures. Separation of the organic-aqueous phases, however, was slower than with the diethyl ether.

c. Separation as a function of pH Models of the three isomers indicate that in each the three fused benzene rings are coplanar, the phthalic anhydride ring being at right angles to this plane. The phthalic anhydride ring was therefore in position for intramolecular hydrogen bonding between the hydroxy protons in the 1' and 8' positions and the oxygen atoms of the phthalic anhydride ring. This intramolecular hydrogen bonding decreases the acidity of the isomer because the proton in more firmly bound between two oxygen atoms and less available to react with base. On this basis, the gamma isomer should be the most acidic, the beta intermediate and the alpha the least acidic.



The gamma isomer should extract into a slightly basic buffer solution, the beta isomer into a more basic buffer solution and the alpha isomer into a strongly basic buffer solution.

A series of extractions from the organic phase into buffers of known pH was made. Each successive extraction was analyzed as to pH of the extracted solution, weight of material extracted and isomer extracted. The isomer extracted was determined by analysis of the NMR spectra, presence or lack of fluorescence, and color of the isomer in basic solution. Four sets of extractions were necessary to obtain pure isomers. Tar was removed by extraction at pH 7, gamma-dimethyldihydroxyfluoran at pH 8.95, beta-dimethyldihydroxyfluoran at pH 10.75 and alpha-dimethyldihydroxyfluoran at pH 13.00.

Extraction of crude material at pH 7, 9, 11 and 13 did give a

trace of unreacted material, pure gamma-dimethyldihydroxyfluoran, pure beta-dimethyldihydroxyfluoran and pure alpha-dimethyldihydroxyfluoran. There was no cross-contamination of isomers in each fraction as determined by NMR analysis.

3. Properties and structure

a. Structure assignments of Orndorff and Allen The original structure assignments of the dimethyldihydroxyfluorans by Orndorff and Allen (12) proved to be correct.

Orndorff and Allen ascribed the fluorescent isomer to be gamma-dimethyldihydroxyfluoran, the -OH groups at positions 3' and 6' in the orcinol ring. Their assignment of structure was based on Meyer's work (10). Meyer had independently synthesized fluorescein and the fluorescent di-methyl structural analog, that is, gamma-dimethyldihydroxyfluoran. Correlation of properties led Meyer to assign similar structures to both compounds.

Orndorff and Allen ascribed the other symmetrical isomer to be alpha-dimethyldihydroxyfluoran, the -OH groups at positions 1' and 8' in the orcinol ring. Their assignment of structure was based on Baeyer's work (2). Baeyer had independently synthesized phenyl-2, 2'-dioxyxanthanol, 1, 8-dinitrofluorescein and 1', 8'-dihydroxy-3', 6'-dimethylfluorescein, that is, alpha-dimethyldihydroxyfluoran. Correlation of properties led Baeyer to assign similar structures to all three compounds.

Orndorff and Allen attributed the remaining unsymmetrical isomer to be beta-dimethyldihydroxyfluoran, the -OH groups at positions 3' and 8' in the orcinol ring. Orndorff and Allen predicted and confirmed

beta-dimethyldihydroxyfluoran to have properties intermediate between those of gamma- and alpha-dimethyldihydroxyfluoran. Specifically, gamma-dimethyldihydroxyfluoran, the strongest acid, readily absorbed two molecules of ammonia per molecule of compound; beta-dimethyldihydroxyfluoran absorbed some ammonia; and alpha-dimethyldihydroxyfluoran, the weakest acid, absorbed none.

b. General The alkaline solution of each of the three isomers was colored differently: gamma-dimethyldihydroxyfluoran was dark red almost black with a green cast; beta-dimethyldihydroxyfluoran red-brown almost black; and alpha-dimethyldihydroxyfluoran dark purple almost black.

The colors of the three isomers in acid solution were also different. Gamma-dimethyldihydroxyfluoran was bright orange when just acidified and changed to pale orange on standing; beta-dimethyldihydroxyfluoran was yellow to ivory; alpha-dimethyldihydroxyfluoran was consistently white.

c. Mass spectroscopy The mass scale up to 450 mass units was scanned. The largest recorded peak found was at 360 a. m. u. for all three isomers. The 360 peak was both the molecular ion and the parent peak. The 360 peak corresponded to the theoretical molecular weight of the anhydrous material. The spectra of alpha-, beta- and gamma-dimethyldihydroxyfluoran were identical in peak position but differed only slightly in relative peak intensity.

The spectra were quite complex but did give a reasonable degradation pattern. Peaks were present at 345, 330 and 312 indicative

of the parent minus one $-CH_3$, minus one H_2O, minus the second $-CH_3$ and minus the second CH_3 plus H_2O.

Phenols give a conspicuous parent peak and, under complex rearrangement, peaks at 77, 78, and 79. These four peaks were observed. The loss of CO at P-28 (parent peak minus 28) at 332, loss of CHO at P-29 at 331, loss of OH at P-17 at 343, loss of H_2O at P-18 at 342 were expected for phenols and were observed.

Aromatic ethers undergo complex rearrangement giving a P-H at 359, P-CO at 332 and P-CHO at 331. Peaks at 359, 332 and 331 were observed.

The complex spectra were in accord with the proposed structures of alpha-, beta- and gamma-dimethyldihydroxyfluoran. The theoretical molecular weight of 360 a.m.u. for each isomer was also confirmed.

d. Infrared spectroscopy The infrared spectra obtained of alpha-, beta- and gamma-dimethyldihydroxyfluoran were very similar. There were small differences in the position of the peaks and in the intensity of the peaks.

The three areas of special interest were those at wavelengths 2.75 to 2.10 microns, 5.70 to 5.80 microns, and 13 to 15 microns.

The region 2.75 to 3.10 microns is characteristic of OH stretching vibrations. The alpha- and gamma-dimethyldihydroxyfluoran gave a sharp peak with a small shoulder, indicative of complex interactions. The beta-dimethyldihydroxyfluoran gave a sharp, single peak.

The peak at 5.74 to 5.80 microns is characteristic of C-O stretching vibrations. The strong C-O stretching mode of esters and

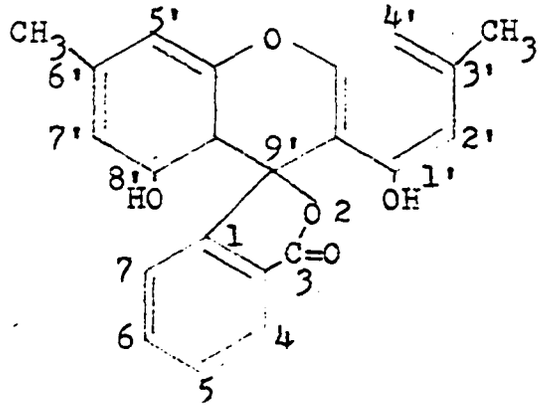
lactones in the 7. 70 and 10. 0 micron region was also present.

The 13 to 15 micron region was characteristic of a multisubstituted aromatic compound.

The three infrared spectra were in accord with the proposed structures of alpha-, beta- and gamma-dimethyldihydroxyfluoran.

e. Nuclear magnetic resonance spectroscopy Ideally, samples for NMR analysis should be at least 1 M inconcentration. This ideal situation rarely presented itself in the current work. The isomers were only slightly soluble in common organic solvents. The isomers were slightly more soluble in aqueous base than in dimethyl sulfoxide. However, the D_2O-NaOD exchanged with acidic protons, here phenol, and thus the acidic protons did not appear on the spectrum. The isomers were somewhat soluble in trifluoroacetic acid.

Observed in the NMR spectra of alpha-dimethyldihydroxyfluoran in D_2O-NaOD plus Tier's Salt was a 1:2:1:4:6 proton ratio; in dimethyl sulfoxide plus TMS a 2:1:2:1:2:2:6 proton ratio; and in trifluoroacetic acid plus TMS a 1:2:2:1:2:6 proton ratio. The results are summarized in Table 2 and Figure 9.



alpha-dimethyldihydroxy-fluoran

Table 2. The peak positions, the proton integrations and the group bearing the proton obtained from the NMR spectrum of alpha-dimethyldihydroxyfluoran in D_2O-NaOD, dimethyl sulfoxide and trifluoroacetic acid

Solvent	Peak position (ppm)	Proton integration	Group bearing protons
D_2O-NaOD	2.13	6	two $-CH_3$ protons
	6.02	4	2', 4', 5', 7' protons of orcinol ring
	7.13	1	7 proton of phthalate ring
	7.54	2	5, 6 protons of phthalate ring
	7.97	1	4 proton of phthalate ring
d_6-dimethyl sulfoxide	2.22	6	two $-CH_3$ protons
	6.28	2	4', 5' protons of orcinol ring
	6.55	2	2', 7' protons of orcinol ring
	6.98	1	7 proton of phthalate ring
	7.46	2	5, 6 protons of phthalate ring
	7.70	1	4 proton of phthalate ring
	9.60	2	1', 8' -OH protons
trifluoro-acetic acid	2.67	6	two $-CH_3$ protons
	6.95	2	4', 5' protons of orcinol ring
	7.47	2	2', 7' protons of orcinol ring
	7.18	1	7 proton of phthalate ring
	7.80	2	5, 6 protons of phthalate ring
	8.42	1	4 proton of phthalate ring

Figure 9. A proton magnetic resonance spectrum of alpha-dimethyldihydroxyfluoran in trifluoroacetic acid

A. Methyl group protons (6)

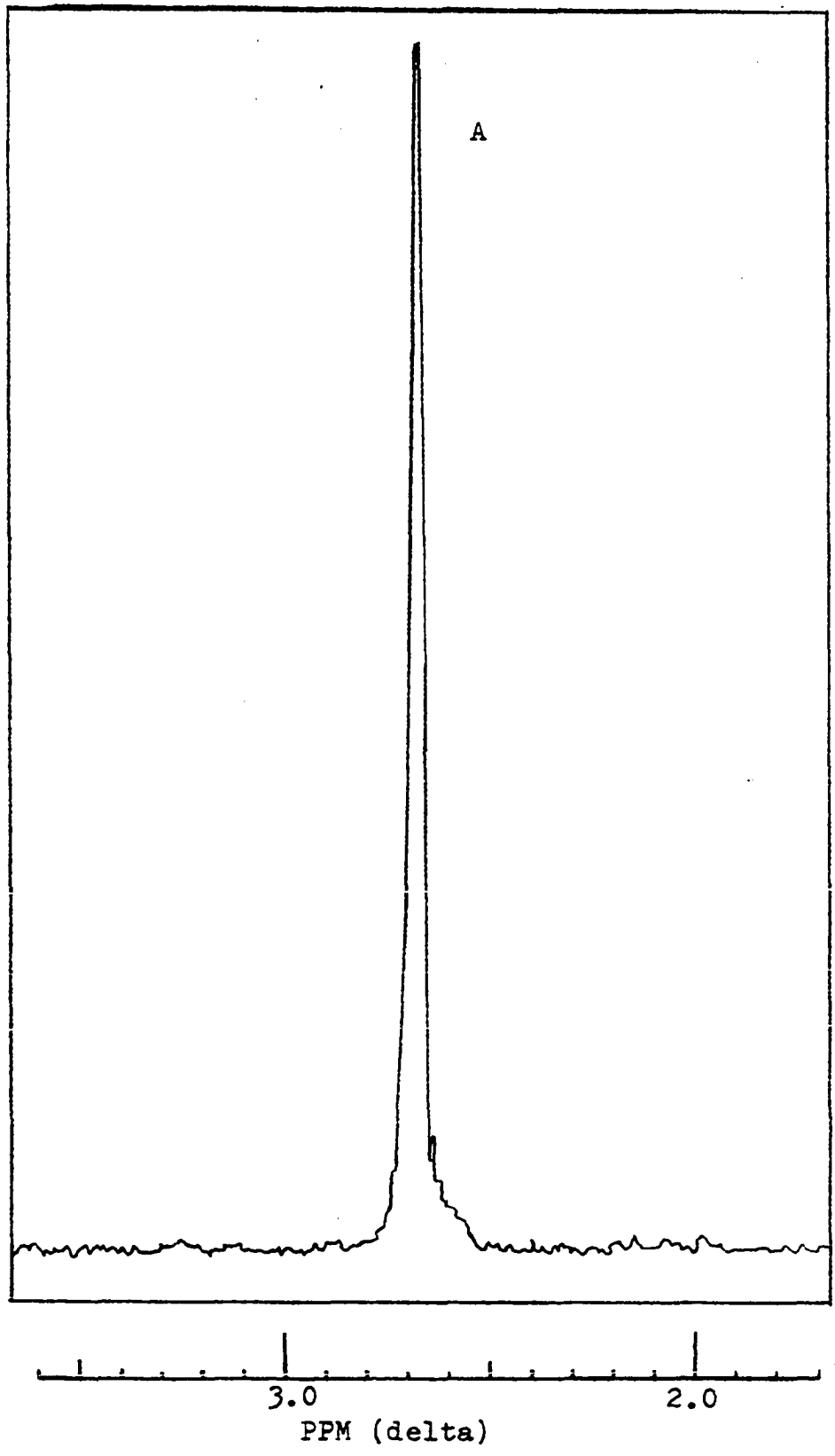
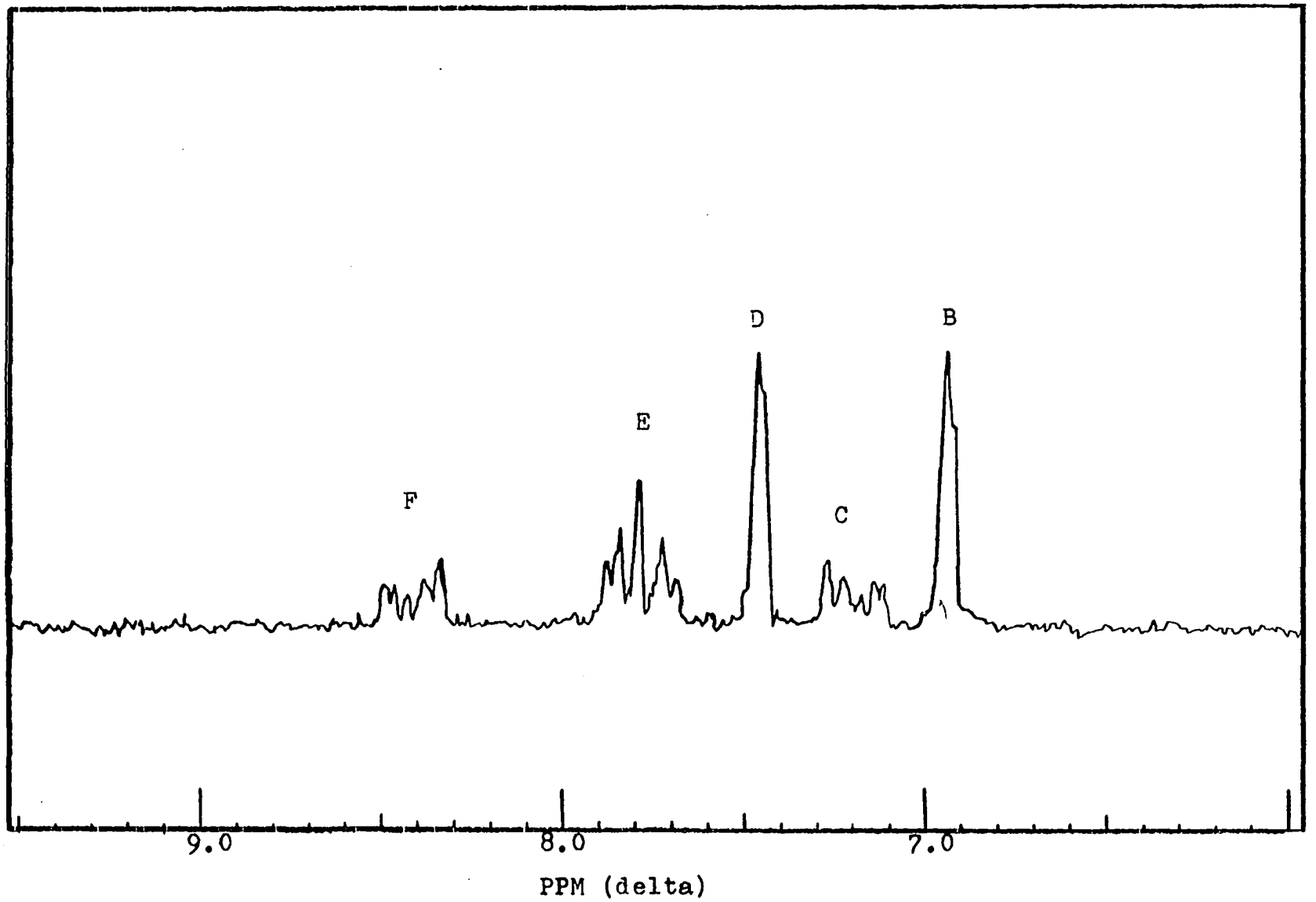

A
3.0
2.0
PPM (delta)

Figure 9. (Continued)

B. 4', 5' protons of the orcinol ring (2)
C. 7 proton of the phthalate ring (1)
D. 2', 7' protons of the orcinol ring (2)
E. 5, 6 protons of the phthalate ring (2)
F. 4 proton of the phthalate ring (1)


D
B
E
F
C
9.0
8.0
7.0
PPM (delta)

The singlet integrating to six protons at 2.13 delta in D_2O-NaOD, at 2.22 delta in dimethyl sulfoxide and at 2.67 delta in trifluoroacetic acid was attributed to the two methyl group protons. By comparison, the methyl group of toluene neat falls at 2.32 delta (3).

The singlet integrating to four protons at 6.02 delta in D_2O-NaOD was attributed to the orcinol ring protons.

The two singlets at 6.28 delta and 6.55 delta in dimethyl sulfoxide and at 6.95 delta and 7.47 delta in trifluoroacetic acid were attributed to the orcinol ring protons. Protons 4' and 5' are in identical environments as are protons 2' and 7'. The 4' and 5' protons are each ortho to a methyl group and an ether group. The 2' and 7' protons are each ortho to a methyl group and a hydroxy group. The hydroxy group has a stronger deshielding effect than the ether group. Therefore, the 2' and 7' protons are further downfield than the 4' and 5' protons. The 2' and 7' protons are thus assigned to the singlet at 6.55 delta (dimethyl sulfoxide) and at 7.47 delta (trifluoroacetic acid); and the 4' and 5' protons at 6.28 delta (dimethyl sulfoxide) and at 6.95 delta (trifluoroacetic acid).

The multiplets integrating to 1:2:1 protons were attributed to the phthalate ring protons. The phthalate ring protons were observed at 7.13 delta, 7.54 delta and 7.97 delta in D_2O-NaOD; at 6.98 delta,

7.46 delta and 7.70 delta in dimethyl sulfoxide; and at 7.18 delta, 7.80 delta and 8.42 delta in trifluoroacetic acid.

Inspection of the phthalate ring proton spectra indicates that two protons are each ortho to a central group. That is, an AB_2C system is present. The 4 proton is splitting the 5 and 6 protons into a doublet. The 7 proton is splitting the 5 and 6 protons into a doublet.

The 4 proton, being ortho to a $-CO_2R$ group, is the most deshielded and therefore appears the furthest downfield. The peak at 7.97 delta (D_2O-NaOD), at 7.70 delta (dimethyl sulfoxide) and at 8.42 delta (trifluoroacetic acid) was attributed to the 4 proton of the phthalate ring.

The 5 and 6 protons are less intensely but about equally deshielded by the carbonyl. The peak integrating to two protons at 7.54 delta (D_2O-NaOD), at 7.46 delta (dimethyl sulfoxide) and at 7.80 delta (trifluoroacetic acid) was attributed to the 5 and 6 protons.

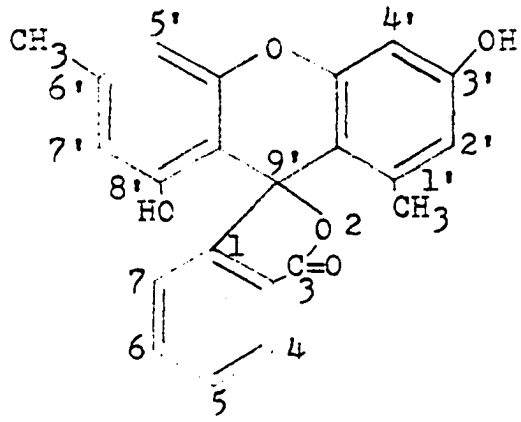
The 7 proton was attributed to the multiplet at 7.13 delta (D_2O-NaOD), 6.98 delta (dimethyl sulfoxide) and at 7.18 delta (trifluoroacetic acid).

The singlet at 9.60 delta in dimethyl sulfoxide integrating to two protons was attributed to the 1' and 8' -OH group protons. No -OH protons were observed in D_2O-NaOD due to rapid exchange with the solvent. No -OH protons were observed in trifluoroacetic acid because the -COOH proton and the -OH proton appear in the same place in the spectrum.

The positions and integrations of the protons were in accord

with the proposed structure of alpha-dimethyldihydroxyfluoran.

Observed in the NMR spectra of beta-dimethyldihydroxyfluoran in D_2O-NaOD plus Tier's Salt was a 1:2:1:2:2:3:3 proton ratio; in dimethyl sulfoxide plus TMS a 1:1:2:1:2:2:3:3 proton ratio; and in trifluoroacetic acid a 1:2:2:1:2:3:3 proton ratio. The results are summarized in Table 3 and Figure 10.



beta-dimethyldihydroxy-fluoran

The singlet integrating to three protons at 2.15 delta in D_2O-NaOD, at 2.20 in dimethyl sulfoxide; and at 2.65 in trifluoroacetic acid was attributed to the 6' methyl group protons. The two methyl groups of alpha-dimethyldihydroxyfluoran at 2.13 delta (D_2O-NaOD), at 2.22 delta (dimethyl sulfoxide) and at 2.67 delta (trifluoroacetic acid) are structurally equivalent to the 6' methyl group of beta-dimethyldihydroxyfluoran.

The singlet integrating to three protons at 1.45 delta in

Table 3. The peak positions, the proton integrations and the group bearing the proton obtained from the NMR spectrum of beta-dimethyldihydroxyfluoran in D_2O-NaOD, dimethyl sulfoxide and trifluoroacetic acid

Solvent	Peak position (ppm)	Proton integration	Group bearing protons
D_2O-NaOD	1.45	3	1' -CH_3 protons
	2.15	3	6' -CH_3 protons
	6.05	2	4', 5' protons of orcinol ring
	6.40	2	2', 7' protons of orcinol ring
	7.13	1	7 proton of phthalate ring
	7.54	2	5, 6 protons of phthalate ring
	7.97	1	4 proton of phthalate ring
d_6-dimethyl sulfoxide	1.56	3	1' -CH_3 protons
	2.20	3	6' -CH_3 protons
	6.36	2	4', 5' protons of orcinol ring
	6.58	2	2', 7' protons of orcinol ring
	7.05	1	7 proton of phthalate ring
	7.58	2	5, 6 protons of phthalate ring
	7.87	1	4 proton of phthalate ring
	9.60	1	8' -OH proton
trifluoro-acetic acid	1.97	3	1' -CH_3 protons
	2.65	3	6' -CH_3 protons
	6.93	2	4', 5' protons of orcinol ring
	7.22	3	7 proton of phthalate ring
	7.40		2', 7' protons of orcinol ring
	7.92	2	5, 6 protons of phthalate ring
	8.50	1	4 proton of phthalate ring

Figure 10. A proton magnetic resonance spectrum of beta-dimethyldihydroxyfluoran in trifluoroacetic acid

A. 1' Methyl group protons (3)
B. 6' Methyl group protons (3)

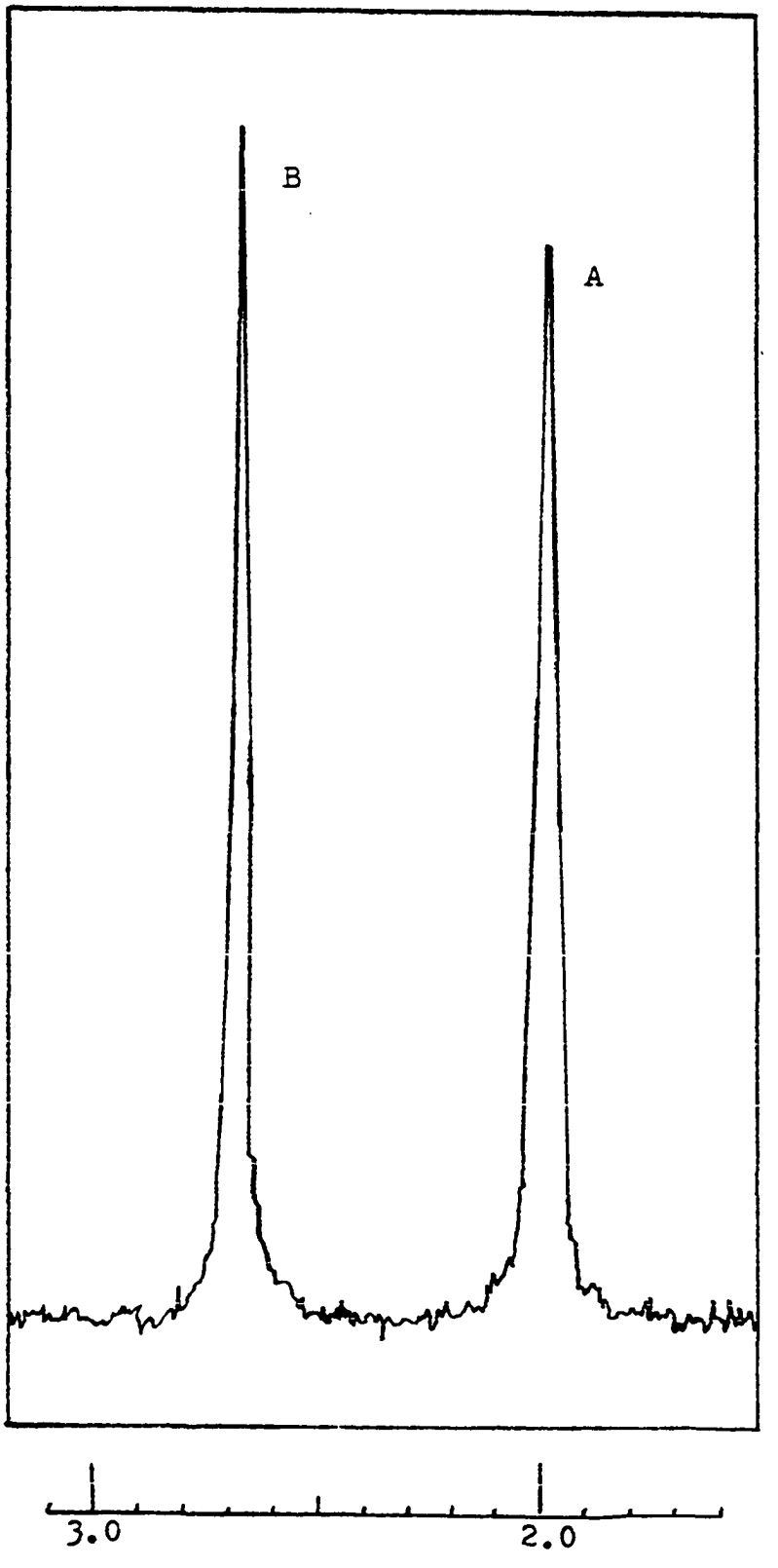
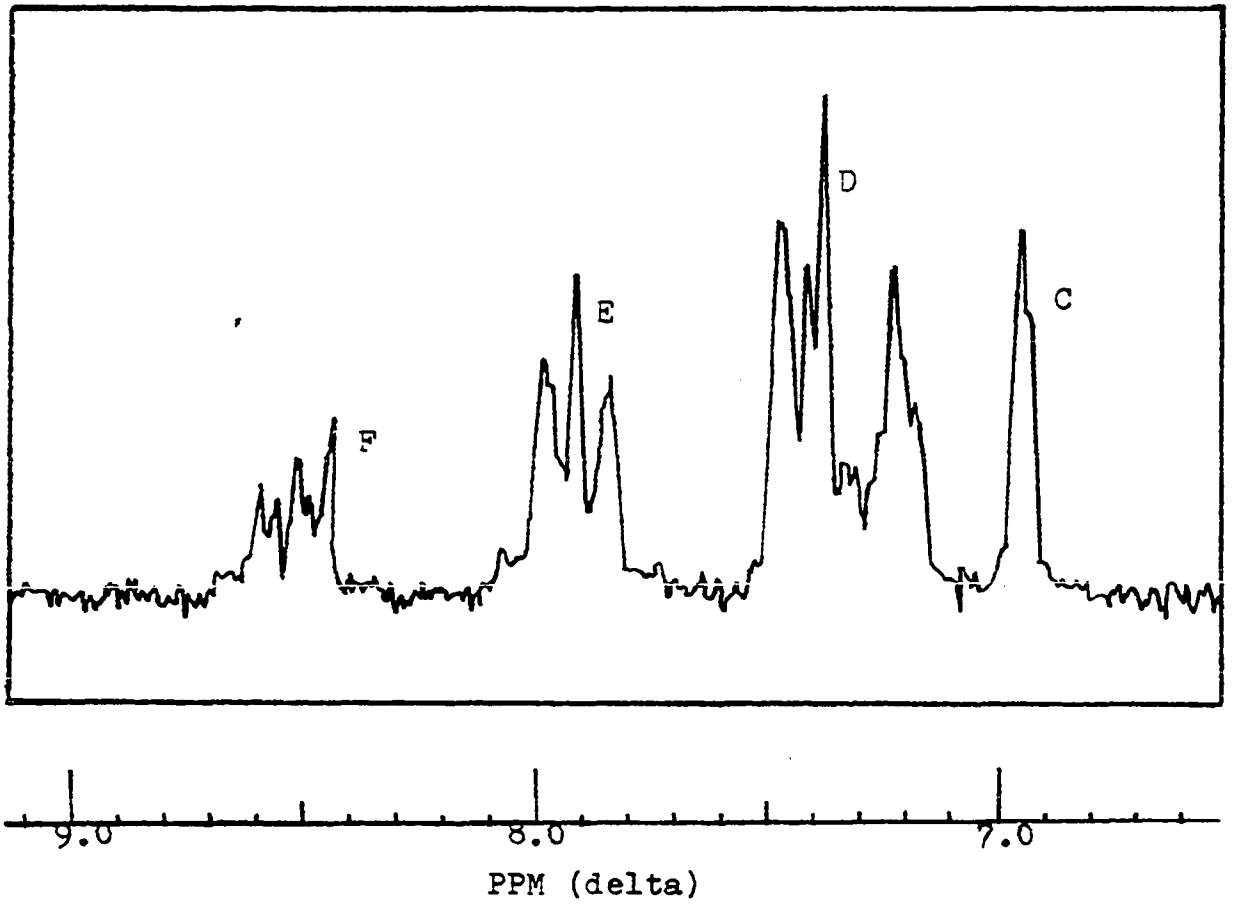

B
A
3.0
2.0
PPM (delta)

Figure 10. (Continued)

C. 4', 5' Protons of the orcinol ring (2)
D. 7 Proton of the phthalate ring (1)
   2', 7' Protons of the orcinol ring (2)
E. 5, 6 Protons of the phthalate ring (2)
F. 4 Proton of the phthalate ring (1)


F
E
D
C
9.0
8.0
7.0
PPM (delta)

D_2O-NaOD, at 1.56 delta in dimethyl sulfoxide, and at 1.97 delta in trifluoroacetic acid was attributed to the 1' methyl group. On building models of the three isomers, it was found that the phthalic anhydride ring is perpendicular to the plane of the other three fused aromatic rings, that is, the orcinol ring. The phthalic anhydride ring is in proximity to shield, that is move upfield, any methyl groups in the 1' and 8' positions. This was borne out by the NMR spectra of gamma-dimethyldihydroxyfluoran.

The singlet integrating to two protons at 6.40 delta and the multiplet integrating to two protons at 6.05 delta in D_2O-NaOD were attributed to the 2' and 7' protons and to the 4' and 5' protons. The singlet integrating to two protons at 6.58 delta and the doublet integrating to two protons at 6.36 delta in dimethyl sulfoxide were attributed to the 2' and 7' protons and to the 4' and 5' protons. The singlet integrating to two protons at 6.93 delta in trifluoroacetic acid was attributed to the 4' and 5' protons.

The multiplet composed of two overlapping multiplets centered at 7.22 delta and 7.40 delta integrating to a total of three protons was observed in the spectra in trifluoroacetic acid. By inspection of the overlapping peaks, the upfield peak was attributed to the 7 proton of the phthalate ring and the downfield peak was attributed to the 2' and 7' protons of the orcinol ring.

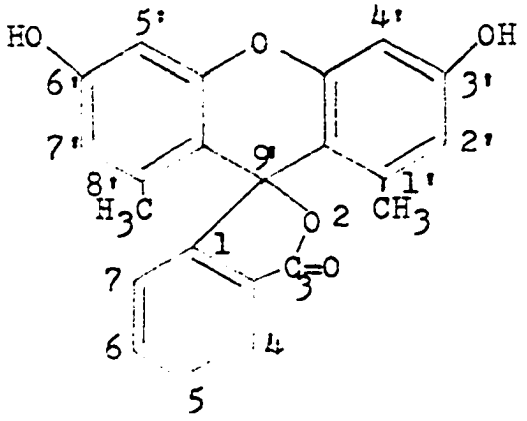
The phthalate ring protons in D_2O-NaOD appeared identical in splitting and position to those of alpha-dimethyldihydroxyfluoran in D_2O-NaOD. In dimethyl sulfoxide, multiplets at 7.05 delta, 7.58 delta

and 7.87 delta integrating to 1:2:1 protons were attributed to the 7, 5 and 6, and 4 protons, respectively. In trifluoroacetic acid, the multiplets at 7.92 delta and 8.50 delta were attributed to the 5 and 6 protons and the 4 proton, respectively.

The singlet at 9.60 delta in dimethyl sulfoxide integrating to one proton was attributed to the 8' OH proton of the orcinol ring. The 3'-OH proton is in position to rapidly exchange with the solvent protons. Also, the intra-molecular hydrogen bonding to the solvent molecules results in a very broad, singlet. In this case, the singlet was so broad as to be indistinguishable from the base line.

The positions and integrations of the protons were in accord with the proposed structure of beta-dimethyldihydroxyfluoran.

Observed in the NMR spectra of gamma-dimethyldihydroxy-fluoran in D_2O-NaOD plus Tier's Salt was a 1:2:5:6 proton ratio; in dimethyl sulfoxide plus TMS a 1:2:1:4:6 proton ratio; and in trifluoroacetic acid a 1:2:3:2:6 proton ratio. The results are summarized in Table 4 and Figure 11.



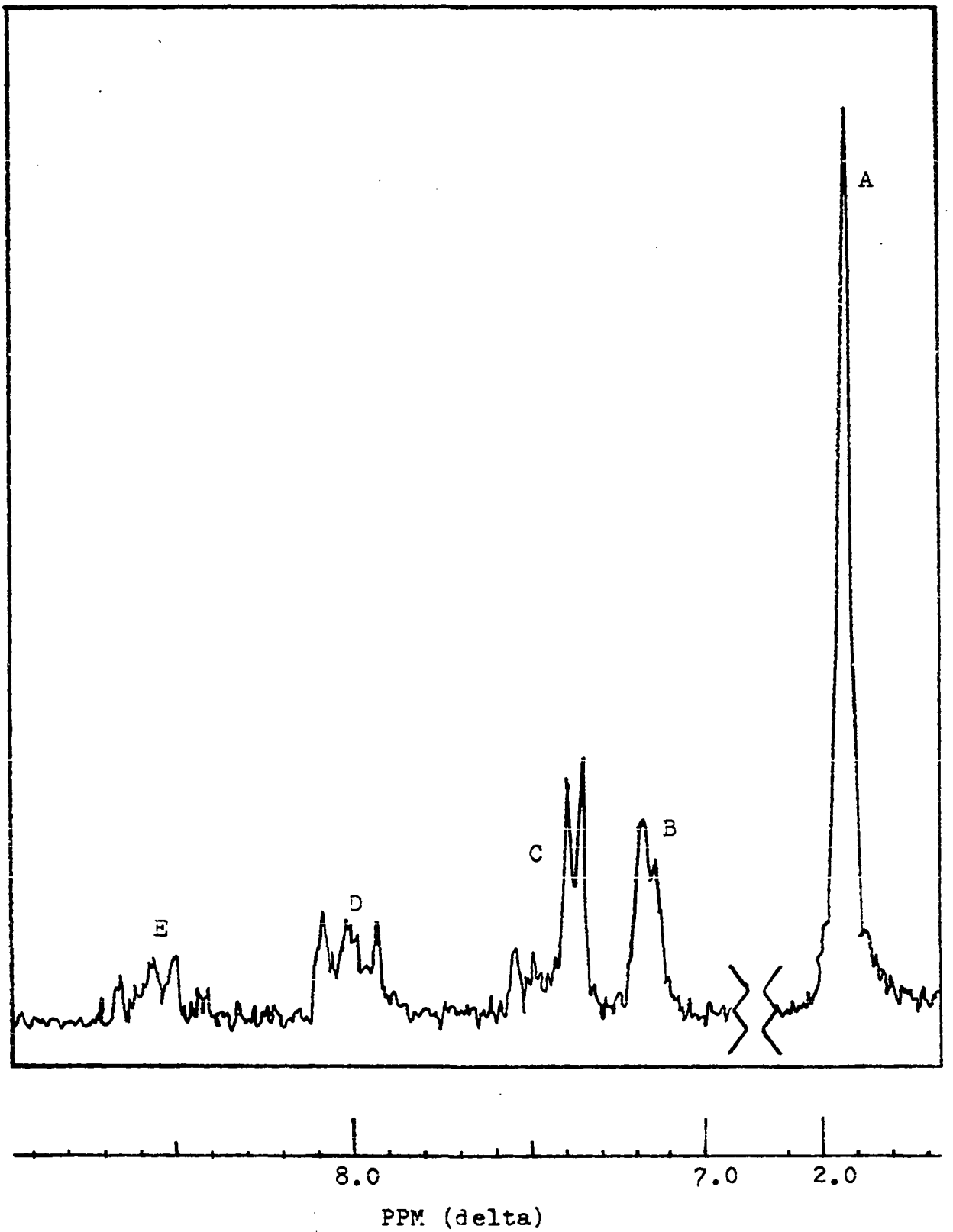
gamma-dimethyldihydroxy-fluoran

Table 4. The peak positions, the proton integrations and the groups bearing the proton obtained from the NMR spectrum of gamma-dimethyldihydroxyfluoran in D_2O-NaOD, dimethyl sulfoxide and trifluoroacetic acid

Solvent	Peak position (ppm)	Proton Integration	Group bearing protons
D_2O-NaOD	1.45	6	two $-CH_3$ protons
	6.52	5	2', 4', 5', 7' protons of orcinol ring 7 proton of phthalate ring
	7.43	2	5, 6 protons of phthalate ring
	8.03	1	4 proton of phthalate ring
d_6-dimethyl sulfoxide	1.58	6	two $-CH_3$ groups
	6.47	4	2', 4', 5', 7' protons of orcinol ring
	6.76	1	7 proton of phthalate ring
	7.42	2	5, 6 protons of phthalate ring
	7.91	1	4 proton of phthalate ring
trifluoro-acetic acid	1.90	6	two $-CH_3$ protons
	7.20	2	4', 5' protons of orcinol ring
	7.40	3	2', 7' protons of orcinol ring 7 proton of phthalate ring
	8.05	2	5, 6 protons of phthalate ring
	8.88	1	4 proton of phthalate ring

Figure 11. A proton magnetic resonance spectrum of gamma-dimethyldihydroxyfluoran in trifluoroacetic acid

A. Methyl protons (6)
B. 4', 5' Protons of the orcinol ring (2)
C. 2', 7' Protons of the orcinol ring (2)
   7 Proton of the phthalate ring (1)
D. 5, 6 Protons of the phthalate ring (2)
E. 4 Proton of the phthalate ring (1)


A
B
C
D
E
8.0
7.0
2.0
PPM (delta)

The singlet integrating to six protons at 1.45 delta in D_2O-NaOD, at 1.58 delta in d_6-dimethyl sulfoxide and at 1.90 delta in trifluoroacetic acid was attributed to the two methyl group protons. The shielding affect of the phthalic anhydride ring moved the aromatic methyl groups upfield from their usual position at 2.32 delta (ref. toluene, neat, at 2.32 delta). By comparison, the 1' methyl group of beta-dimethyldihydroxyfluoran structurally equivalent to the two methyl groups of gamma-dimethyldihydroxyfluoran was observed at 1.45 delta in D_2O-NaOD, at 1.56 delta in d_6-dimethyl sulfoxide and at 1.97 delta in trifluoroacetic acid.

The multiplet integrating to five protons at 6.52 delta in D_2O-NaOD was attributed to the orcinol ring protons plus the 4 proton of the phthalate ring. The multiplets integrating to 1:2 protons at 8.03 delta and 7.43 delta in D_2O-NaOD were attributed to the 7 proton and to the 5 and 6 protons of the phthalate ring.

In dimethyl sulfoxide, the multiplet integrating to four protons at 6.47 delta was attributed to the orcinol ring protons. The multiplets integrating to 1:2:1 protons at 6.76 delta, 7.42 delta and 7.91 delta were attributed to the 7, 5 and 6, and 4 protons of the phthalate ring.

In trifluoroacetic acid, the doublet integrating to two protons at 7.20 delta was attributed to the 4' and 5' protons of the orcinol ring.

The multiplet composed of a doublet and multiplet overlapping centered at 7.40 delta integrating to a total of three protons was attributed to the 2' and 7' protons of the orcinol ring and to the 7 proton of the phthalate ring. The multiplets at 8.05 delta and 8.88 delta integrating to 2:1 protons were attributed to the 5 and 6 protons and to the 4 proton of the phthalate ring. In dimethyl sulfoxide, no phenolic protons were observed. This is in accord with the proposed structure. The 3' and 6' OH group protons are in position to exchange with the protons of the solvent. Also, both protons are in position to intra-molecular hydrogen bond to the solvent molecules. This will result in a very broad singlet. In this case, the singlet was so broad as to be indistinguishable from the base line.

The positions and integrations of the protons were in accord with the proposed structure of gamma-dimethyldihydroxyfluoran.

f. Melting point data The melting point data for alpha-, beta- and gamma-dimethyldihydroxyfluoran was inconclusive. At 250° all three isomers showed some rearrangement of crystal form, however, no phase change was discernable. At 280°, some charring was noted, and by 300° charcoal was the only product.

g. Fluorescence Alkaline aqueous solutions of gamma-dimethyldihydroxyfluoran were strongly fluorescent. Beta- and alpha-dimethyldihydroxyfluoran were non-fluorescent.

h. Elemental analysis Alpha-, beta- and gamma-dimethyldihydroxyfluoran were analyzed for percentage carbon and hydrogen. Oxygen was determined by difference. The results are

summarized in Table 5. The percentage carbon, hydrogen and oxygen determined experimentally was consistent with the theoretical percentage for anhydrous gamma- and beta-dimethyldihydroxyfluoran and with hemihydrate alpha-dimethyldihydroxyfluoran.

i. Residue on ignition The residue on ignition of 0.5 grams each of alpha-, beta- and gamma-dimethyldihydroxyfluoran was found to be less than 0.1 mg. The isomers were therefore devoid of non-volatile impurities.

j. Solubility in non-aqueous solvents The solubility of alpha-, beta- and gamma-dimethyldihydroxyfluoran was qualitatively determined in a number of solvents.

Of the solvents tested, alpha-dimethyldihydroxyfluoran was slightly less soluble than beta-dimethyldihydroxyfluoran in a given solvent; and gamma-dimethyldihydroxyfluoran was slightly more soluble than beta-dimethyldihydroxyfluoran.

The solubility of beta-dimethyldihydroxyfluoran in a number of solvents is summarized in Table 6.

In general, the isomers were highly insoluble in the many solvents tested. Each solvent and solid was allowed to stand for four hours at each dissolution attempt.

As expected from the structure assignments of the isomers, the isomers were somewhat soluble only in polar solvents and in solvents capable of hydrogen bonding. It was also found that the solubility greatly decreased in the presence of a small amount of water.

The two OH groups of gamma-dimethyldihydroxyfluoran are in

Table 5. Percentage carbon, hydrogen and oxygen of alpha-, beta- and gamma-dimethyldihydroxyfluoran and of $C_{22}H_{16}O_5$, $C_{22}H_{16}O_5 \cdot 1/2\ H_2O$, and $C_{22}H_{16}O_5 \cdot H_2O$

Compound	Percentage carbon	Percentage hydrogen	Percentage oxygen
alpha-dimethyl-dihydroxyfluoran	70.77	4.93	24.40
beta-dimethyl-dihydroxyfluoran	73.24	4.66	22.19
gamma-dimethyl-dihydroxyfluoran	73.27	4.68	22.15
$C_{22}H_{16}O_5$	73.33	4.48	22.20
$C_{22}H_{16}O_5 \cdot 1/2\ H_2O$	71.54	4.64	23.82
$C_{22}H_{16}O_5 \cdot H_2O$	69.84	4.80	25.37

Table 6. Solubility of beta-dimethyldihydroxyfluoran in various solvents

Solvent	Solubility[a]
Alcohols	
Methyl	VS
Ethyl	VS
n-Propyl	VS
n-Butyl	VS
iso-Pentyl	VS
Ketones	
Acetone	S
di-n-Propyl	S
Methyl iso-butyl	VS
Cyclohexanone	VS
Ethers	
di-Ethyl	VS
di-n-Propyl	VS
di-n-Butyl	VS
1, 4-Dioxane	VS
Furan	S
Tetrahydrofuran	S
Amides and Amines	
Dimethylformamide	S
Pyridine	VS
Aliphatic organic	
Acetonitrile	SS
Dimethyl sulfoxide	SS (S on heating)
Chloroform	I
Carbon tetrachloride	I
Carbon disulfide	I
Ethylene dichloride	I
Ethyl acetate	VS
Aromatic organic	
Benzene	I
Toluene	I
m-Xylene	I
Chlorobenzene	I

[a]VS = very soluble
S = soluble
SS = slightly soluble
I = insoluble

position to hydrogen bond with a solvent; gamma-dimethyldihydroxy-fluoran was found to be the most soluble in dimethylsulfoxide. One OH group of beta-dimethyldihydroxyfluoran is in position to hydrogen bond with a solvent; beta-dimethyldihydroxyfluoran was found to be somewhat soluble in dimethyl sulfoxide. Neither of the OH groups of alpha-dimethyldihydroxyfluoran is in position to hydrogen bond with a solvent; alpha-dimethyldihydroxyfluoran was least soluble in dimethyl sulfoxide.

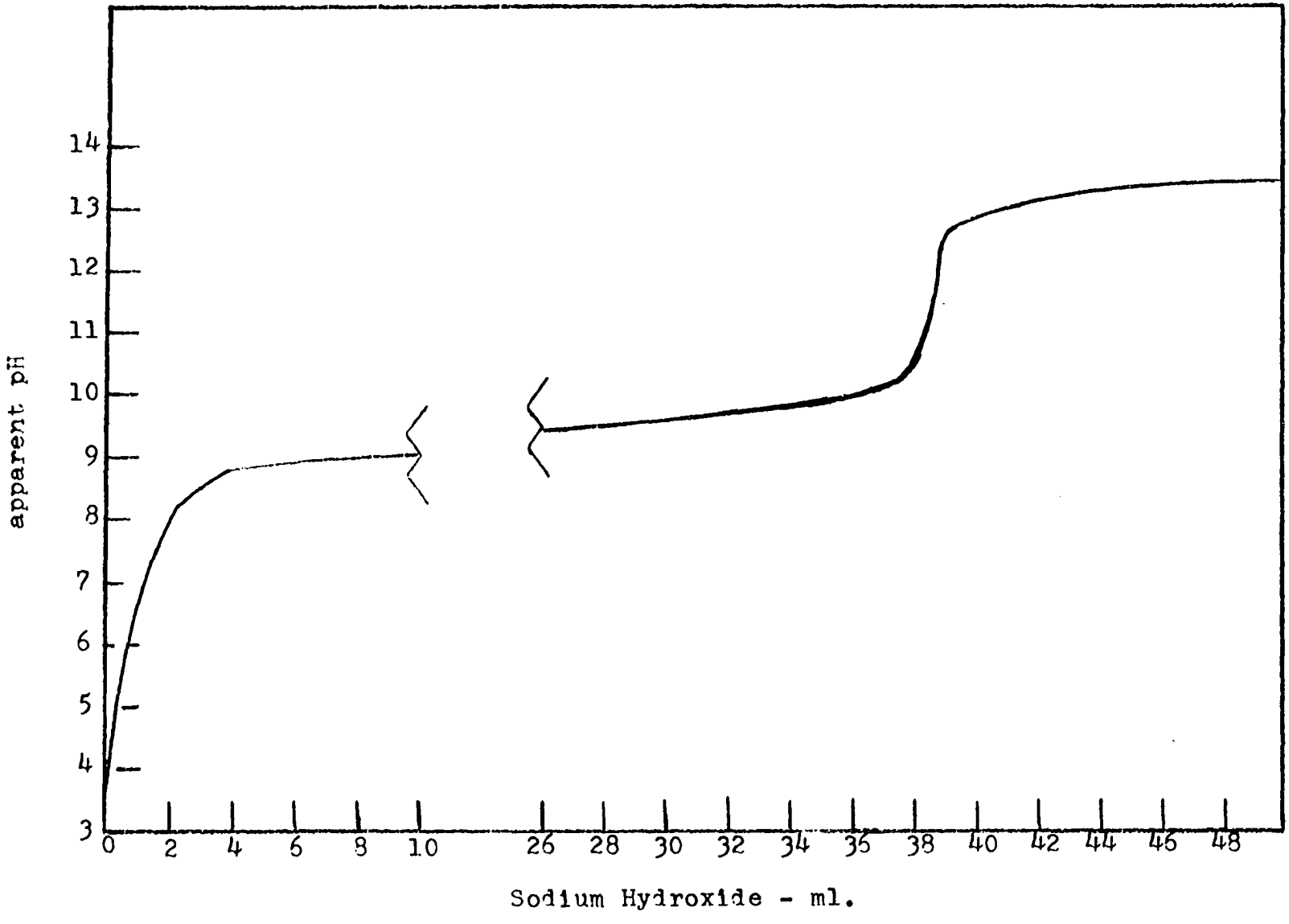
k. Titrations in non-aqueous solvents Many acids too weak or insoluble for determination in aqueous media can be titrated in appropriate non-aqueous solvents. The choice of solvent is determined by that solvent's acid and base properties, dielectric constant and solubility of the solute.

Alpha-dimethyldihydroxyfluoran, beta-dimethyldihydroxyfluoran, and gamma-dimethyldihydroxyfluoran were soluble in the polar solvents ethanol and pyridine. The purity of each isomer was determined by non-aqueous titration in ethanol or pyridine by potentiometric titration with standard sodium hydroxide.

Gamma-dimethyldihydroxyfluoran was dissolved in absolute ethanol and titrated potentiometrically with standard sodium hydroxide in ethanol. The data was plotted as apparent pH versus volume titrant. One good end point was observed. (Figure 12.) A first derivative plot was constructed to determine the end point of the titration. The apparent pH at 0 per cent titration was 3.50, at 50 per cent 9.23 and at 100 per cent 12.45. The experimental equivalent weight was 179.91. The theoretical equivalent weight, assuming two

Figure 12. Non-aqueous potentiometric titration of gamma-dimethyldihydroxyfluoran in ethanol with sodium hydroxide

0.7436 g. acid
0.1073 N sodium hydroxide


apparent pH
14
13
12
11
10
9
8
7
6
5
4
3
0
2
4
6
8
10
26
28
30
32
34
35
38
40
42
44
46
48
Sodium Hydroxide - ml.

replaceable hydrogens per molecule, was 180.18. This gave a purity of 99.9 per cent for gamma-dimethyldihydroxyfluoran.

Beta-dimethyldihydroxyfluoran was dissolved in absolute ethanol and titrated potentiometrically with standard sodium hydroxide in ethanol. The data was plotted as apparent pH versus volume titrant. (Figure 13.) One end point was observed. The change in apparent pH in the end point region was smaller than that observed with gamma-dimethyldihydroxyfluoran, structurally proposed to be the stronger acid. A first derivative plot was constructed to determine the end point of the titration. The apparent pH at 0 per cent titration was 6.10, at 50 per cent 11.21 and at 100 per cent 12.59. At 50 per cent titration, pH equals pKa in aqueous titrimetry. At 50 per cent titration of beta-dimethyldihydroxyfluoran the apparent pH was 11.21 whereas of gamma-dimethyldihydroxyfluoran was 9.23. This indicated that gamma-dimethyldihydroxyfluoran was a stronger acid than beta-dimethyldihydroxyfluoran. The experimental equivalent weight of beta-dimethyldihydroxyfluoran was 180.06, the theoretical 180.18, the calculated purity 99.9 per cent.

The attempted titration of alpha-dimethyldihydroxyfluoran in ethanol with standard sodium hydroxide in ethanol gave only dilution, no titration. (Figure 14.) Therefore, alpha-dimethyldihydroxyfluoran was a weaker acid than both gamma- and beta-dimethyldihydroxyfluoran in ethanol, as expected from structure assignment.

Alpha-dimethyldihydroxyfluoran was dissolved in pyridine, a solvent more basic than ethanol and titrated with standard sodium hydroxide. One end point in the graph of apparent pH versus volume

Figure 13. Non-aqueous potentiometric titration of beta-dimethyldihydroxyfluoran in ethanol with sodium hydroxide

0.6424 g. acid
0.1073 N sodium hydroxide

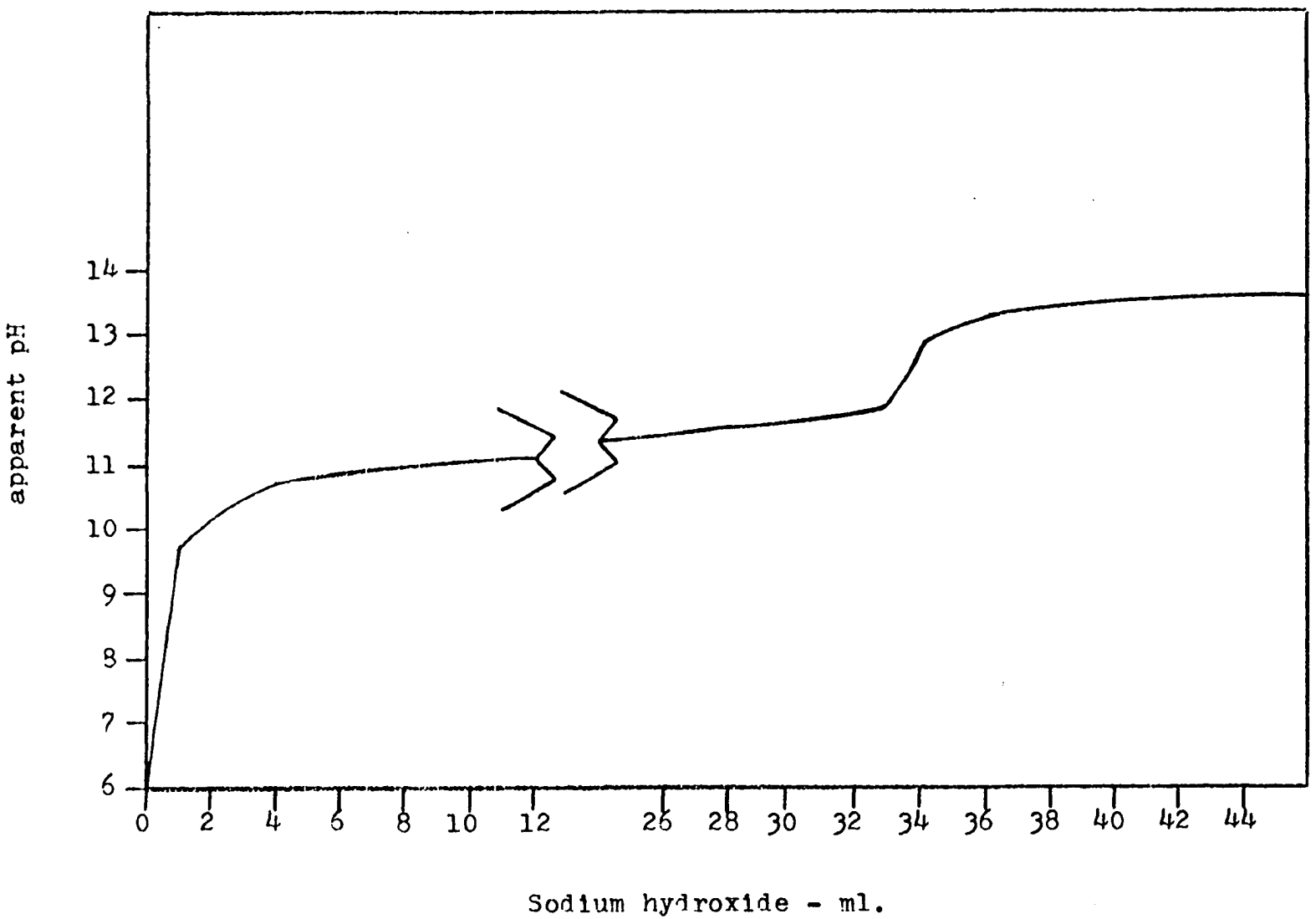
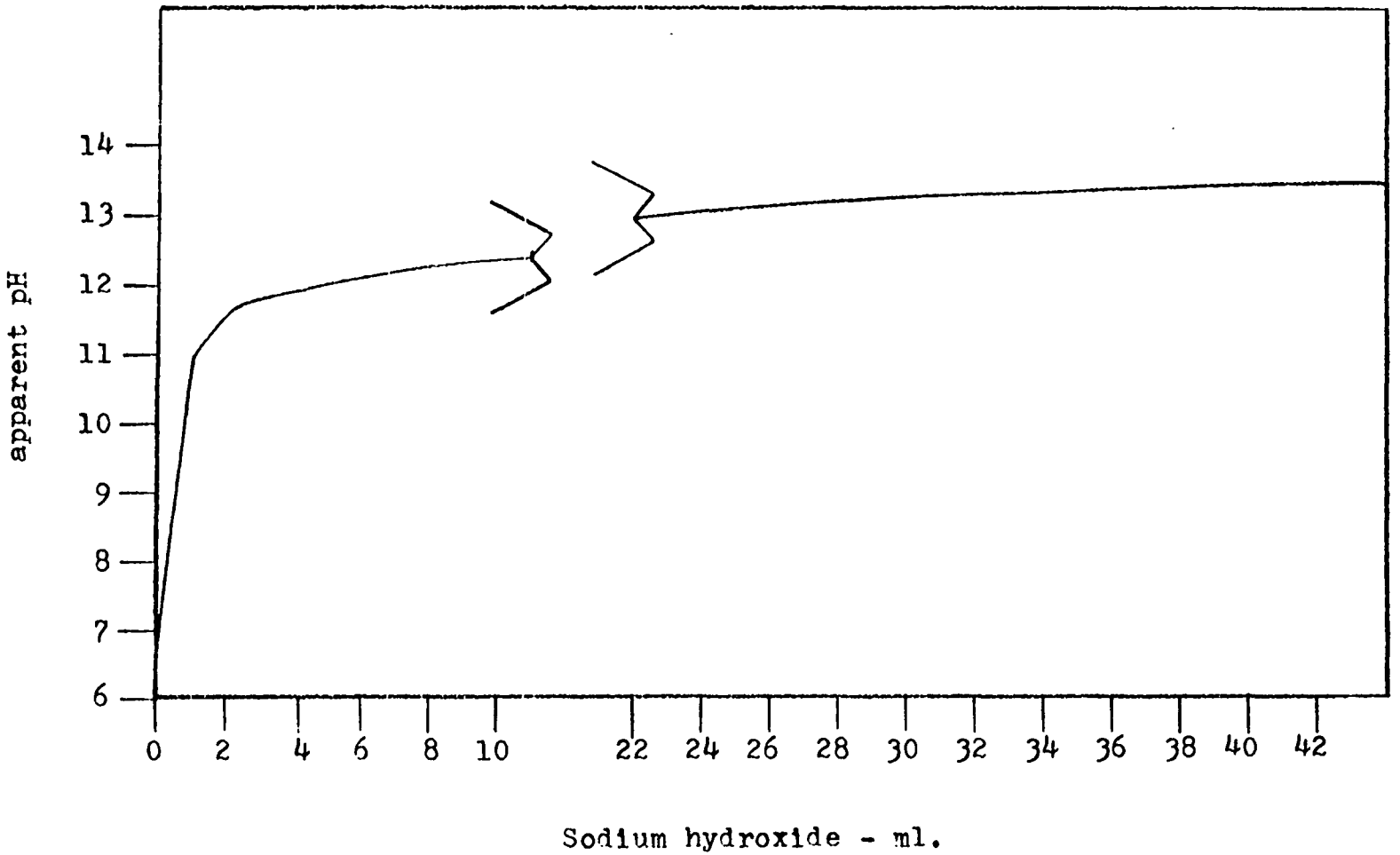

apparent pH
14
13
12
11
10
9
8
7
6
0
2
4
6
8
10
12
26
28
30
32
34
36
38
40
42
44
Sodium hydroxide - ml.

Figure 14. Attempted non-aqueous potentiometric titration of alpha-dimethyldihydroxyfluoran in ethanol with sodium hydroxide

0.6229 g. acid
0.1073 N sodium hydroxide


apparent pH
14
13
12
11
10
9
8
7
6
0
2
4
6
8
10
22
24
26
28
30
32
34
36
38
40
42
Sodium hydroxide - ml.

titrant was observed. (See Figure 15.) A first derivative plot was constructed to determine the end point of the titration. The experimental equivalent weight was 180.23, the theoretical 180.18, the purity 100.0 per cent.

By means of non-aqueous titrimetry, the equivalent weight of alpha-, beta- and gamma-dimethyldihydroxyfluoran was determined. All were 180.18. This corresponded to the titration of two replaceable hydrogen atoms per molecule.

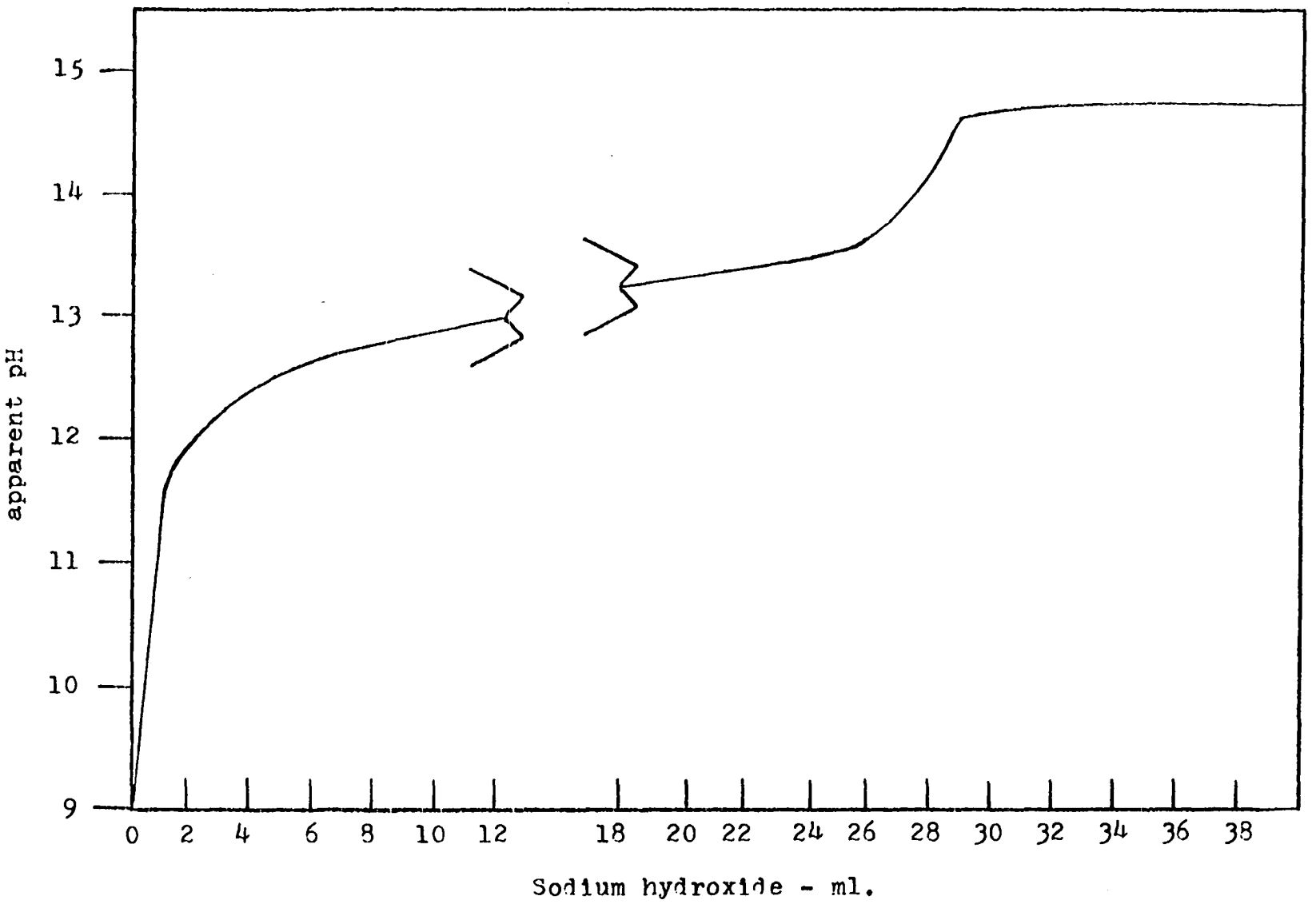
The purity of alpha-, beta- and gamma-dimethyldihydroxyfluoran was 99.9 per cent plus. The amount of water, organic solvent of crystallization, or any entrapped inorganic salt was negligible.

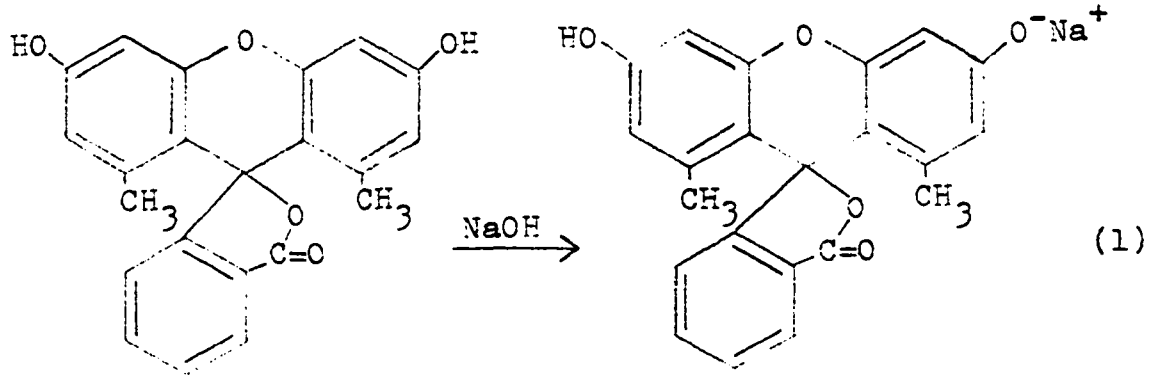
The structure assignments from predicted acidity was borne out by the solvent necessary for the non-aqueous titrations and by the apparent pH at 50 per cent titration in a given solvent.

1. Titrations in water The solubility in aqueous media and the first acid dissociation constant of alpha-, beta- and gamma-dimethyldihydroxyfluoran were determined by aqueous potentiometric microtitration. The determination was hampered by the low solubility and the weak acidic nature of the compounds.

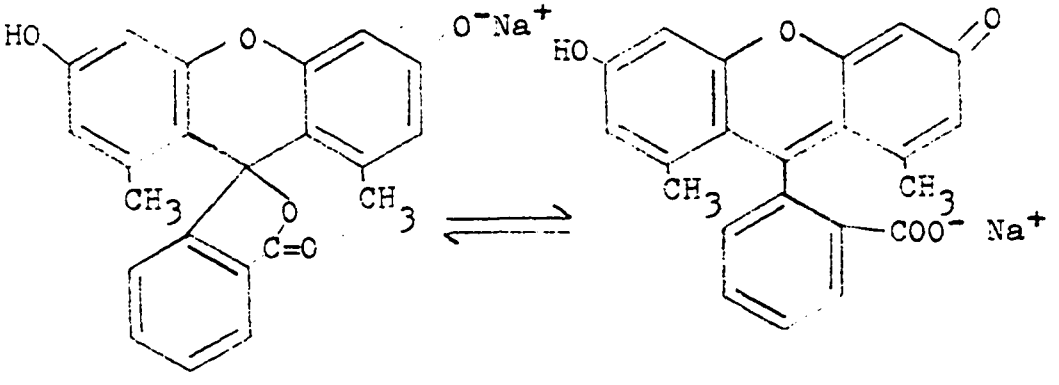
Figure 15. Non-aqueous potentiometric titration of alpha-dimethyldihydroxyfluoran in pyridine with sodium hydroxide

0.5347 g. acid
0.1075 N sodium hydroxide

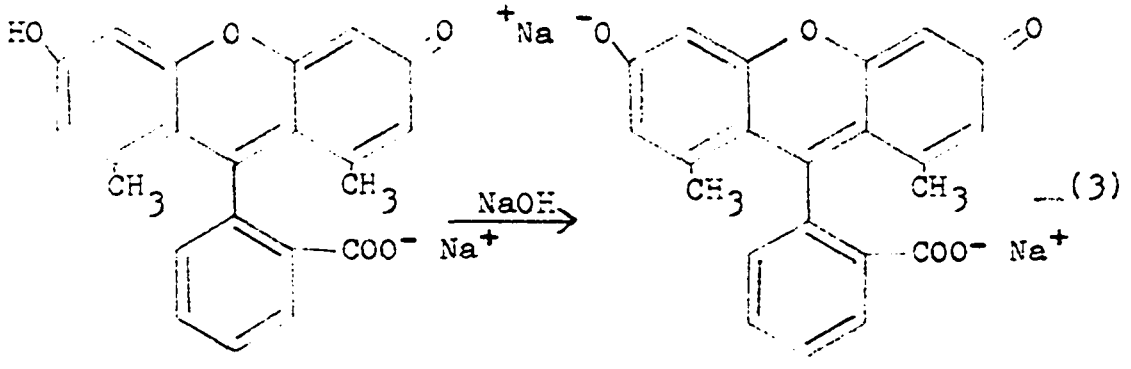

15
14
13
12
11
10
9
apparent pH
0
2
4
6
8
10
12
18
20
22
24
26
28
30
32
34
36
38
Sodium hydroxide - ml.


HO
O
OH
CH3
CH3
O
C=O
NaOH
HO
O
O⁻Na⁺
CH3
CH3
O
C=O

(1)


HO
O
O⁻Na⁺
CH3
CH3
O
C=O
HO
O
O
CH3
CH3
COO⁻ Na⁺

(2)


HO
O
O
⁺Na ⁻O
CH3
CH3
COO⁻ Na⁺
NaOH
O
O
CH3
CH3
COO⁻ Na⁺

(3)

The product of reaction (1) is further stabilized by resonance - reaction (2). Reaction (3) taking place would necessitate the removal of a second phenolic proton from an already weak acid. Such a product would not be further stabilized by resonance. The titration of the second phenolic group was not possible in water solution. The limited solubility in water was also a major drawback.

The micro-titration data was plotted as pH versus volume titrant. The pKa was assumed to equal the pH at the midpoint of the respective titration. (See Figures 16, 17, 18). Knowing the exact initial volume, the exact volume and concentration of reagent added and the end point, the solubility of each isomer was calculated. (See Table 7.)

As expected, gamma-dimethyldihydroxyfluoran proved to be a stronger acid than beta- and the latter stronger than alpha-. Likewise, gamma-dimethyldihydroxyfluoran was the most soluble and alpha- the least soluble in water. This data was in accord with the proposed structures.

m. Acid dissociation constants by solubility measurements

The acid dissociation constant of one of the phenolic protons of beta-dimethyldihydroxyfluoran was determined by the method of Krebs and Speakman (9). This method is based on the solubility of an acid as a function of pH. The data obtained is summarized in Table 8. This method is directly applicable to a monobasic acid. Beta-dimethyldihydroxyfluoran has two phenolic groups, however, only one is involved in the neutralization over the pH range 6.08 to 8.00.

For a monobasic acid, the theory can be summarized as follows: For a given monobasic acid, HA,

$$HA = H^+ + A^- \qquad (1)$$

and

$$K_a = \frac{(H^+)(A^-)}{(HA)} \qquad (2)$$

The acid is present in solution in the two forms HA and A^-. The relative amount of HA and A^- present is a function of pH. The total

Figure 16. Aqueous potentiometric microtitration of alpha-dimethyldihydroxyfluoran with sodium hydroxide

Saturated solution of acid
0.1086 N sodium hydroxide

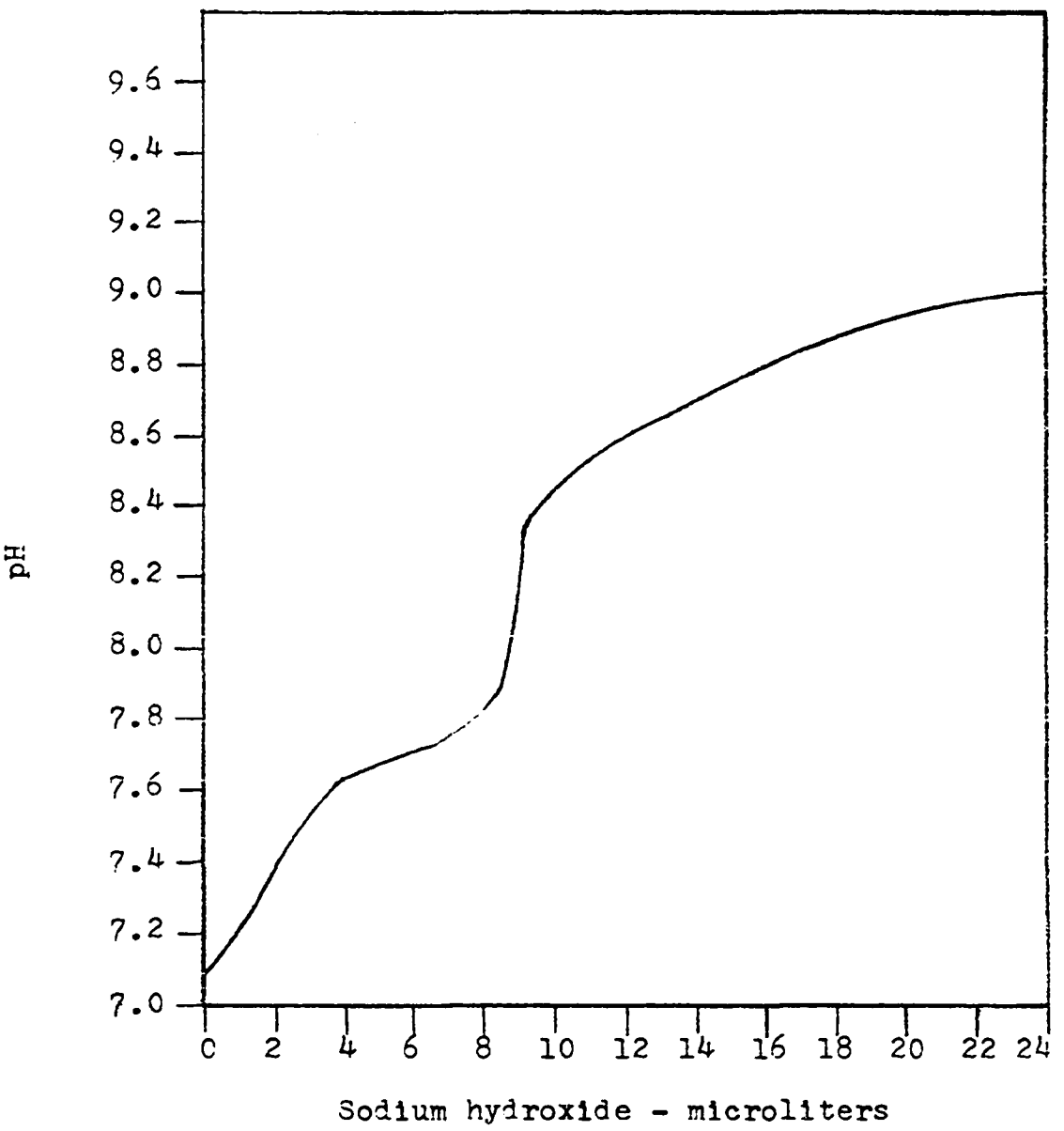

pH
9.6
9.4
9.2
9.0
8.8
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
0
2
4
6
8
10
12
14
16
18
20
22
24
Sodium hydroxide - microliters

Figure 17. Aqueous potentiometric microtitration of beta-dimethyldihydroxyfluoran with sodium hydroxide

Saturated solution of acid
0.1086 N sodium hydroxide

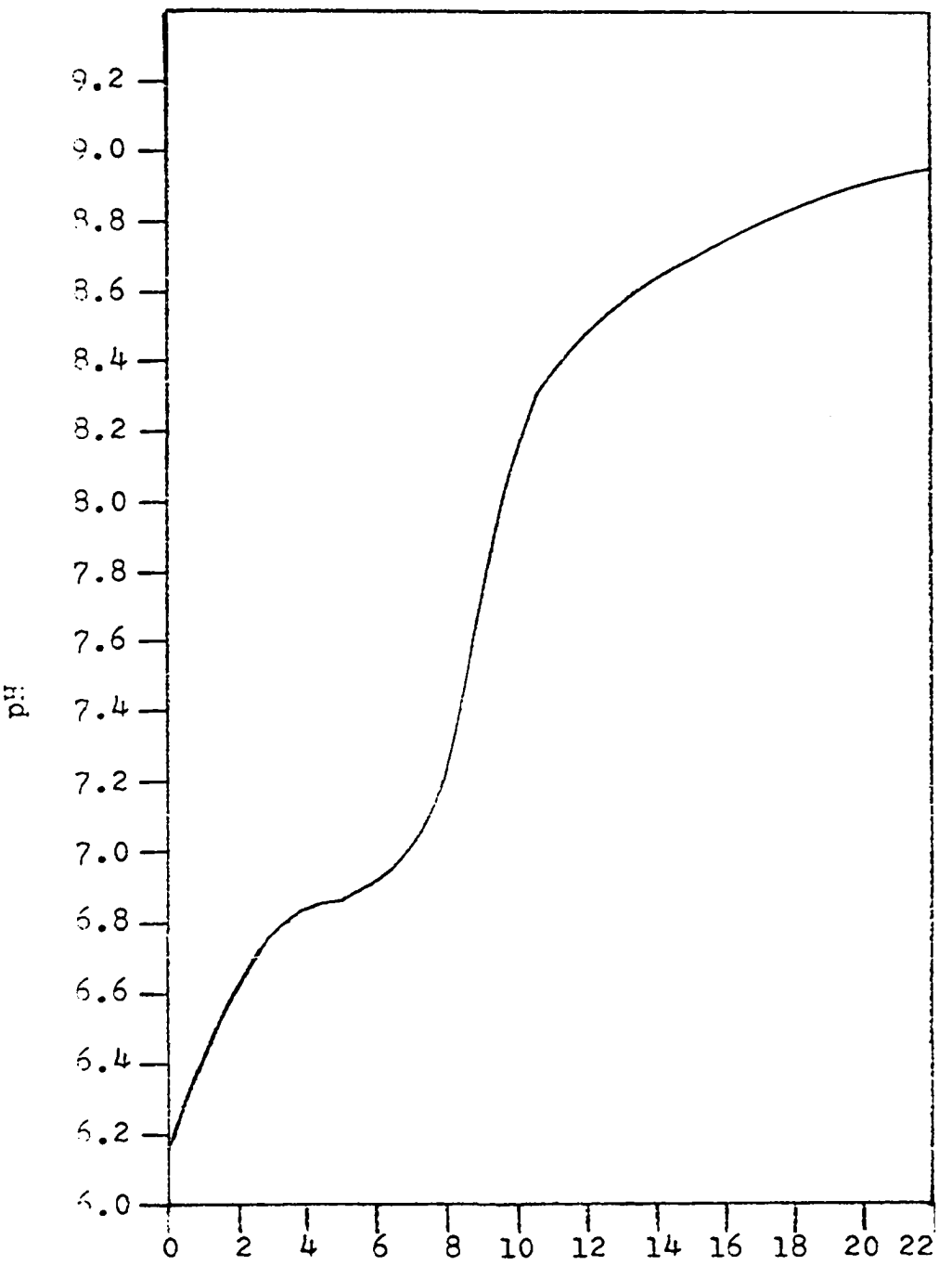

9.2
9.0
8.8
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
pH
0
2
4
6
8
10
12
14
16
18
20
22
Sodium hydroxide - microliters

Figure 18. Aqueous potentiometric microtitration of gamma-dimethyldihydroxyfluoran with sodium hydroxide

Saturated solution of acid
0.1086 N sodium hydroxide

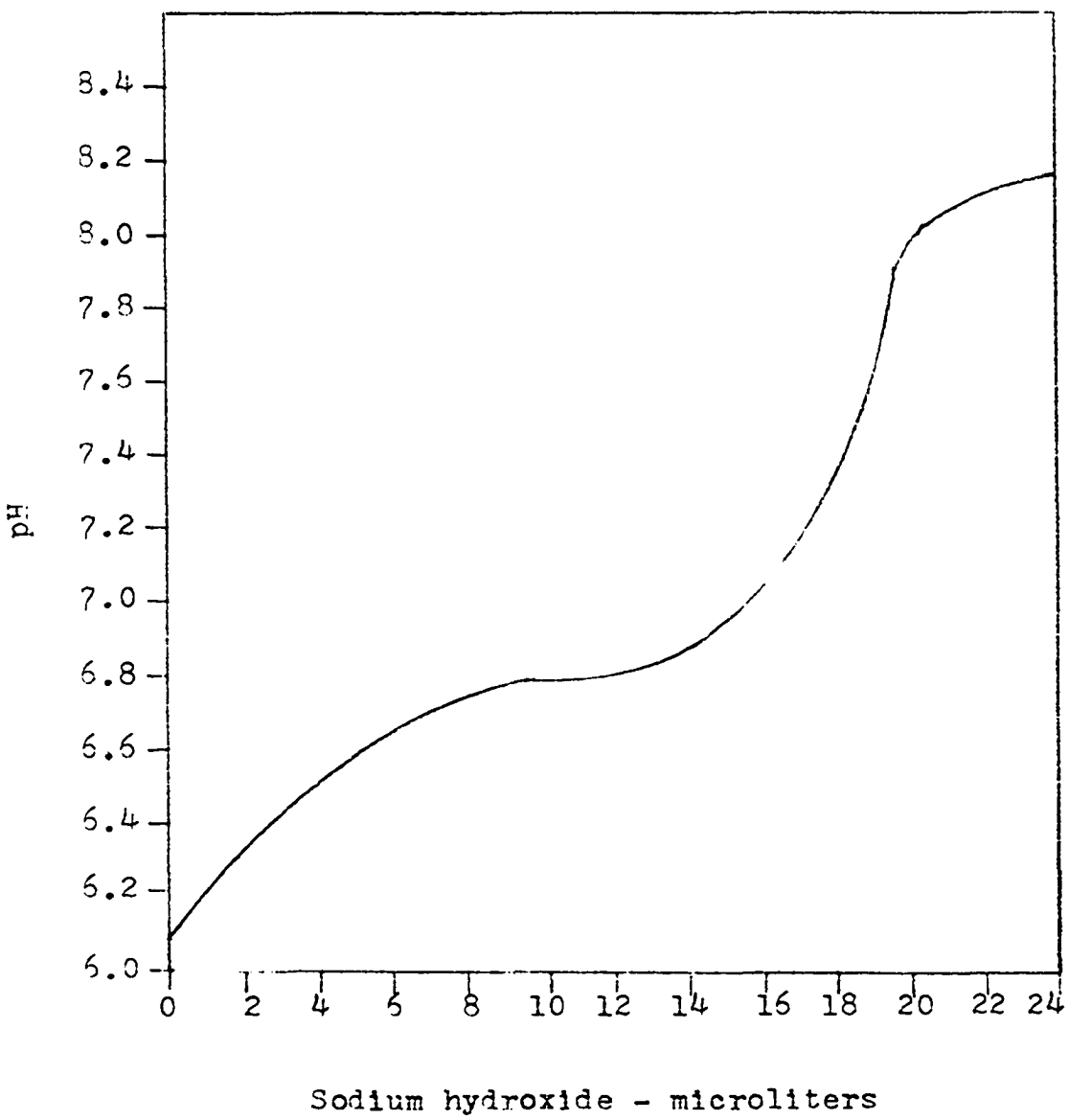

8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
pH
0
2
4
6
8
10
12
14
16
18
20
22
24
Sodium hydroxide - microliters

Table 7. The acid dissociation constants of the phenolic group proton and the solubility of alpha-, beta- and gamma-dimethyldihydroxyfluoran determined from potentiometric microtitration measurements

Compound	Ka	pKa	Solubility	
			mmoles/ml.	mg./100 ml
alpha-dimethyldihydroxyfluoran	2.19×10^{-8}	7.66	1.80×10^{-5}	6.48
beta-dimethyldihydroxyfluoran	1.12×10^{-7}	6.95	1.86×10^{-5}	6.70
gamma-dimethyldihydroxyfluoran	1.62×10^{-7}	6.79	3.80×10^{-5}	13.68

Table 8. Acid dissociation constants of the phenolic acid group proton of beta-dimethyl-dihydroxyfluoran determined from solubility measurements

Compound	S_i	pH	S_o (mmoles/ml.)	pKa (Equation 9)	pKa (graph)
beta-dimethyl-dihydroxyfluoran	4.50×10^{-6} (0.162 mg/100 ml.)	8.00	14.90×10^{-6}	7.64	
		7.50	7.53×10^{-6}	7.67	
		7.00	5.64×10^{-6}	7.60	
		6.08	4.63×10^{-6}	7.62	7.64
			(Average)	7.63	

material in solution, the solubility, S_o, is the sum of the neutral molecule and of the anion in solution.

$$S_o = (HA) + (A^-) \qquad (3)$$

It is assumed that the solubility of the unionized acid is constant over the pH range. The solubility of the neutral molecule, HA, is the intrinsic solubility, S_i. Such that,

$$S_i = (HA) \qquad (4)$$

and

$$S_o = S_i + (A^-) \qquad (5)$$

From the equilibrium expression,

$$(A^-) = \frac{Ka\,(HA)}{(H^+)} \qquad (6)$$

such that

$$S_o = S_i + \frac{K_a\, S_i}{(H^+)} \qquad (7)$$

At high hydrogen ion concentration, S_i approaches S_o. The activities of the neutral molecule and the anion are assumed to be one. Rearrangement of Equation (7) gives

$$S_o = S_i \left[1 + \text{antilog}\,(pH - pK_a)\right] \qquad (8)$$

Rearranging Equation (8) gives

$$pK_a = pH - \log\left(\frac{S_o}{S_i} - 1\right) \qquad (9)$$

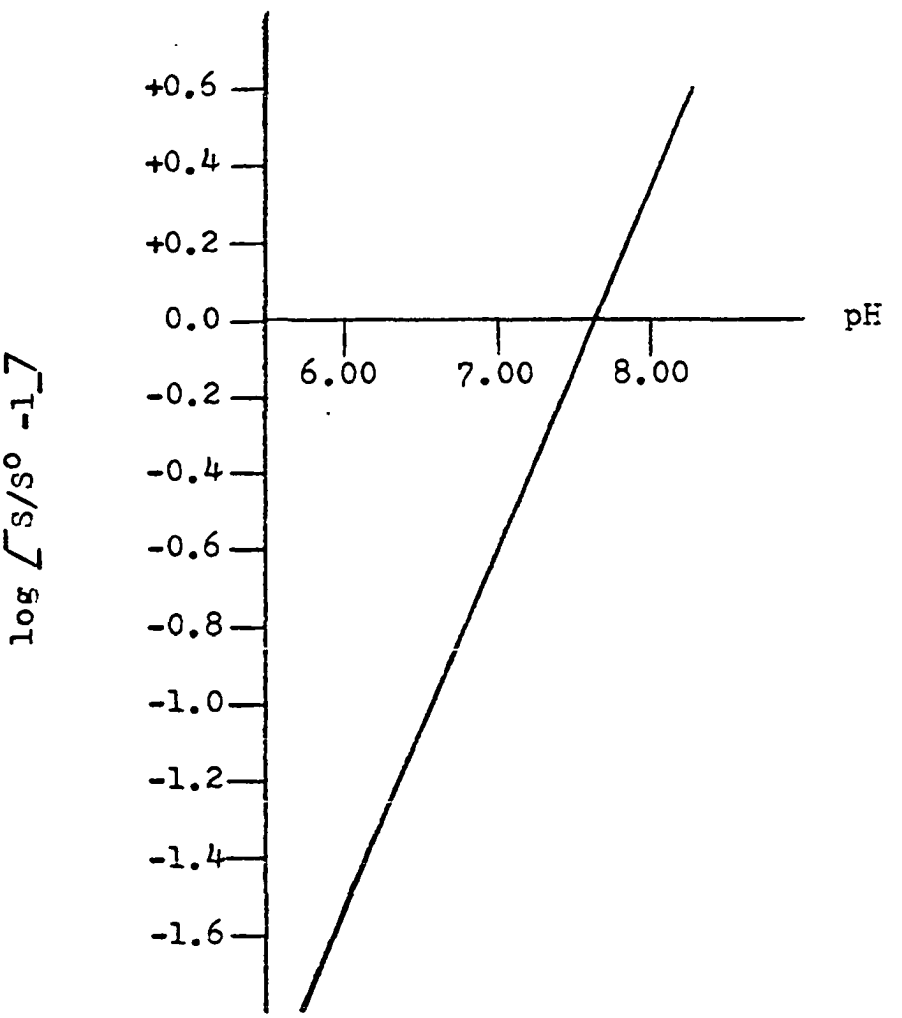
Prior to use of Equation (9), S_i must be determined. S_i can be determined by plotting S_o versus $1/(H^+)$. According to Equation (7), extrapolation of $1/(H^+)$ to 0 gives S_i. (Figure 2.) S_i for beta-dimethyl-dihydroxyfluoran was found to be 0.162 mg./100 ml. The acid dissociation constant was then calculated using Equation (9) and by plotting $\log\left[S_o/S_i - 1\right]$ versus pH. In the latter case, the pK_a is the

pH at which the straight line intersects the abscissa. Both methods yielded essentially identical results. The results are summarized in Table 8. The intercept of the line in Figure 19 is 7.62, that is, pK_1 7.62. The average pK value obtained by use of Equation (8) is 7.63, $K_1 = 2.34 \times 10^{-8}$.

n. Acid dissociation constants by spectrophotometric measurements The first acid dissociation constant of the phenolic proton of alpha- and gamma-dimethyldihydroxyfluoran was determined spectrophotometrically. The absorption spectra in the ultraviolet and visible wavelength regions of alpha- and gamma-dimethyldihydroxyfluoran are shown in Figures 3, 4, 5 and 6. In acid solution, one wavelength of maximum absorption in the ultraviolet region was observed; there was no absorbance in the visible wavelength region. In basic solution, one wavelength of maximum absorption in the ultraviolet and visible region was observed. The measured absorbance and the change in absorbance in the pH range numerically equal to the estimated pKa in the ultraviolet region were of much smaller magnitude than in the visible. Thus the absorbance in the visible wavelength region as a function of pH was used in the calculation of pK_1.

The absorbance in the visible wavelength region changed markedly with pH. The absorbance at 528 nm. and 486 nm. increased with increasing pH. This change in absorption spectra was attributed to the neutralization of one phenolic proton. Only one point of inflection was present in the curve in the given pH range. The pH at the point of

Figure 19. Change in log $[S/S^{o} - 1]$ of beta-dimethyldihydroxyfluoran with pH


+0.6
+0.4
+0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
6.00
7.00
8.00
pH
log [S/S° -1]

maximum slope is a good approximation of pKa. Knowing the pKa, the log-ratio method was used to accurately determine the first acid dissociation constant of the phenolic proton of alpha- and gamma-dimethyldihydroxyfluoran.

The log-ratio method can be derived as follows. For the reaction

$$HA = H^+ + A^- \qquad (10)$$

and acid dissociation constant is defined as

$$Ka = \frac{(H^+)(A^-)}{(HA)} \qquad (11)$$

The ratio of $(A^-)/(HA)$ can be described by the ratio of the absorbances

$$\frac{A_{mixture} - A_{HA}}{A_{A^-} - A_{mixture}} \qquad (12)$$

Substituting Equation(12) into Equation(11) and taking the negative logarithms

$$pKa = pH - \log \left(\frac{A_{mixture} - A_{HA}}{A_{A^-} - A_{mixture}} \right) \qquad (13)$$

By substituting the proper values of pH and absorbances into Equation (13) the pK_a values were obtained. The values calculated for the first acid dissociation constant of the phenolic proton of alpha- and gamma-dimethyldihydroxyfluoran are given in Table 9.

Table 9. Acid dissociation constants of the phenolic protons of alpha- and gamma-dimethyldihydroxyfluoran determined from spectroscopic measurements

Compound	pH	pKa
alpha-dimethyl-dihydroxyfluoran	10.40	9.97
	10.30	9.96
	10.10	9.97
	9.90	9.94
	9.70	9.95
	9.40	9.96
		9.96 (Average)
gamma-dimethyl-dihydroxyfluoran	7.11	6.65
	7.00	6.67
	6.80	6.67
	6.60	6.66
	6.40	6.64
	6.10	6.66
		6.66 (Average)

III. THE METHYLENEIMINODIACETIC ACID DERIVATIVES OF THE DIMETHYLDIHYDROXYFLUORANS

A. Experimental Work

1. Synthesis

a. Apparatus and reagents Infrared spectra were obtained using a Perkin-Elmer Model 21 Infra Red Spectrophotometer.

Nuclear magnetic resonance spectra were obtained using a Varian Associates A-60 Nuclear Magnetic Resonance Spectrometer.

Mass spectra were obtained using an Atlas CH4 Mass Spectrometer.

Clear, precision ground glass NMR tubes of 0.5 cm. o.d. were used for all NMR work.

Measurements of pH were made using a Leeds and Northrup pH meter equipped with a Corning No. 476024 Triple-Purpose glass electrode and a Corning sleeve-type saturated calomel electrode.

Glycine, 99.5 per cent minimum, was obtained from the J. T. Baker Chemical Company.

Iminodiacetic acid, disodium salt, monohydrate, was obtained from the Geigy Chemical Company, and purified as described on pages 104 and 105.

J. T. Baker Chemical Company reagent-grade sodium hydroxide was used to prepare a 50 per cent stock solution. The solution was allowed to stand for two days to allow the sodium carbonate to precipitate out. Approximately 5.5 ml. of the stock solution was then

diluted with freshly boiled deionized water to give a carbonate-free 0.1 N solution. The 0.1 N solution of sodium hydroxide was standardized against primary standard potassium biphthalate using phenolphthalein indicator.

Alpha-, beta- and gamma-dimethyldihydroxyfluoran, respectively, were synthesized, separated and purified as described on pages 8-9 and 12.

Formaldehyde solution containing 37 per cent formaldehyde was obtained from the J. T. Baker Chemical Company.

All the water used was distilled and deionized by passage through Amberlite MB-1 ion exchange resin.

All reagent chemicals were of reagent grade quality.

b. Purification and analysis of glycine and iminodiacetic acid

Commercial glycine was dissolved in hot water and precipitated by the addition of absolute ethanol. The material was recrystallized twice, filtered and dried under reduced pressure at 45° for 24 hours. The glycine, a white powder, was stored in a desiccator over magnesium perchlorate.

Commercial iminodiacetic acid, disodium salt monohydrate, (IDA), was dissolved in hot water and precipitated by the addition of absolute ethanol. The material was recrystallized three times. The first mother liquor was dirty-grey-yellow in color; the final mother liquor was colorless. The material was vacuum dried under reduced pressure at 45° for 24 hours. The IDA, a white crystalline solid, was stored in a desiccator over magnesium perchlorate.

Iminodiacetic acid was prepared from the iminodiacetic acid,

disodium salt monohydrate. Dilute hydrochloric acid was slowly added to an aqueous solution of IDA until the pH of the solution measured 2. A white, crystalline solid precipitated. This solid was filtered, washed, filtered and vacuum dried under reduced pressure at 45° for 24 hours. The free acid was stored in a desiccator over magnesium perchlorate.

The purity and composition of the recrystallized glycine and IDA were determined by:

1. Loss on heating
2. Melting point
3. Infrared spectroscopy
4. Nuclear magnetic resonance spectroscopy
5. Mass spectroscopy
6. Nitrogen analysis by the Kjeldahl method
7. Equivalent weight by neutralization

1. Loss on heating A known weight on purified IDA was heated for 24 hours at 110°.

2. Melting point Melting point determinations were made with a mercury thermometer by placing the sample between two cover glasses on the stage of a polarizing microscope which was heated electrically at a constant rate.

3. Infrared spectroscopy Infrared spectra were obtained on potassium bromide pellets of IDA and glycine. Spectra were run on the commercial IDA as obtained, the purified IDA, the commercial glycine, and the purified glycine. A spectra of ethanol, neat, was also obtained.

4. Nuclear magnetic resonance spectroscopy A solution of iminodiacetic acid, disodium salt monohydrate, and glycine, respectively, in D_2O, dimethyl sulfoxide, acetone and trifluoroacetic acid, respectively, were used to obtain the spectra. The reference standard was either Tier's Salt when D_2O was used as a solvent or tetramethyl silane (TMS) when other organic solvents were used.

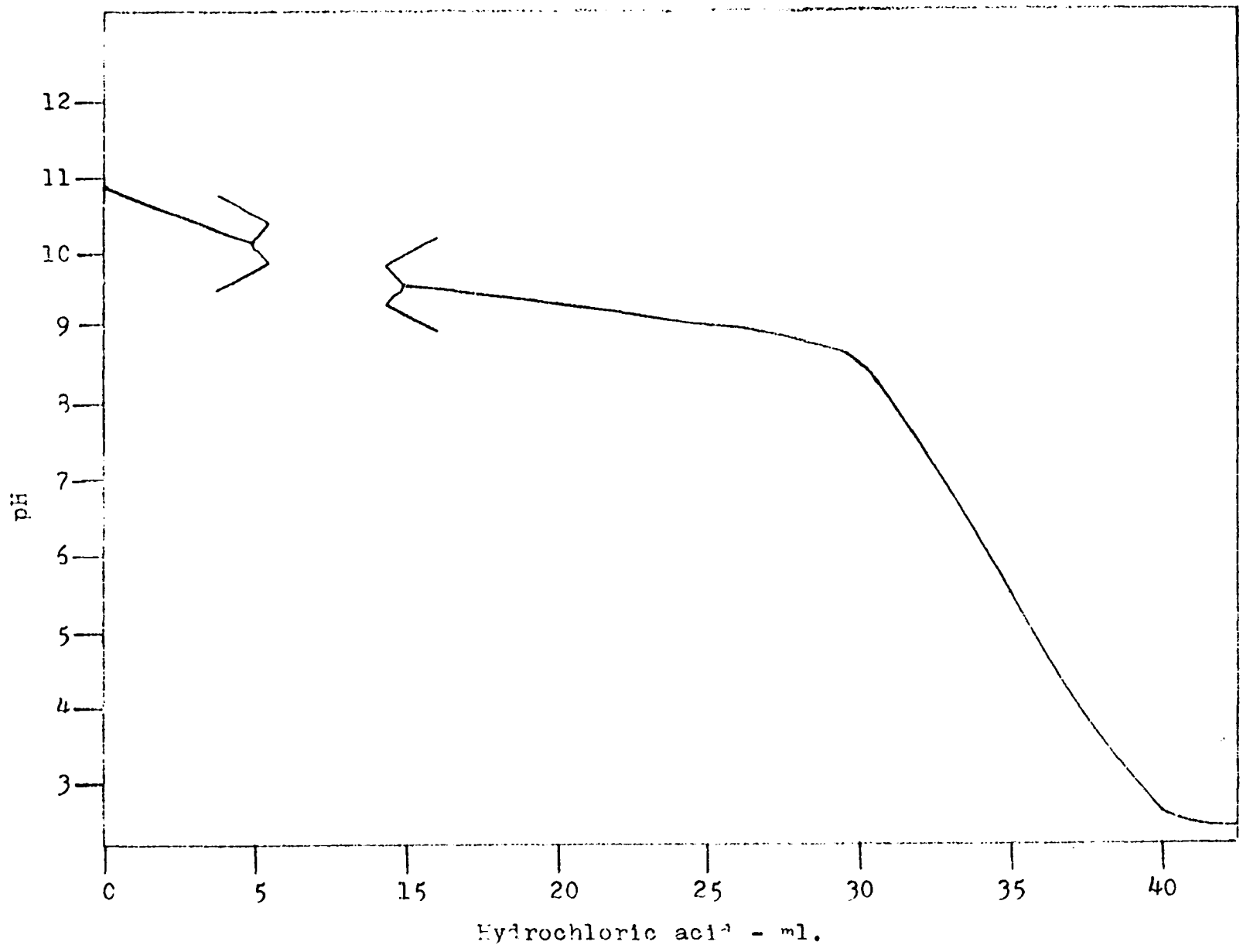
5. Mass spectroscopy Mass spectra of glycine, iminodiacetic acid, disodium salt monohydrate, and iminodiacetic acid (free acid) were obtained at an electron beam energy of 70 electron volts.

6. Nitrogen analysis by the Kjeldahl method To ascertain purity, glycine and iminodiacetic acid disodium salt monohydrate were analyzed for percentage nitrogen by the Kjeldahl method. Weighed samples were digested with concentrated sulfuric acid with copper selenite as catalyst and silicon carbide boiling chips. The ammonium sulfate thus produced was distilled as ammonia gas, by the addition of 40 per cent sodium hydroxide, into a measured excess of 0.1007 N hydrocholoric acid. The solution containing the distilled ammonia and excess hydrochloric acid was back-titrated with 0.1086 N sodium hydroxide.

7. Equivalent weight by neutralization The neutralization equivalent weight of iminodiacetic acid disodium salt monohydrate was obtained by titrating a known weight of the material dissolved in 50 ml. of deionized water with 0.1007 N hydrochloric acid. The titration curve obtained is shown in Figure 20-21.

Figure 20-21. Aqueous potentiometric titration of iminodiacetic acid disodium salt monohydrate with hydrochloric acid

0.7050 g. base
0.1007 N hydrochloric acid


12
11
10
9
8
7
6
5
4
3
pH
0
5
15
20
25
30
35
40
Hydrochloric acid - ml.

c. Condensation of alpha-, beta- and gamma-dimethyldihydroxyfluoran with iminodiacetic acid and formaldehyde

To 80 ml. of glacial acetic acid at 70° was added 0.009 moles (3.24 g.) of the respective alpha-, beta- or gamma-dimethyldihydroxyfluoran and 0.030 moles (5.85 g.) of disodium iminodiacetate monohydrate with stirring. This was followed by the dropwise addition of 0.027 moles (2.22 ml.) of 37 per cent formaldehyde. The reaction was allowed to proceed at 68-73° for two hours with constant stirring. The alpha-dimethyldihydroxyfluoran mixture became dark brown, the beta-dimethyldihydroxyfluoran mixture brown-black, and the gamma-dimethyldihydroxyfluoran mixture red-green within a few minutes of mixing. The solution was filtered although very little insoluble material was present. The mixture was then poured into two liters of water at 70°. The mixture containing the alpha-dimethyldihydroxyfluoran derivative became brown, the beta-derivative yellow-brown and the gamma-derivative red-orange. Dilute hydrochloric acid (3M) was added dropwise until the pH of the mixture reached 1.60, the pH of maximum insolubility. The mixture was digested at 70° for two hours to ensure better crystal formation and filtration. The mixture was allowed to stand overnight, then filtered. The solid was recrystallized by dissolution in two liters of water at 70° by adjusting the pH to 7.00 with 3M sodium hydroxide and then precipitating the material by dropwise addition of 3M hydrochloric acid to pH 1.60. The material was digested at 70° for two hours and then allowed to stand overnight. The material was filtered, air dried for four hours and then vacuum dried at room temperature for 24 hours. The material was stored in a

desiccator over magnesium perchlorate.

2. Properties and structure

a. Apparatus and reagents Mass spectra were obtained using an Atlas CH4 Mass Spectrometer.

Infrared spectra were obtained using a Perkin-Elmer Model 21 Infra Red Spectrophotometer.

Nuclear magnetic resonance spectra were obtained using a Varian Associates A-60 Nuclear Magnetic Resonance Spectrometer. Clear, precision ground glass NMR tubes of 0.5 cm. o.d. were used for all NMR work.

A Leeds and Northrup pH meter was used to monitor the non-aqueous and aqueous potentiometric titrations. The glass indicating electrode was a Corning silver-silver chloride internal reference, high alkalinity electrode, catalog #476024. A modified sleeve-type saturated calomel electrode was used as the reference electrode in the non-aqueous potentiometric titrations. A saturated solution of potassium chloride in methanol was used as the internal salt solution. A saturated calomel sleeve-type electrode was used as the reference electrode in the aqueous potentiometric titrations.

Ultraviolet and visible spectra were obtained using a Cary 14 Spectrophotometer. Perkin-Elmer quartz cells of 10.0 mm. internal path length or Aminco quartz cells of 50.017 internal path length were used for all spectrophotometric work.

The presence of fluorescence was detected using radiation from a General Electric 15 watt fluorescent lamp.

The methyleneiminodiacetic acid derivatives of the parent dimethyldihydroxyfluorans were synthesized and purified as described on pages 109-110.

Trifluoroacetic acid was obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin.

Ethylenediamine was obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Pyridine, dimethyl sulfoxide, and isopropyl alcohol were obtained from Mallinckrodt, St. Louis, Missouri.

Tetraethylammonium bromide was obtained from the Matheson, Coleman and Bell Chemical Company, Norwood, Ohio.

All water used was distilled and deionized by passage through Amberlite MB-1 ion exchange resin.

All reagent chemicals were of reagent grade quality.

b. Mass spectroscopy Mass spectra were obtained at an electron beam energy of 70 electron volts. The mass scale up to 700 mass units was scanned. The highest recorded peaks for each isomer were 650 and 651 mass units.

c. Infrared spectroscopy Infrared spectra were obtained on potassium bromide pellets of the methyleneiminodiacetic acid derivatives of the respective alpha-, beta- and gamma-dimethyldihydroxyfluoran.

d. Nuclear magnetic resonance spectroscopy A solution of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneimino-diacetic acid, respectively, in trifluoroacetic acid was used to obtain the spectra. The reference standard was tetramethyl silane (TMS).

e. Elemental analysis Alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid were analyzed directly for carbon, hydrogen and nitrogen. Oxygen was determined by difference.

f. Residue on ignition The non-volatile metal content of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid was determined by ignition. Samples of approximately 0.5 grams of each isomer were ignited in platinum crucibles at red heat with ample access to air. The weight of the residue was determined.

g. Fluorescence The presence of fluorescence was qualitatively detected using radiation from a General Electric 15 watt fluorescent lamp.

h. Solubility in non-aqueous solvents The solubility of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid was qualitatively determined in a number of solvents. The solubility was determined by adding a semimicro spatula tip of the derivative (approximately 0.1 grams) to 2 ml. of the solvent and shaking the mixture in a test tube. The mixture was allowed to stand for four hours and then the extent of dissolution was noted. The mixture in a test tube was then held in a hot to boiling water bath for four hours and the extent of dissolution was then noted.

i. Titrations in non-aqueous solvents A known amount of derivative, 200 to 270 mg. corresponding to 0.31 to 0.42 mmoles, was dissolved in the appropriate organic solvent.

Gamma- and alpha-dimethyldihydroxyfluoranmethyleneiminodiacetic acid were dissolved in ethylenediamine; beta-dimethyldihydroxy-

fluoranmethyleneiminodiacetic acid was dissolved in pyridine. Gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid dissolved rapidly, five minutes; beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid dissolved slower, one hour; and alpha-dimethyldihydroxyfluoranmethyleneiminodiacetic acid dissolved very slowly, nine hours.

The respective mixture was titrated potentiometrically. The titrant, tetraethylammonium hydroxide, was in the same solvent as the derivative being titrated.

Response of the pH meter was slow in the non-aqueous media. A thirty second time delay per addition of titrant was observed.

The tetraethylammonium hydroxide was prepared by reacting tetraethylammonium bromide with silver oxide in either methyl or isopropyl alcohol for two hours. After filtration, the solution was diluted to one liter with the appropriate solvent. This titrant was standardized daily

j. Acid dissociation constants by potentiometric titration

The second, third and fourth acid dissociation constants of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid were determined by indirect potentiometric titration. To a 115 to 290 mg. sample of derivative was added 25.00 ml. of 0.1086 M sodium hydroxide, an excess of the amount necessary to titrate the derivative. The resulting solution was then titrated potentiometrically with standard hydrochloric acid.

Beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid was also titrated by the addition of 50.00 ml. of standard sodium hydroxide

and back titrated with standard hydrochloric acid.

Each derivative was titrated until an excess of hydrochloric acid had been added. Using the total amount of derivative present, and the amount of sodium hydroxide added, the volume of hydrochloric acid required to titrate the excess sodium hydroxide and the derivative was calculated. The titration curves obtained are shown in Figures 22,23,24,25.

k. Acid dissociation constants by solubility measurements

The solubility of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid over the pH range 2.13 to 3.18, 2.31 to 3.05 and 2.10 to 3.47 was determined by buffering solutions containing an excess of derivative at specific pH values and shaking for twelve hours to ensure complete equilibration. Appropriate volumes of the filtrates containing alpha-, beta-, and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid were adjusted to pH 10.50, 10.00 and 10.00, the pH of maximum absorbance, diluted to 100 ml. in volumetric flasks with 0.10 M potassium chloride, and shaken for twelve hours to ensure complete equilibration. The amount of derivative in each solution was determined spectrophotometrically on a Carey 14 spectrophotometer. The absorbance was measured at 545 nm., 435 nm. and 493 nm. The absorbance of these solutions was related to concentration by using a calibration curve prepared by making absorbance measurements on standard solutions. The results are shown in Table 10. A graph of solubility versus $1/[H^+]$, shown in Figures 26, 27, 28, yielded the value for the intrinsic solubility of alpha-, beta-, and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid. From the values of solubility at known pH and from the intrinsic solubility, the

Figure 22. Potentiometric titration of alpha-dimethyldihydroxy-fluoranmethyleneiminodiacetic acid in excess standard sodium hydroxide with standard hydrochloric acid

0.1150 g. acid
25.00 ml. of 0.1086 N sodium hydroxide
0.1007 N hydrochloric acid

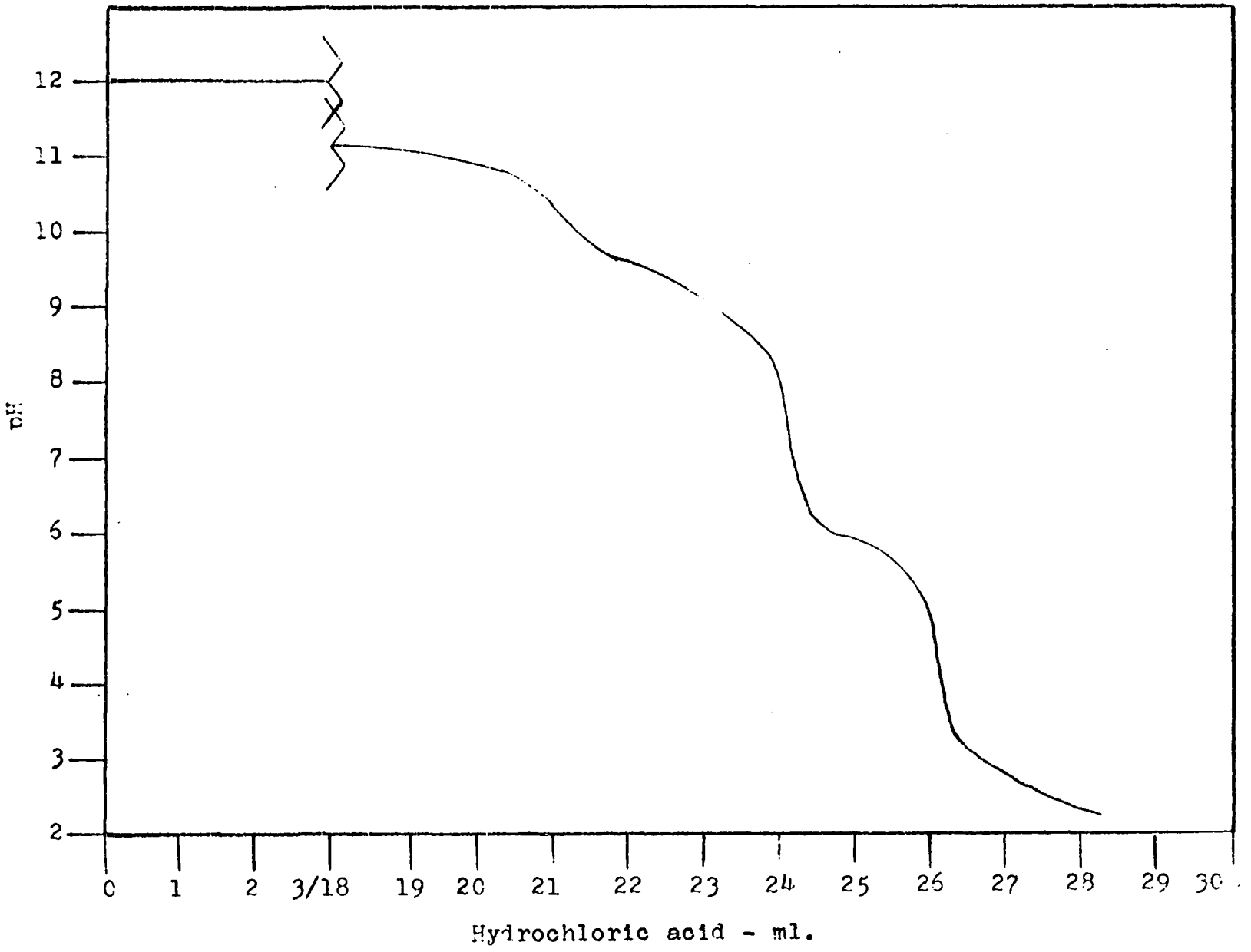

pH
12
11
10
9
8
7
6
5
4
3
2
0
1
2
3/18
19
20
21
22
23
24
25
26
27
28
29
30
Hydrochloric acid - ml.

Figure 23. Potentiometric titration of beta-dimethyldihydroxy-fluoranmethyleneiminodiacetic acid in excess standard sodium hydroxide with standard hydrochloric acid

0.2744 g. acid
25.00 ml. of 0.1086 N sodium hydroxide
0.1007 N hydrochloric acid

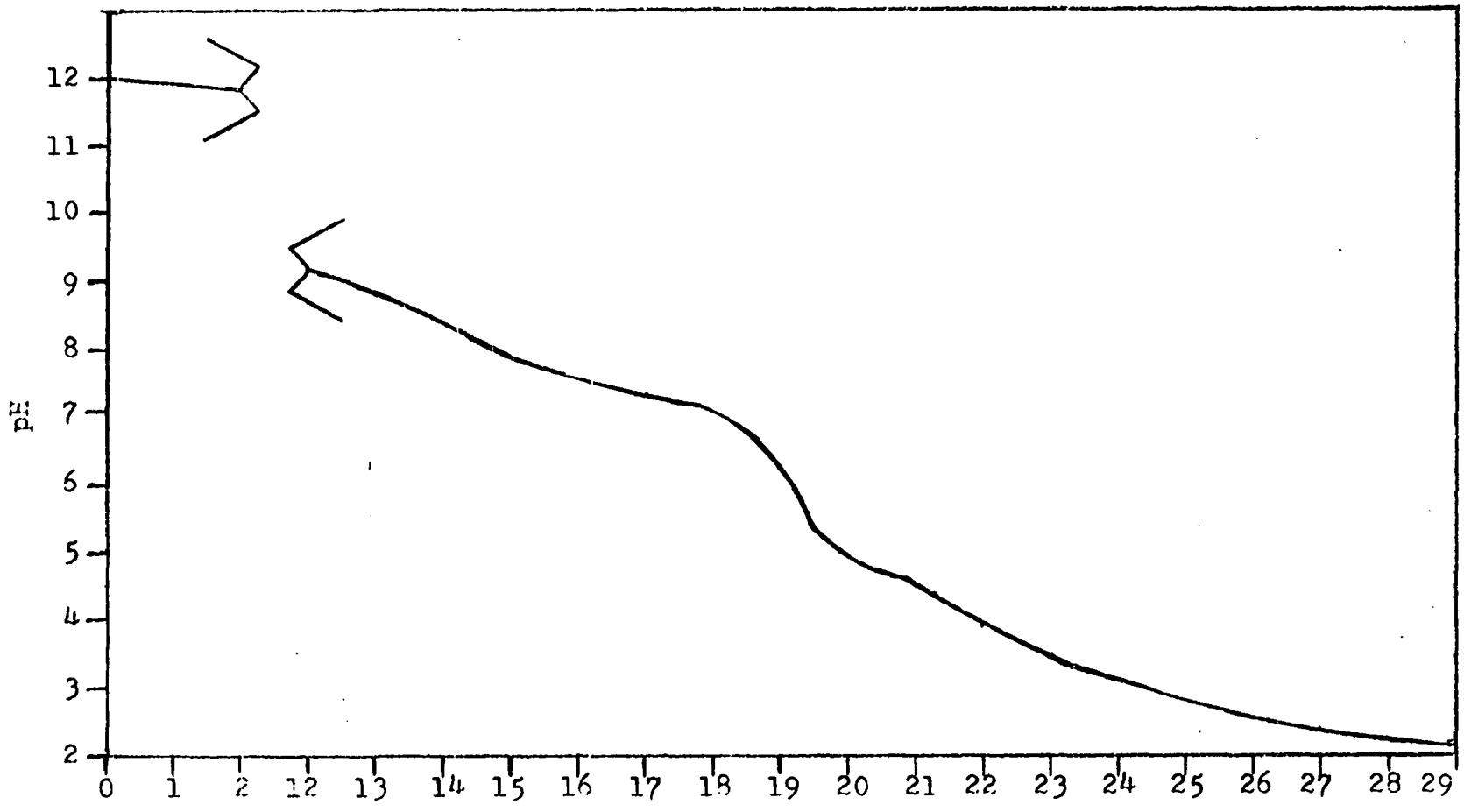

pH
12
11
10
9
8
7
6
5
4
3
2
0
1
2
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
Hydrochloric acid - ml.

Figure 24. Potentiometric titration of gamma-dimethyldihydroxyfluoran-methyleneiminodiacetic acid in excess standard sodium hydroxide with standard hydrochloric acid

0.1952 g. acid
25.00 ml. 0.1086 N sodium hydroxide
0.1007 N hydrochloric acid

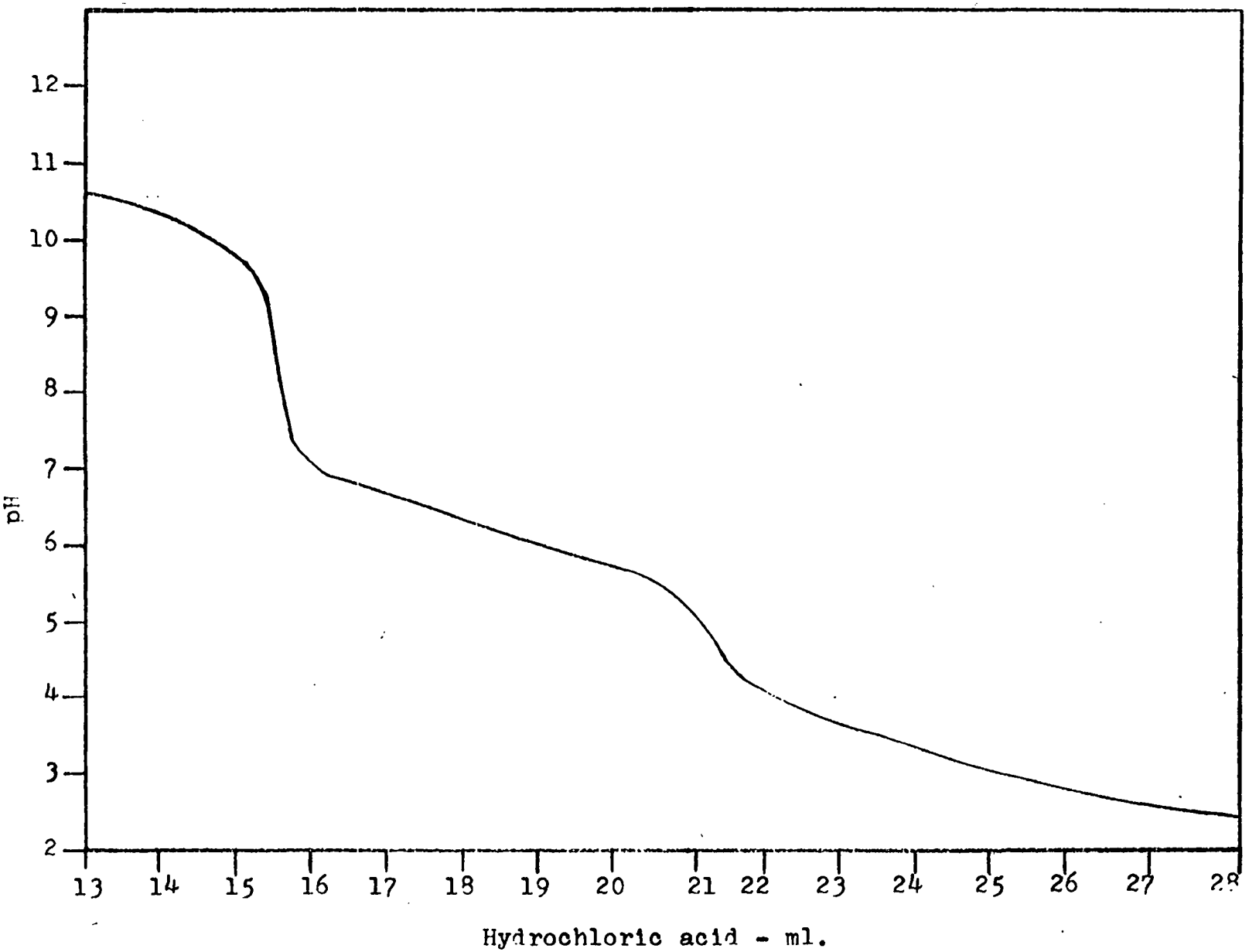

12
11
10
9
8
7
6
5
4
3
2
pH
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
Hydrochloric acid - ml.

Figure 25. Potentiometric titration of beta-dimethyldihydroxyfluoran-methyleneiminodiacetic acid in excess standard sodium hydroxide with standard hydrochloric acid

0.2895 g. acid
50.00 ml. 0.1086 N sodium hydroxide
0.1007 N hydrochloric acid

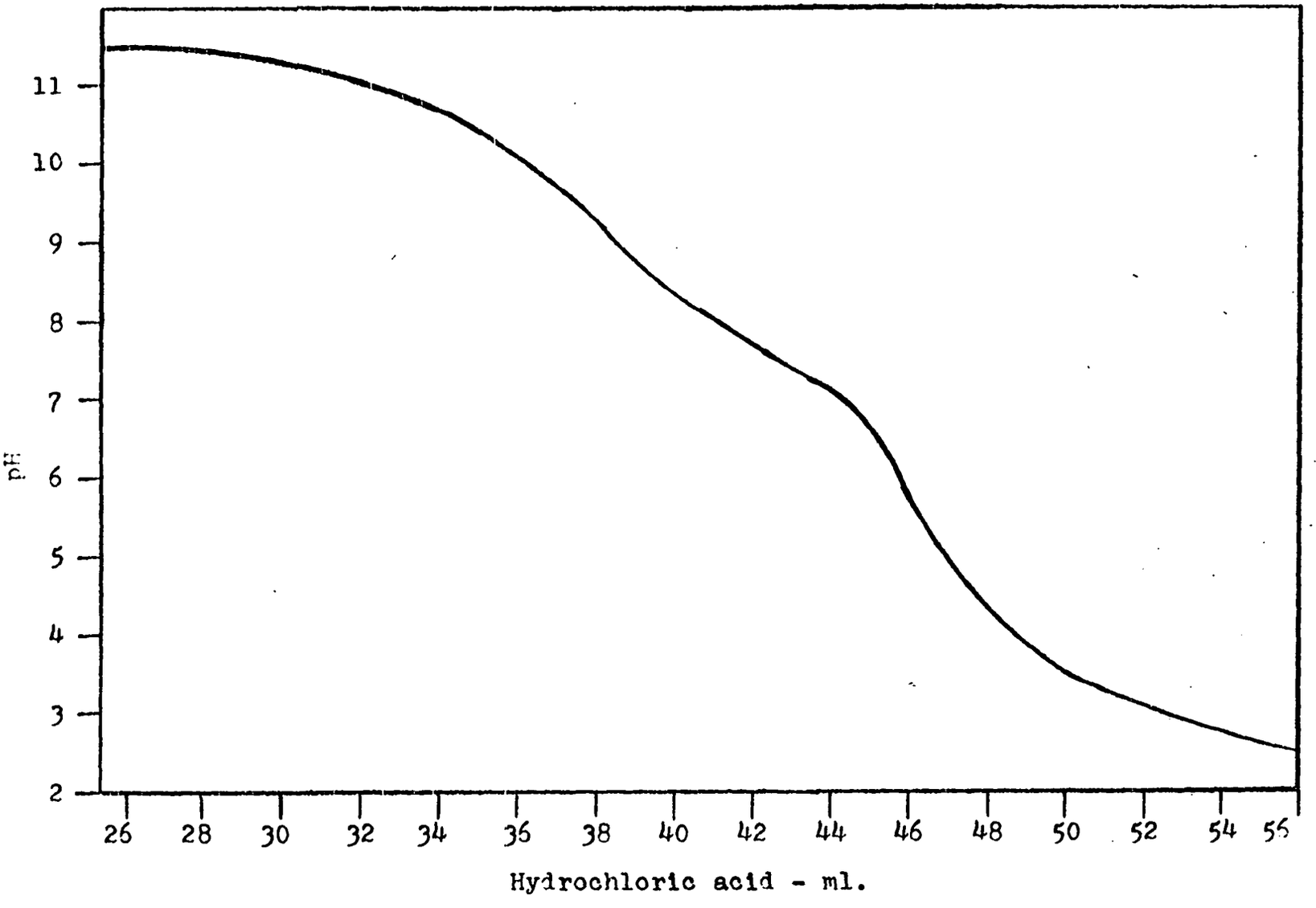

pH
11
10
9
8
7
6
5
4
3
2
26
28
30
32
34
36
38
40
42
44
46
48
50
52
54
56
Hydrochloric acid - ml.

Table 10. Solubility of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid as a function of pH

Compound	pH	Solubility (mg./100 ml.)
alpha-dimethyldihydroxy-fluoranmethyleneimino-diacetic acid	2.13	1.031
	2.56	1.197
	2.67	1.258
	2.95	1.577
beta-dimethyldihydroxy-fluoranmethyleneimino-diacetic acid	2.31	5.014
	2.55	5.256
	2.80	5.738
	3.05	6.623
gamma-dimethyldihydroxy-fluoranmethyleneimino-diacetic acid	2.48	19.10
	2.90	21.73
	3.22	27.91
	3.47	37.15

Figure 26. Change in solubility of alpha-dimethyldihydroxyfluoranmethylene-iminodiacetic acid with pH

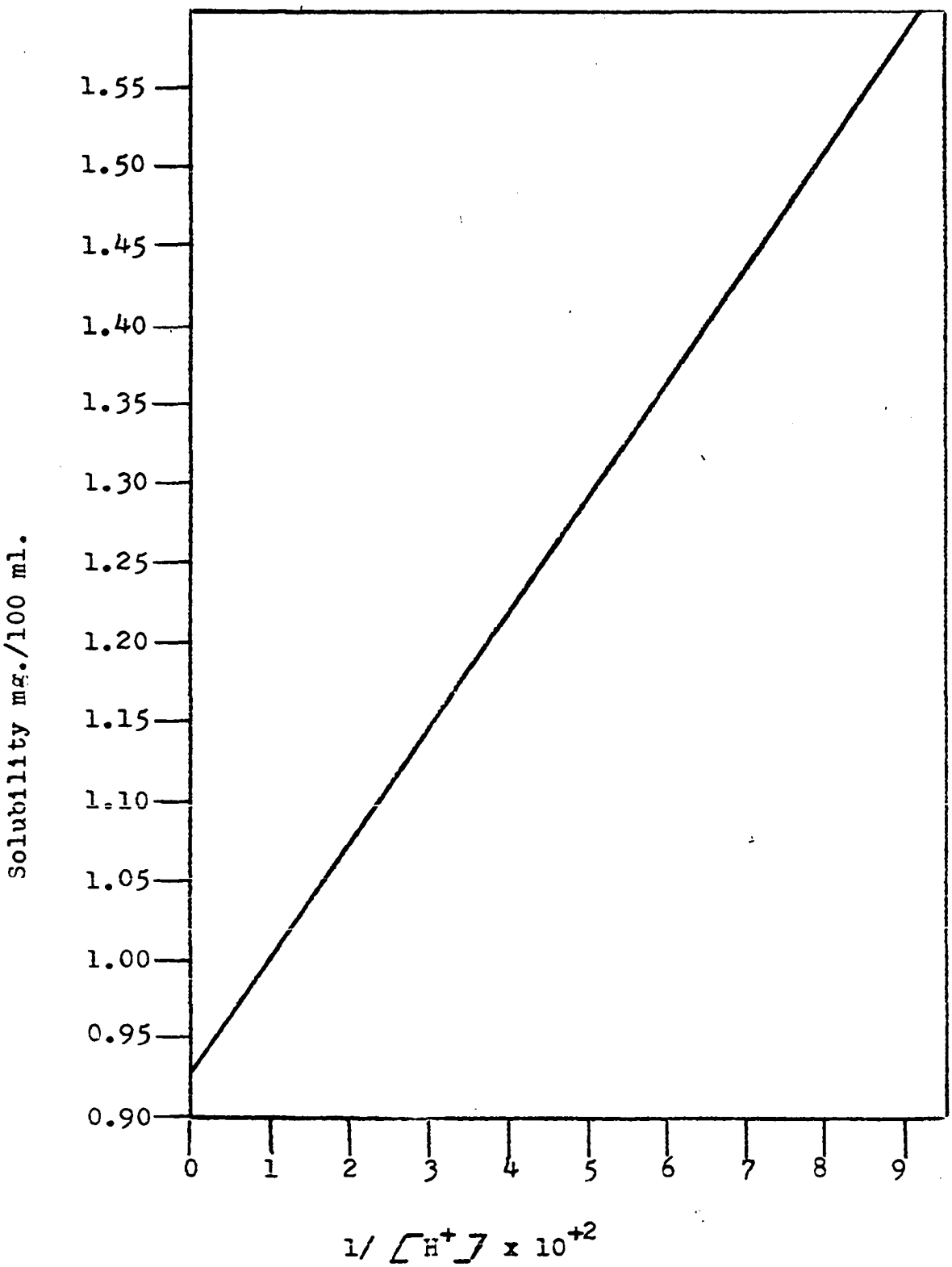

1.55
1.50
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
Solubility mg./100 ml.
0
1
2
3
4
5
6
7
8
9
1/ [H+] x 10+2

Figure 27. Change in solubility of beta-dimethyldihydroxy-fluoranmethyleneiminodiacetic acid with pH

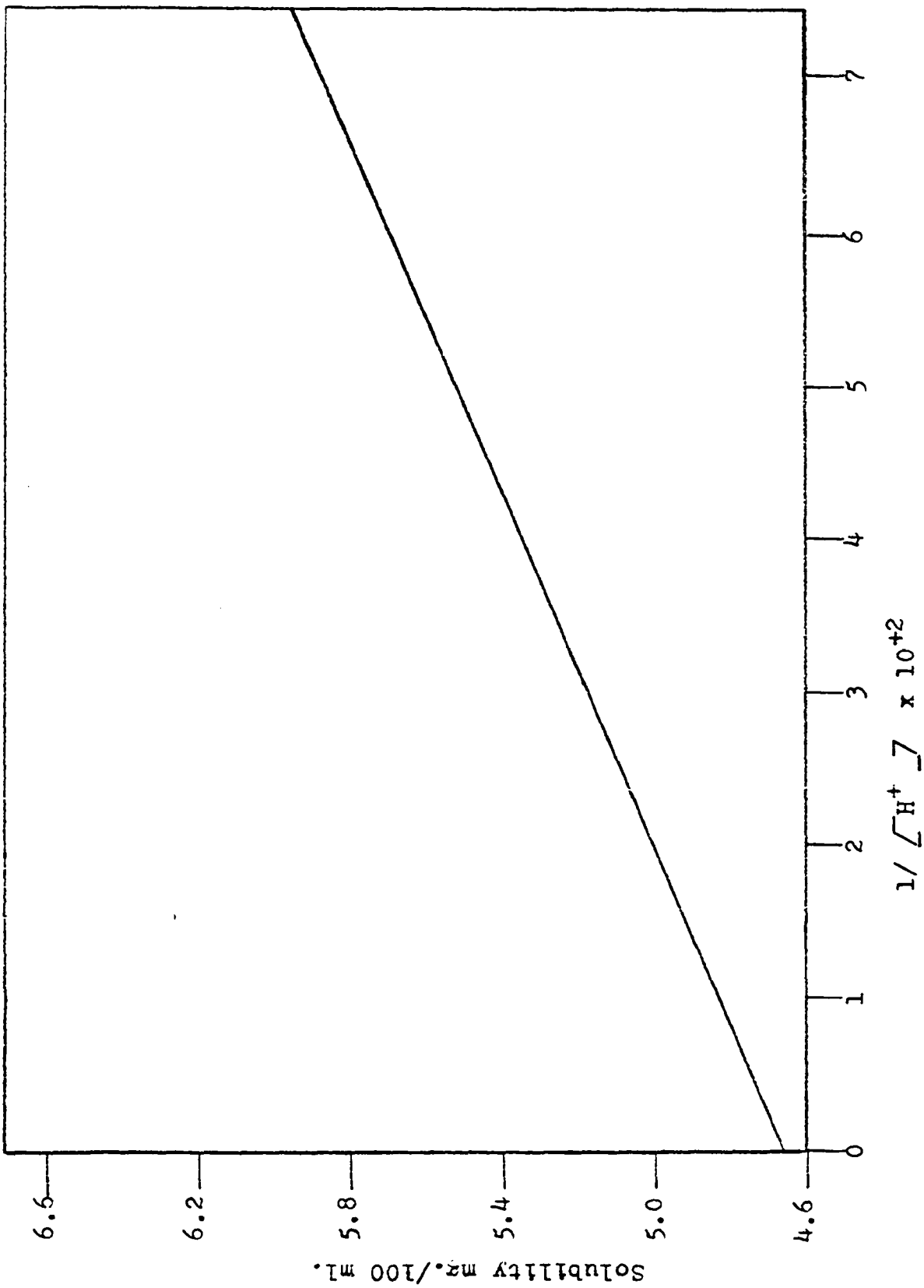
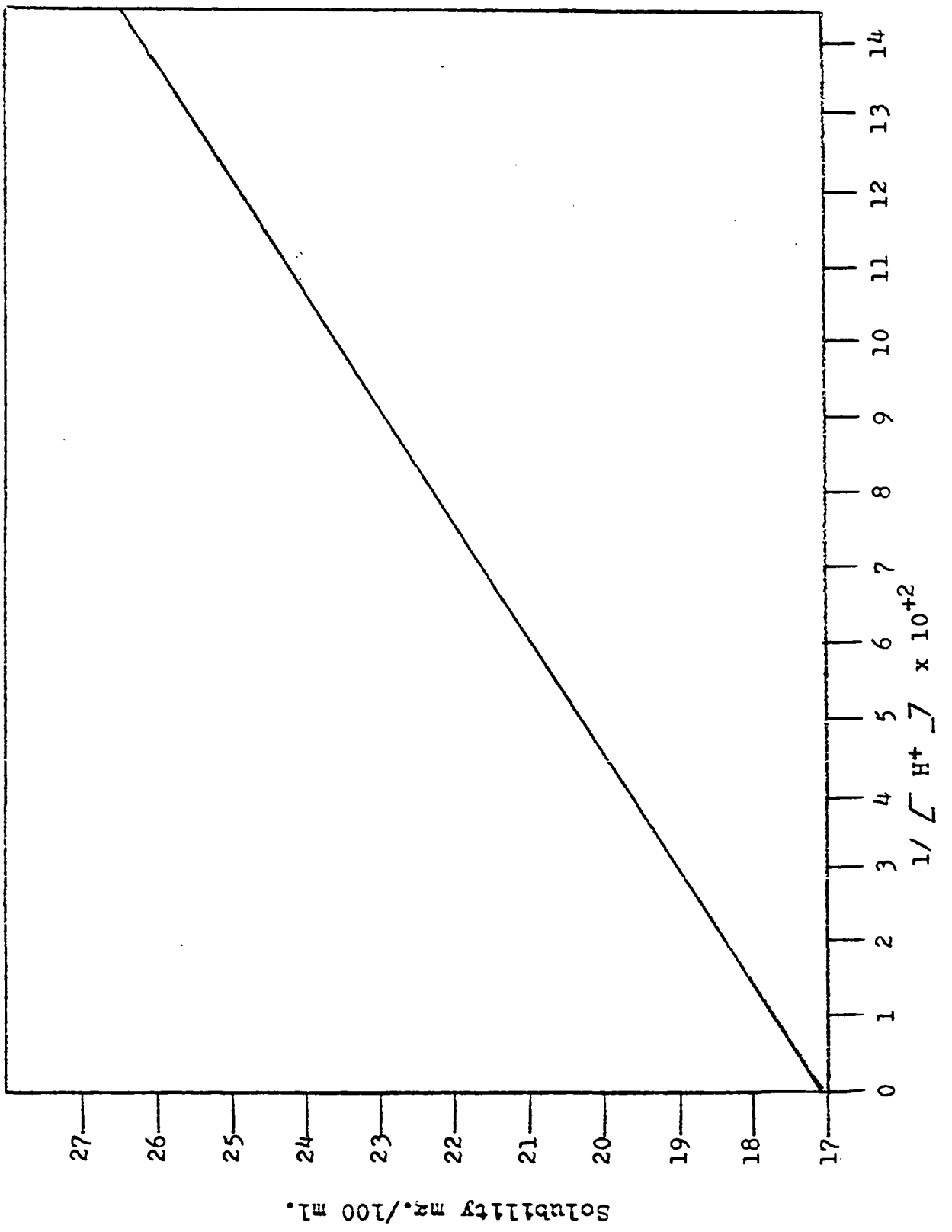

Solubility mg./100 ml.
6.6
6.2
5.8
5.4
5.0
4.6
0
1
2
3
4
5
6
7
1/ [H+] x 10+2

Figure 28. Change in solubility of gamma-dimethyldihydroxy-fluoranmethyleneiminodiacetic acid with pH


Solubility mg./100 ml.
27
26
25
24
23
22
21
20
19
18
17
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
1/ [H+] x 10+2

first acid dissociation constant of the carboxyl group proton was determined for alpha-, beta- and gamma-dimethyldihydroxyfluoran-methyleneiminodiacetic acid.

B. Results and Discussion

1. Synthesis

a. Purification and analysis of glycine and iminodiacetic acid

Iminodiacetic acid, disodium salt monohydrate, (IDA) was used in subsequent reaction with the parent dimethyldihydroxyfluorans and formaldehyde to synthesize the di(methyleneiminodiacetic acid) derivatives. The IDA was a non-stock item and was therefore purified and subjected to careful investigation to establish the final purity. As a cross check on purity and as a possible impurity in the IDA, a structurally similar compound, glycine, readily available commercially at high purity underwent similar analysis.

The commercial glycine was assayed by the manufacturer at 99.5 per cent minimum. The purified material appeared more crystalline than the commercial material.

glycine $NH_2CH_2CO_2H$ F.W. 75.07

The IDA was an off-white powder as received commercially and a pure white, crystalline solid after purification.

iminodiacetic acid, disodium salt, monohydrate $NH(CH_2CO_2Na)_2H_2O$ F.W. 195.08

1) Loss on heating IDA lost 1.005 moles of water per mole of compound, confirming that the material was a monohydrate.

2) Melting point The purified IDA and glycine did not melt sharply but slowly decomposed and charred at temperatures above 225°.

The following melting point behavior for glycine was found in the literature:

Reference	Behavior on heating
The Merck Index, Eighth Edition, Merck and Company, Inc., 1968	starts to decompose at 233° completely sintered at 290°
1969-1970 Aldrich Chemical Co. Catalog #14, The Aldrich Chemical Company	245° decomposes
Lange, Handbook of Chemistry, McGraw-Hill, 1961	232-236° decomposes

The melting point behavior observed on the glycine purified in this work was inconclusive.

3) Infrared spectroscopy The infrared spectra of commercial and purified glycine were obtained using the potassium bromide disc technique. The spectra were identical and of highly pure compounds as evidenced by the sharp absorption bands. Assignments of various absorption bands in the spectra are given in Table II.

Free primary amino acids are characterized by a broad strong NH^{+}_{3} stretching band in the 3.23 to 3.85 micron region (15). The strong band observed in the spectra of glycine at 3.20 to 3.85 microns was attributed to the NH^{+}_{3} stretching vibration.

Also characteristic is the presence of the weak asymmetric NH^{+}_{3} bending band in the 6.03 to 6.21 micron region and the fairly strong symmetrical bending band in the 6.45 to 6.73 micron region (15).

Table 11. Assignments of infrared absorption bands of glycine

Assignment	Band position (microns)	Intensity[a]
NH_3^+ stretch	3.20 - 3.85	S
NH_3^+ bend	6.30	S
C-O asymmetrical stretch	6.30	S
NH_3^+ bend	6.55 - 6.65	S
C-O symmetrical stretch	7.10	S
combination of C-O stretch and OH in-plane bend	7.50	M
C-C-O stretch	9.70	W

[a] W = weak,
M = medium,
S = strong.

The strong bands observed at 6.30 microns and at 6.55 to 6.65 microns was attributed to the respective NH^{+}_{3} bending bands.

The carboxylic anion absorbs strongly in the 6.25 to 6.29 micron region and more weakly at 7.15 microns due to the asymmetrical and symmetrical C-O stretching, respectively (15). The strong bands observed at 6.30 microns and at 7.10 microns were attributed to the asymmetrical and symmetrical C-O stretching bands.

The C-O stretch coupled with the OH in-plane-bend absorbs in the 7.04 to 7.52 micron region(15) . The medium band observed at 7.50 microns was attributed to this C-O stretch and OH in-plane-bend.

The C-O stretch coupled with the adjacent C-C stretch, that is, the assymmetrical C-C-O stretch, absorbs in the 7.93 to 10.00 micron region (15). A weak band observed at 9.70 microns was attributed to the C-C-O stretch.

The infrared spectra of commercial and purified iminodiacetic acid disodium salt monohydrate were obtained using the potassium bromide disc technique. The absorption bands of the commercial IDA were slightly broader than those of the purified IDA indicating that some purification was effected. Otherwise, the spectra were identical. Assignments of various absorption bands in the spectra are given in Table 12.

The carboxylate ion of the sodium salt of an amino acid gives rise to two characteristic bands: a strong asymmetrical stretching band from 6.25 to 6.29 microns, and a weaker symmetrical stretching band at 7.15 microns (15). Bands observed in the spectrum of IDA at

Table 12. Assignments of infrared absorption bands of iminodiacetic acid disodium salt monohydrate

Assignment	Band position (microns)	Intensity[a]
N-H stretch	2.80 - 3.25	S
$-COO^-$ asymmetric stretch	6.30	S
$-COO^-$ symmetric stretch	7.00 - 7.15	M

[a] M = medium,
S = strong.

6.30 microns and 7.00 to 7.15 microns were attributed to the carboxylate anion.

Sodium salts of amino acids show the normal N-H stretching vibrations at 2.94 to 3.13 microns common to other amines (15). A strong band observed in the spectrum of IDA at 2.80 to 3.25 microns was attributed to this normal N-H stretching vibration.

The spectrum of IDA was simpler than that of glycine and characteristic of a sodium salt of an alpha-amino acid.

4) Nuclear magnetic resonance spectroscopy A strong singlet peak was present in the proton magnetic spectra of iminodiacetic acid and of glycine at 3.18 delta and 3.57 delta, in D_2O, respectively. This peak is due to the $-CH_2-$ protons. The position of the peak depends on the solvent and pH.

No peaks corresponding to $\overset{\oplus}{N}H_3^-$ and $\overset{\oplus}{N}H_2=$ appeared in glycine and IDA, respectively. The ^{14}N nucleus has a spin number $I = 1$ and, in accordance with the formula $2I + 1$, should cause a proton attached to it and a proton on an adjacent carbon atom to show three equally intense peaks. There are two complicating factors - the rate of exchange of the proton on the nitrogen atom and the electrical quadrupole moment of the ^{14}N nucleus.

The proton on a nitrogen atom may undergo rapid, intermediate, or slow exchange. If the exchange is rapid, the NH proton(s) is decoupled from the nitrogen atom and from protons on adjacent carbon atoms. The NH peak is therefore a sharp singlet, and the adjacent CH

protons are not split by NH. Such is the case for most aliphatic amines. At an intermediate rate of exchange, the NH proton is partially decoupled and a broad NH peak results. The adjacent CH protons are not split by the NH proton. Such is the case for N-methyl aniline. If the NH exchange rate is slow, the NH peak is still broad because the electrical quadrupole moment of the nitrogen nucleus induces a moderately efficient spin relaxation and thus an intermediate lifetime for the spin states of the nitrogen nucleus. The proton thus sees three spin states of the nitrogen nucleus (spin number equals one) which are changing at a moderate rate, and the proton responds by giving a broad peak. In this case, coupling of the NH proton to the adjacent proton is observed. Such is the case for pyrroles, indoles, secondary and tertiary amides and carbamates.

Aliphatic and cyclic amine NH protons absorb from 3.0 to 0.5 delta. Because amines are subject to hydrogen bonding, the shift position depends on concentration, solvent, pH and temperature.

Protons on the nitrogen atom of an amine salt exchange at a slow rate; they are seen as a broad peak downfield (8.5 to 6.0 delta), and they are coupled to protons on adjacent carbon atoms. The alpha-protons are recognized by their downfield position in the salt compared with that in the free amine.

The nuclear magnetic resonance spectra showed no glycine was present in the IDA. The nuclear magnetic resonance spectra also showed the glycine, and IDA, respectively, to have a purity of 95 per cent plus.

5) Mass spectroscopy Mass spectra of glycine, iminodiacetic acid disodium salt, and iminodiacetic acid (free acid) were obtained at an electron beam energy of 70 electron volts.

The mass spectrum of glycine confirmed the molecular weight for the structure $NH_2CH_2CO_2H$. Up to 200 m/e units were scanned. The highest peak found was at 77 m/e, the P + 2 peak.

m/e	assignment	experimental relative intensity	theoretical relative intensity
75	P	100.00	100.00
76	P + 1	2.60	2.70
77	P + 2	0.38	0.43

The P + 1 and P + 2 peaks experimental relative intensities were in excellent agreement with the empirical formula for glycine, $C_2 H_5 NO_2$. The parent peak was very weak but discernible. This is characteristic of straight chain monocarboxylic acids and also of aliphatic monoamine entities.

Cleavage resulted at the carbon-carbon bonds successively removed from the nitrogen atom with retention of charge on the nitrogen-containing fragment. This is almost conclusive evidence for a straight chain primary amine. This gave rise to the base peak at m/e 30. The peak at 75 m/e was the parent peak, due to $NH_2CH_2CO_2H$. The peak at 60 m/e was due to the loss of NH_2 from the parent compound, leaving $CH_3 C(-OH) (= OH^+)$. The peak at 57 m/e was due to the loss of H_2O from the parent compound, $NH_2\text{-}CH = C = O$ remaining. The peaks at 46, 45 and 44 m/e were due to the HCO_2H^+, CO_2H^+ and CO^+_2 fragments, respectively. The peak at 41 m/e was due to the loss of NH_2 from the 57 m/e fragment, leaving $CH = C = O$. The peak at

31 m/e was due to the H_2NCH_3 fragment. The peak at 30 m/e was due to the loss of CO_2H from the parent compound, leaving $H_2N^+ = CH_2$. The peaks at 29 and 28 m/e were due to the H_2NCH and N_2 fragments, respectively.

The mass spectrum was characteristic of glycine.

The mass spectrum of iminodiacetic acid disodium salt monohydrate was inconclusive. The spectrum was poor. This was attributed to the very high boiling point of the compound, thus making it difficult to vaporize sufficient compound at low temperatures. At higher temperatures, thermal decomposition of iminodiacetic acid disodium salt made the spectrum quite complex and meaningless.

No parent peak in the spectrum of iminodiacetic acid (free acid) was observed. The theoretical molecular weight of iminodiacetic acid is 133 a.m.u. The parent peak of a carboxylic acid is weak, that of an aliphatic monoamine is very weak and that of a tri-substituted amine nondiscernible.

Characteristic of carboxylic acids is the P-CO_2H peak (here at 133 - 45 = 88 m/e) which was observed. This left the $C_3H_6O_2N$ fragment attributed to $CH_2 = \overset{+}{N}HCH_2CO_2H$. Characteristic of secondary amines are the m/e 30 and 44 fragments attributed to the $CH_2 = \overset{+}{N}H_2$ and $CH_2 = \overset{+}{N}HCH_3$ fragments. Both fragements were observed.

Although no parent peak was observed, the spectrum was in accord with a carboxylic acid and secondary amine with a molecular weight of 133. The spectrum was characteristic of iminodiacetic acid.

6) Nitrogen analysis by the Kjeldahl method Five

determinations of nitrogen in glycine by the Kjeldahl method gave an average of 18.66 per cent; calculated 18.67 per cent.

Six determinations of nitrogen in iminodiacetic acid disodium salt monohydrate gave an average of 7.186 per cent; calculated 7.180 per cent.

7) Equivalent weight by neutralization Iminodiacetic acid disodium salt monohydrate was titrated potentiometrically with 0.1007 N hydrochloric acid. One break was present in the titration curve, Figure 21 , corresponding to one mole of acid added per mole of the base. The equivalent weight found was 196.7; the molecular weight of iminodiacetic acid disodium salt monohydrate is 195.1. This corresponds to a purity of 99.2 per cent.

b. Condensation of alpha-, beta- and gamma-dimethyldihydroxyfluoran with iminodiacetic acid and formaldehyde

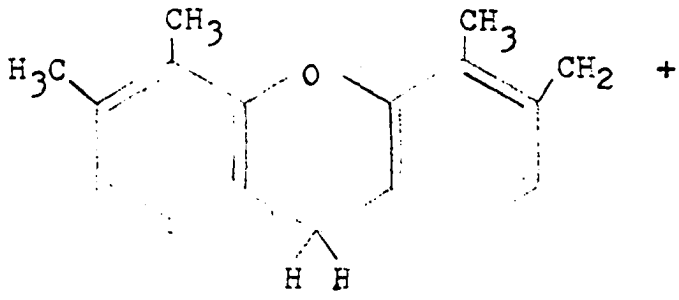
The di(methyleneiminodiacetic acid) derivatives of alpha-, beta- and gamma-dimethyldihydroxyfluoran, respectively, were synthesized by the Mannich condensation of the respective dimethyldihydroxyfluoran, iminodiacetic acid and formaldehyde using acetic acid as solvent. The reactions were allowed to proceed at 68-73° for two hours. The desired compounds were obtained in yields of 64 per cent, 73 per cent, and 52 per cent, respectively, for the alpha-, beta- and gamma-dimethyldihydroxyfluoran derivatives. Alpha-dimethyldihydroxyfluoranmethyleneiminodiacetic acid is an off-white solid; beta- a yellow-brown solid; and gamma- a bright orange solid.

2. Properties and Structure

a. Mass spectroscopy The mass scale up to 700 mass units was scanned. The largest recorded peaks found for all three isomers were at 650 and 651 a. m. u. The 650 peak was the molecular ion. The 651 peak was the P + 1 peak. From the proposed structural formula corresponding to $C_{32}H_{30}O_{13}N_2$, the molecular weight was calculated to be 650.593 a. m. u. The 650 and 651 peaks were very weak but discernible. The spectra of the three isomers varied only slightly in the relative intensity of the various peaks.

The peak at 474 m/e was due to the loss of four CO_2 groups from the methyleneiminodiacetic acid entity of the molecule, leaving $C_{28}H_{30}O_5N_2$. The peak at 382 m/e was due to the loss of two $H_2N(CH_2CO_2H)_2$ groups from the parent compound, $C_{24}H_{14}O_5$ remaining. This cleavage was beta to the aromatic ring leaving a benzyl type group. The peak at 356 m/e was due to the loss of two $N(CH_2CO_2H)_2$ and two CH_3 groups from the parent compound, leaving $C_{22}H_{12}O_5$. The peak at 332 m/e was due to the loss of CO from the precursor compound, that is, the alpha-, beta- or gamma-dimethyldihydroxyfluoran, M. W. 360. This 360 -CO peak is characteristic of diphenyl ethers by complex rearrangement. The peak at 298 m/e was due to the loss of CO_2 and H_2O from the precursor compound. The peak at 280 m/e was due to the loss of CO_2 and two H_2O from the precursor compound. The peak at 270 m/e was due to the loss of CO_2, H_2O and CO from the precursor compound, leaving $C_{22}H_{16}O_5$. The peak at 237 m/e was due to the loss of the two OH groups and the

phthalate ring, leaving , $C_{17}H_{17}O$.



The peak at 222 m/e was due to the loss of CH_3 from the 237 m/e fragment. The peak at 209 m/e was due to the loss of CO from the 237 fragment. The loss of CO by complex rearrangement is characteristic of diphenyl ethers. The peak at 207 m/e was due to the loss of CH_3 from the 222 fragment. There was a large abundance of lower m/e peaks.

The complex spectra were in accord with the proposed structures of the di(methyleneiminodiacetic acid) derivatives of alpha-, beta- and gamma-dimethyldihydroxyfluoran, respectively. The theoretical molecular weight of 650 a.m.u. for each isomer was also confirmed.

b. Infrared spectroscopy The infrared spectra of the methyleneiminodiacetic acid derivative of the respective alpha-, beta- and gamma-dimethyldihydroxyfluoran were obtained using the potassium bromide disc technique. Assignments of various absorption bands in the

spectra are given in Table 13.

A weak, broad band at 3.73 to 4.12 microns is characteristic of the tertiary ammonium ion (15). In the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, respectively, this band was obscured by the strong, broad band at 3.00, 2.95 and 3.25 microns, respectively, of the OH group. A second weak band at 7.05 microns is characteristic of the tertiary ammonium ion (15). In the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, respectively, this band was obscured by the strong band at 7.10, 7.10 and 7.13 microns, respectively, of the carboxylate group.

Characteristic of a non-ionized carboxylic acid is the strong carbonyl asymmetric stretch band at 5.80 to 6.06 microns (11). The band observed in the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid at 5.75, 5.75 and 5.74 microns, respectively, was attributed to the non-ionized carboxyl group.

The carboxylate anion absorbs strongly in the 6.21 to 6.45 micron region and more weakly in the 7.04 to 7.69 micron region due to the asymmetric and symmetric C-O stretching, respectively (15). Bands were observed in the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, respectively, at 6.20 and 7.10 microns, at 6.15 and 7.10 microns, and at 6.15 and 7.13 microns, respectively; these bands were attributed to the carboxylate group.

If only one type of acid group were present in alpha-, beta-, and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid,

Table 13. Assignments of infrared absorption bands of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneimino-diacetic acid

Assignment	Band position (microns)			Intensity[a]
	alpha-	beta-	gamma-	
OH stretch (phenol)(COOH)	3.00	2.95	3.25	S
C=O asymmetric stretch (COOH)	5.75	5.75	5.74	S
COO^- asymmetric stretch	6.20	6.15	6.15	S
C=C stretch of aromatic ring	6.85	6.85	6.85	M
COO^- symmetric stretch also OH in-plane bend	7.10	7.10	7.13	S
interaction of OH bend and C-O stretch	7.50	7.40	7.39	M
interaction of OH bend and C-O stretch	7.80	7.80	7.80	M
C-O stretch of phenol	8.05	8.00	8.05	M
C-H in-plane bend of polyaromatic ring	9.00	8.87	9.17	S
C-H out-of-plane ring bend of 1, 2-disubstituted aromatic ring	13.20	13.50	13.22	W
C-H out-of-plane ring bend	14.50	14.40	14.35	W

[a] W = Weak,
MW = Medium weak,
M = Medium,
MS = Medium strong,
S = Strong.

respectively, there could be one or two bands observed in each spectrum. If only the free acid groups were present, only one or two closely spaced bands at 5.80 to 6.06 microns would be present. If only the carboxylate groups were present, two bands at 6.21 to 6.45 and 7.04 to 7.69 microns would be present. However, in the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, respectively, three bands were observed; at 5.75, 6.20 and 7.10 microns; at 5.75, 6.15 and 7.10 microns; and at 5.74, 6.15 and 7.13 microns, respectively. The presence of three bands is indicative of the presence of both free carboxyl and carboxylate anion groups. The amine and carboxylate bands thus confirm the zwitter ion structure.

The shift of the O-H stretching band to longer wavelengths (2.78 to 3.25 microns) and broadening of the band are indicative of strong hydrogen bonding (15). The strong, broad band observed in the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, respectively, at 3.00, 2.95 and 3.25 microns, respectively, was attributed to the OH stretching band.

Solid samples of phenols absorb at 7.20 to 7.52 microns and at 7.93 to 8.48 microns. These bands result from interaction of O-H bending and C-O stretching (15). Such bands were observed in the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, respectively, at 7.50 and 8.05 microns, at 7.40 and 8.00 microns, and at 7.39 and 8.05 microns, respectively.

The O-H in-plane bending vibration occurs at 7.04 to 7.52 microns (15). The bands observed in the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid,

respectively, at 7.10 and 7.50 microns, at 7.10 and 7.40 microns, and at 7.13 and 7.39 microns, respectively, could be attributed to the OH in-plane bending vibration.

In-plane bending bands of C-H of poly-aromatic hydrocarbons occur at 7.70 to 10.00 microns (15). The bands observed in the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneimino-diacetic acid, respectively, at 9.00, at 8.85, and at 8.87 and 9.17 microns, respectively, were attributed to the in-plane bending bands of C-H.

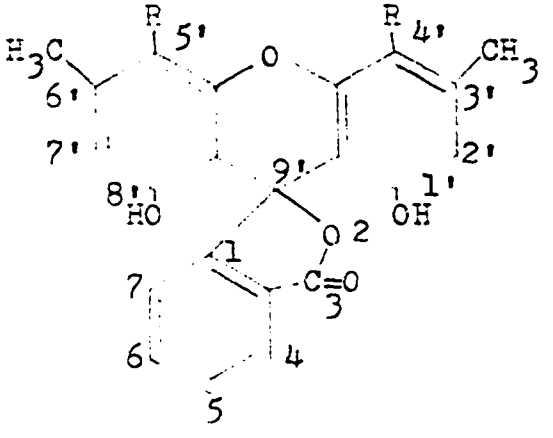
In-plane bending bands of 1,2-disubstituted mono-aromatics occur at 13.00 to 13.50 microns (15). The bands observed in the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethylene-iminodiacetic acid, respectively, at 13.20, 13.50 and 13.22 microns, respectively, were attributed to the phthalate ring.

Out-of-plane ring bending vibrations of C-H occur at 14.08 to 14.81 microns (15). The bands observed in the spectrum of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, respectively, at 14.50, 14.40 and 14.35 microns, respectively, were attributed to the out-of-plane ring bending vibrations of C-H.

c. Nuclear magnetic resonance spectroscopy A solution of alpha-, beta-, and gamma-dimethyldihydroxyfluoranmethyleneimino-diacetic acid, respectively, in trifluoroacetic acid was used to obtain the spectra. The reference standard was tetramethyl silane (TMS). The NMR work was hampered by the insolubility of the derivatives in the many solvents tried (p. 72-75). At best, the derivatives were only slightly soluble in the trifluoroacetic acid used as solvent in the NMR

work. The results are summarized in Tables 14, 15, 16 and Figures 29, 30, and 31.

Observed in the NMR spectrum of alpha-dimethydihydroxy-fluoranmethyleneiminodiacetic acid was a 1:2:2:1:4:8:6 proton ratio.



R= $CH_2N(CH_2CO_2H)_2$

The singlet integrating to six protons was centered at 2.83 delta. This singlet was attributed to the two methyl group hydrogen atoms. By comparison, the two methyl group hydrogen atoms of the parent fluoran in trifluoroacetic acid appeared at 2.67 delta.

The singlet integrating to eight protons was centered at 5.63 delta. This singlet was attributed to the eight methylene hydrogen atoms of the four acetic acid groups.

The singlet integrating to four protons was centered at 6.17 delta. This singlet was attributed to the two methylene groups linking the amino acid to the aromatic ring.

Table 14. The peak positions, the proton integrations and the groups bearing the proton obtained from the NMR spectra of alpha-dimethyldihydroxyfluoran and alpha-dimethyldihydroxyfluoran-methyleneiminodiacetic acid in trifluoroacetic acid

Compound	Peak Position (ppm)	Proton integration	Group bearing protons
alpha-dimethyl-dihydroxyfluoran	2.67 (S)[a]	6	two $-CH_3$
	6.95 (S)	2	4', 5' protons of orcinol ring
	7.18 (M)	1	7 proton of phthalate ring
	7.47 (S)	2	2', 7' protons of orcinol ring
	7.80 (M)	2	5, 6 protons of phthalate ring
	8.42 (M)	1	4 proton of phthalate ring
alpha-dimethyl-dihydroxyfluoran-methyleneimino-diacetic acid	2.83 (S)	6	two $-CH_3$
	5.63 (S)	8	four = $N-CH_2-COO^-$
	6.17 (S)	4	two $Ar-CH_2-N=$
	7.27 (M)	1	7 proton of phthalate ring
	7.90 (M)	2	2', 7' protons of orcinol ring
	8.25 (M)	2	5, 6 protons of phthalate ring
	9.23 (M)	1	4 proton of phthalate ring

[a](S) representing a singlet,
(M) representing a multiplet.

Table 15. The peak positions, the proton integrations and the groups bearing the proton obtained from the NMR spectra of beta-dimethyldihydroxyfluoran and beta-dimethyldihydroxyfluoran-methyleneiminodiacetic acid in trifluoroacetic acid

Compound	Peak Position (ppm)	Proton integration	Group bearing protons
beta-dimethyl-dihydroxyfluoran	1.97 (S)[a]	3	$-CH_3$ at 1' in orcinol ring
	2.65 (S)	3	$-CH_3$ at 6' in orcinol ring
	6.93 (S)	2	4', 5' protons of orcinol ring
	7.22, 7.40 (M)	3	7 proton of phthalate ring; 2', 7' protons of orcinol ring
	7.92 (M)	2	5, 6 protons of phthalate ring
	8.50 (M)	1	4 proton of phthalate ring
beta-dimethyl-dihydroxyfluoran-methyleneimino-diacetic acid	2.08 (S)	3	$-CH_3$ at 1' in orcinol ring
	3.00 (S)	3	$-CH_3$ at 6' in orcinol ring
	4.87 (S)	8	four $=N-CH_2-COO^-$
	5.67 (S)	4	two $Ar-CH_2-N=$
	7.37 (M)	3	7 proton of phthalate ring; 2', 7' protons of orcinol ring
	8.22 (M)	2	5, 6 protons of phthalate ring
	8.40 (M)	1	4 proton of phthalate ring

[a](S) representing a singlet,
(M) representing a multiplet.

Table 16. The peak positions, the proton integrations and the groups bearing the proton obtained from the NMR spectra of gamma-dimethyldihydroxyfluoran and gamma-dimethyldihydroxyfluoranmethyleneiminoacetic acid in trifluoroacetic acid

Compound	Peak Position (ppm)	Proton integration	Group bearing protons
gamma-dimethyldihydroxyfluoran	1.90 (S)[a]	6	two $-CH_3$
	7.20 (S)	2	4', 5' protons of orcinol ring
	7.40 (M)	3	2', 7' protons of orcinol ring + 7 proton of phthalate ring
	8.05 (M)	2	5, 6 protons of phthalate ring
	8.88 (M)	1	4 proton of phthalate ring
gamma-dimethyldihydroxyfluoran-methyleneiminodiacetic acid	2.24 (S)	6	two $-CH_3$
	5.02 (S)	8	four $-N-CH_2-COO^-$
	5.90 (S)	4	two $Ar-CH_2-N=$
	7.73 (M)	3	2', 7' protons of orcinol ring + 7 proton of phthalate ring
	8.33 (M)	2	5, 6 protons of phthalate ring
	8.87 (M)	1	4 proton of phthalate ring

[a](S) representing a singlet,
(M) representing a multiplet.

Figure 29. The proton magnetic resonance spectrum of alpha-dimethyldihydroxyfluoranmethyleneiminodiacetic acid in trifluoroacetic acid

A. Methylene protons of AR-CH_2-N (4)
B. 7 Proton of the phthalate ring (1)
C. 2', 7' Protons of the orcinol ring (2)
D. 5, 6 Protons of the phthalate ring (2)
E. 4 Proton of the phthalate ring (1)

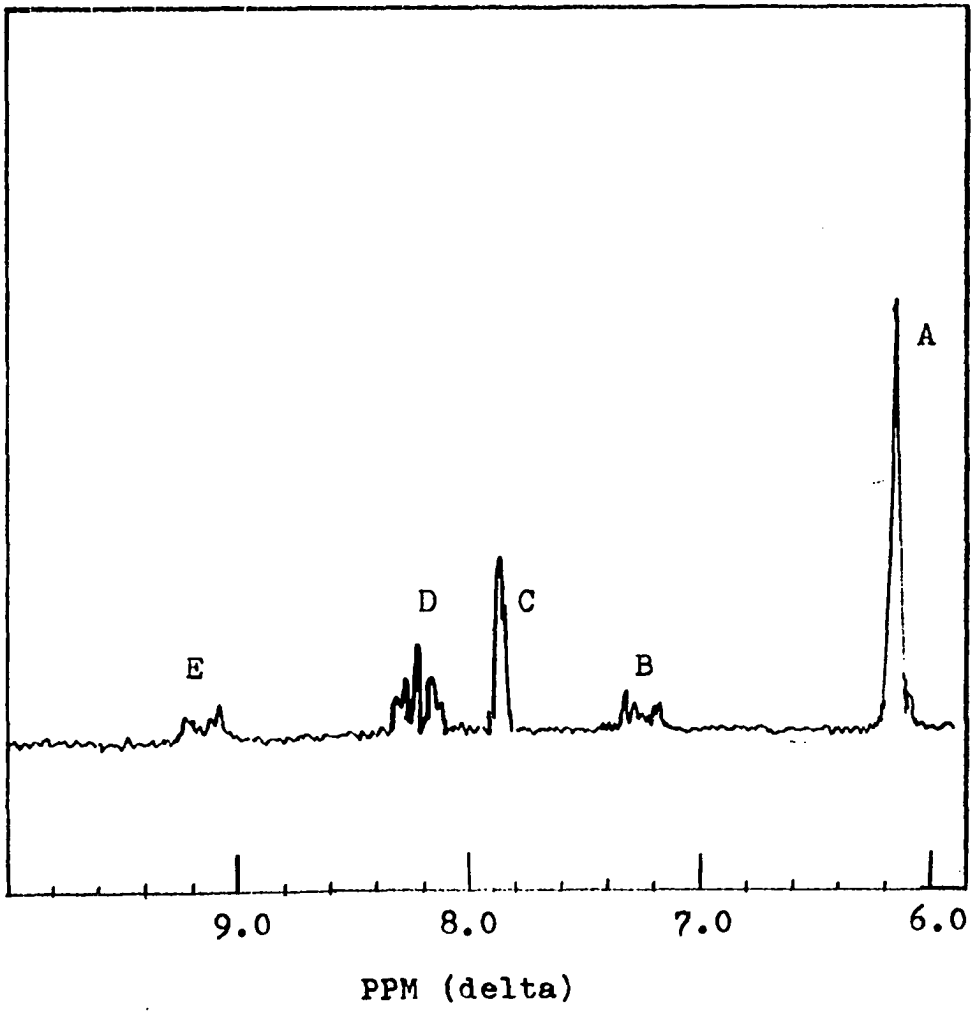

A
B
C
D
E
9.0
8.0
7.0
6.0
PPM (delta)

Figure 30. The proton magnetic resonance spectrum of beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid in trifluoroacetic acid

A. Methylene protons of Ar-CH_2-N (4)
B. 2', 7' Protons of the orcinol ring (2)
   7 Proton of the phthalate ring (1)
C. 5, 6 Protons of the phthalate ring (2)
D. 4 Proton of the phthalate ring (1)

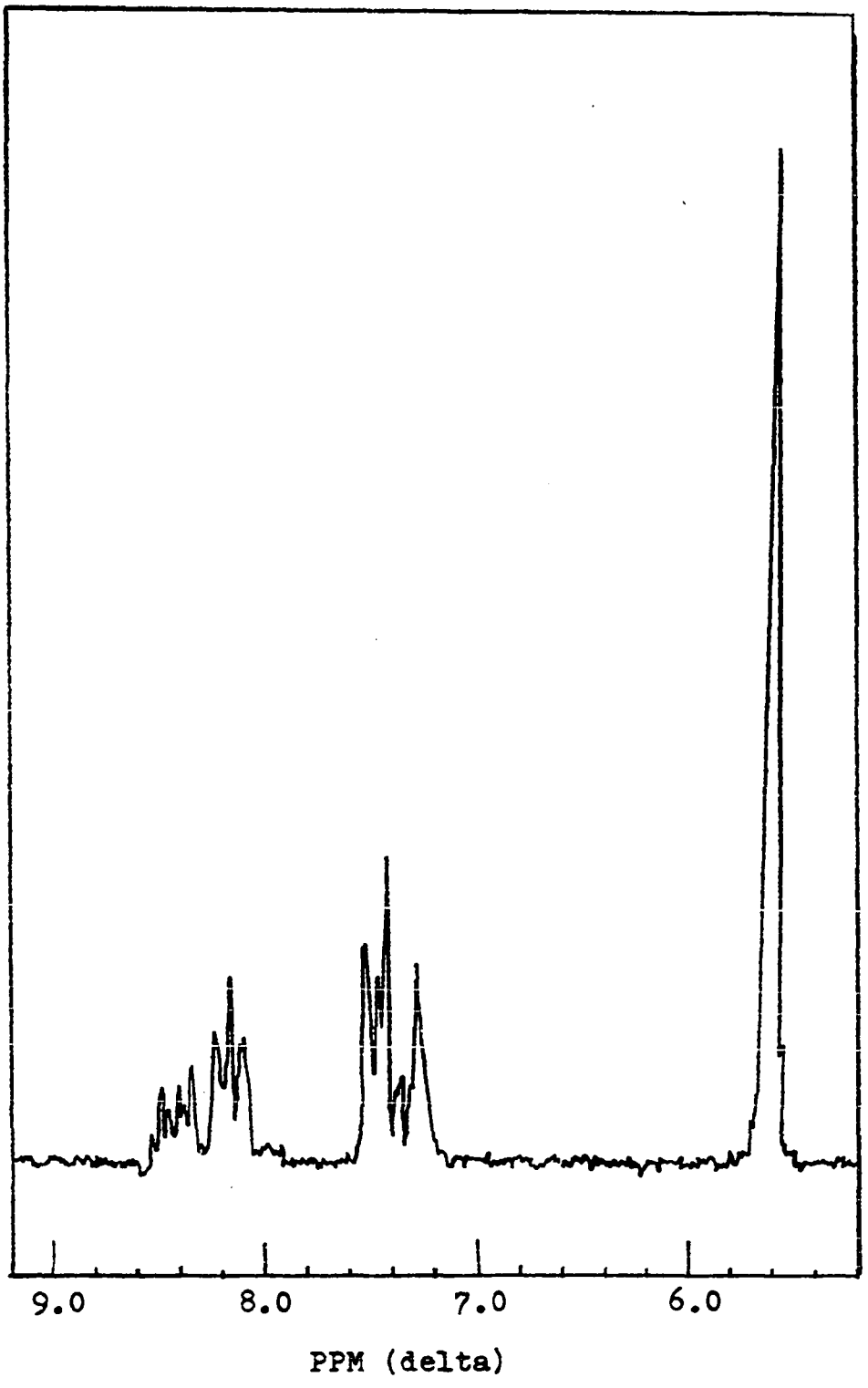
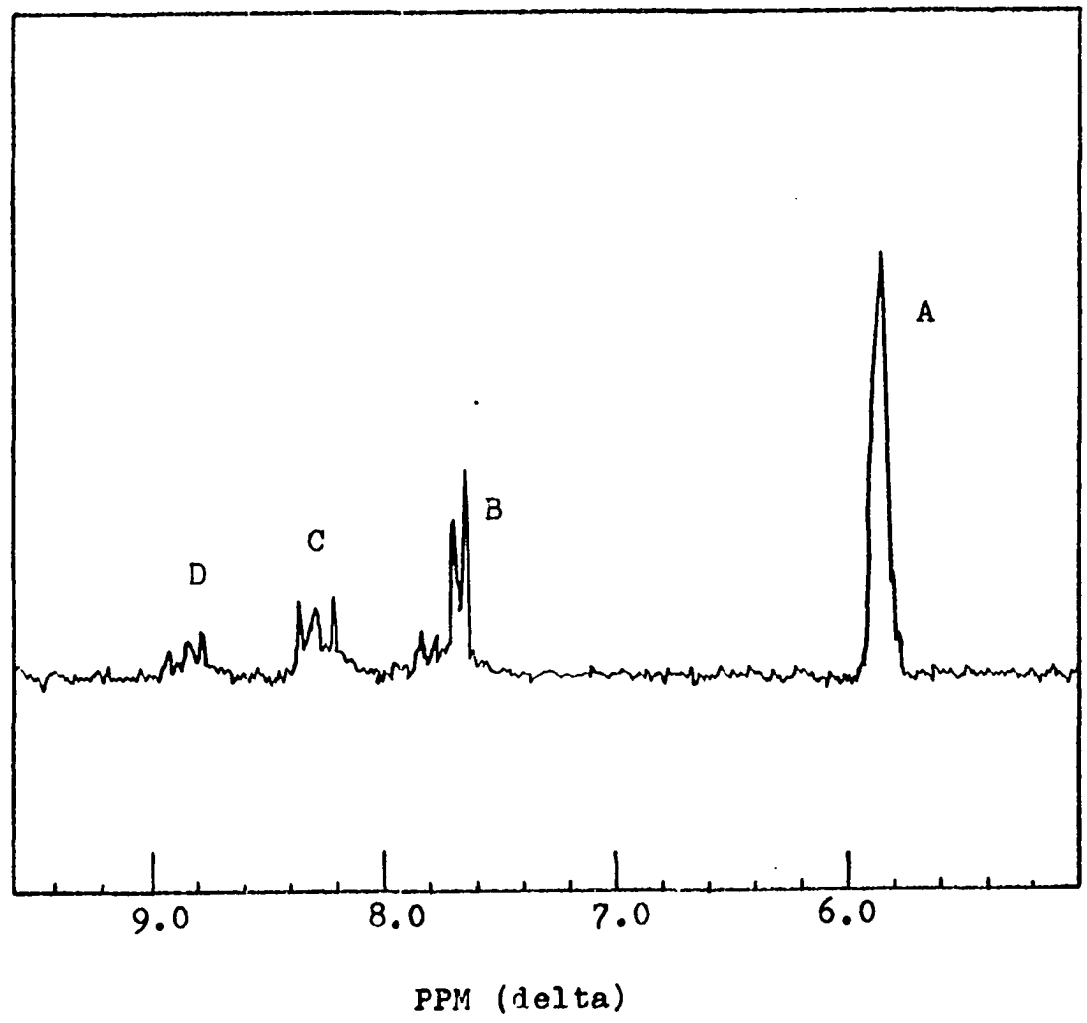

9.0
8.0
7.0
6.0
PPM (delta)

Figure 31. The proton magnetic resonance spectrum of gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid in trifluoroacetic acid

A. Methylene protons of Ar-CH_2-N (4)
B. 7 Proton of the phthalate ring (1)
   2', 7' Protons of the orcinol ring (2)
C. 5, 6 Protons of the phthalate ring (2)
D. 4 Proton of the phthalate ring (1)


A
B
C
D
9.0
8.0
7.0
6.0
PPM (delta)

The multiplets integrating to 1:2:1 protons were centered at 9.23, 8.25 and 7.27 delta. These multiplets were attributed to the phthalate ring hydrogen atoms. By comparison, the phthalate ring protons of the parent fluoran in trifluoroacetic acid appeared as multiplets centered at 8.42, 7.80 and 7.18 delta.

Inspection of the phthalate ring proton spectra of the parent fluoran and of the methyleneiminodiacetic acid derivative indicates that two protons are each ortho to a central group. That is, an AB_2C system is present. The 4 proton is splitting the 5 and 6 protons into a doublet. The 7 proton is splitting the 5 and 6 protons into a doublet.

The 4 proton, being ortho to a $-CO_2R$ group, is the most deshielded (15) and therefore appears the furthest downfield. The peak at 9.23 delta was attributed to the 4 proton of the phthalate ring.

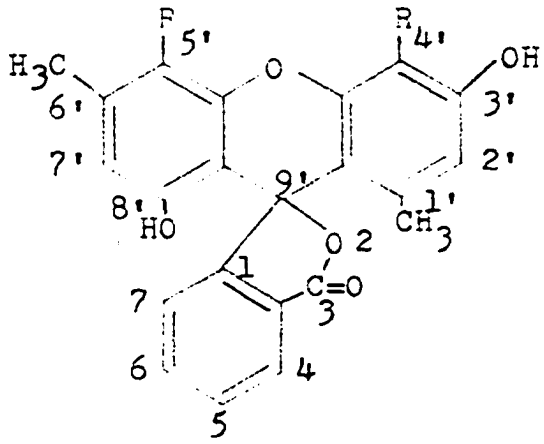
The 5 and 6 protons are less intensely but about equally deshielded by the carbonyl. The peak integrating to two protons centered at 8.25 delta was attributed to the 5 and 6 protons.

The 7 proton was attributed to the multiplet at 7.27 delta.

The multiplet integrating to two protons was centered at 7.90 delta. This multiplet was attributed to the 2' and 7' protons of the orcinol ring. In the NMR spectrum of the parent fluoran, the singlet integrating to two protons centered at 6.95 delta was attributed to the 4' and 5' protons of the orcinol ring. In the NMR spectrum of the parent fluoran, the singlet integrating to two protons centered at 7.47 delta was attributed to the 2' and 7' protons of the orcinol ring. In the NMR spectrum of the parent fluoran, the peak attributed to the 4' and 5' protons (6.95 delta) appeared upfield from the 7 proton (7.18 delta).

In the same spectrum the peak attributed to the 2' and 7' protons (7.47 delta) appeared downfield from the 7 proton (7.18 delta) and upfield from the 5 and 6 protons (7.80 delta). In the NMR spectrum of the methyleneiminodiacetic acid derivative, no aromatic peak was observed upfield from the 7 proton of the phthalate ring. Thus, the 4' and 5' positions had been substituted by the methyleneiminodiacetic acid entity. The peak attributed to the 2' and 7' protons appeared at 7.90 delta. This peak was downfield from the 7 proton of the phthalate ring (7.27 delta) and upfield from the 5 and 6 protons of the phthalate ring (8.25 delta).

Observed in the NMR spectrum of beta-dimethyldihydroxyfluoran-methyleneiminodiacetic acid in trifluoroacetic acid was a 1:2:3:4:8:3:3 proton ratio.



R= $CH_2N(CH_2CO_2H)_2$

The singlets integrating to three protons each were centered at 2.08 delta and 3.00 delta. The singlet at 2.08 delta was attributed to the 1' methyl group. The singlet at 3.00 delta was attributed to the 6' methyl group. By comparison, the 1' and 6' methyl groups of the parent fluoran in trifluoroacetic acid fall at 1.97 delta and 2.65 delta.

The singlet integrating to eight protons was centered at 4.87 delta. This singlet was attributed to the eight methylene hydrogen atoms of the four acetic acid groups.

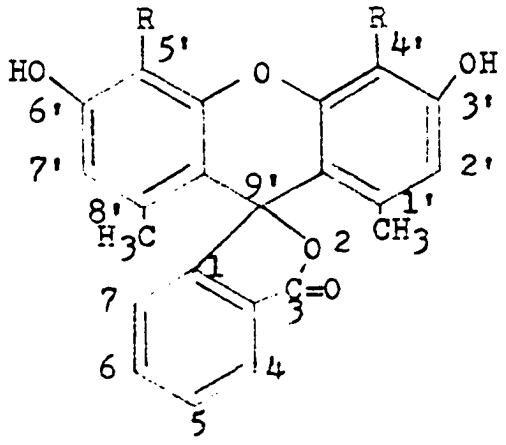
The singlet integrating to four protons was centered at 5.67 delta. This singlet was attributed to the two methylene hydrogen atoms of the two methylene groups linking the amino acid to the aromatic ring.

The multiplets integrating to 1:2:1 protons were centered at 8.40, 8.22 and 7.37 delta. These multiplets were attributed to the phthalate ring hydrogen atoms. By comparison, the phthalate ring protons of the parent fluoran in trifluoroacetic acid appeared as multiplets centered at 8.50, 7.92 and 7.22 delta in a 1:2:1 proton ratio. The peak at 8.40 delta was attributed to the 4 proton of the phthalate ring. The peak at 8.22 delta was attributed to the 5 and 6 protons of the phthalate ring. The peak at 7.37 delta was attributed to the 7 proton of the phthalate ring.

The multiplet centered at 7.37 delta integrated to a total of three protons. One of these protons was attributed to the 7 proton of the phthalate ring. Two of these protons were attributed to the 2' and 7' protons of the orcinol ring. By comparison, in the NMR spectrum of the parent fluoran in trifluoroacetic acid, there was observed a multiplet composed of two overlapping multiplets centered at 7.22 and 7.40 delta,

respectively. This entire peak integrated to three protons. By inspection of the overlapping peaks of the parent fluoran, the upfield peak was attributed to the 7 proton of the phthalate ring and the downfield peak was attributed to the 2' and 7' protons of the orcinol ring. In the NMR spectrum of the parent fluoran, there was observed a singlet at 6.93 delta integrating to two protons. This singlet was attributed to the 4' and 5' protons of the orcinol ring. The 4' and 5' proton peak was upfield from the 7 proton peak of the phthalate ring (7.22 delta). In the NMR spectra of the methyleneiminodiacetic acid derivative, no aromatic peak was observed upfield from the 7 proton of the phthalate ring absorption. Thus, the 4' and 5' positions of the methyleneiminodiacetic acid derivative had been substituted by the methyleneiminodiacetic acid groups.

Observed in the NMR spectrum of gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid in trifluoroacetic acid was a 1:2:3:4:8:6 proton ratio.



R= $CH_2N(CH_2CO_2H)_2$

The singlet integrating to six protons was centered at 2.24 delta. This singlet was attributed to the two methyl group hydrogen atoms. By comparison, the two methyl group hydrogen atoms of the parent fluoran in trifluoroacetic acid fall at 1.90 delta.

The singlet integrating to eight protons was centered at 5.02 delta. This singlet was attributed to the eight methylene hydrogen atoms of the four acetic acid groups.

The singlet integrating to four protons was centered at 5.90 delta. This singlet was attributed to the four methylene hydrogen atoms of the two methylene groups linking the amino acid to the aromatic ring.

The multiplets integrating to 1:2:1 protons were centered at 8.87, 8.33 and 7.73 delta. These multiplets were attributed to the 4 proton, the 5 and 6 protons, and the 7 proton of the phthalate ring. By comparison, the phthalate ring protons of the parent fluoran in trifluoroacetic acid appeared as multiplets centered at 8.88, 8.05 and 7.40 delta in a 1:2:1 proton ratio.

The multiplet centered at 7.73 delta integrated to a total of three protons. One of these protons was attributed to the 7 proton of the phthalate ring. Two of these protons were attributed to the 2' and 7' protons of the orcinol ring. By comparison, in the NMR spectrum of the parent fluoran in trifluoroacetic acid, there was observed a multiplet at 7.40 delta of two overlapping multiplets. The entire peak integrated

to three protons. By inspection of the overlapping peaks, the downfield peak was attributed to the 7 proton of the phthalate ring, and the upfield peak was attributed to the 2' and 7' protons of the orcinol ring. In the NMR spectrum of the parent fluoran, there was observed a multiplet at 7.20 delta integrating to two protons. This peak was attributed to the 4' and 5' protons of the orcinol ring. The 4' and 5' proton peak was upfield from the peak at 7.40 delta due to the 7 proton of the phthalate ring and the 2' and 7' protons of the orcinol ring. In the NMR spectrum of the methyleneiminodiacetic acid derivative, no aromatic peak was observed upfield from the 7, 2', 7' proton peak. Thus, the 4' and 5' positions of the methyleneiminodiacetic acid derivative had been substituted by the methyleneiminodiacetic acid group.

In conclusion, analysis of the NMR spectra of the parent fluorans and of their methyleneiminodiacetic acid derivatives has shown that no substitution occurred in the phthalate ring of the derivatives as evidenced by the presence of a 1:2:1 proton ratio in all of the spectra. Integration and position of the aliphatic hydrogen atom peaks showed the presence of the respective methyl hydrogen atom peaks in both the parent fluoran and the derivative, the presence of the eight methylene hydrogen atoms attributed to the methylene groups of the four acetic acid groups present in the derivative, and the presence of four methylene hydrogen atoms attributed to the methylene groups linking the amino acid to the ring in the derivative. Integration and position of the one aromatic peak in the derivative as compared with the two aromatic peaks in the parent fluoran assigned the one peak to the 2' and 7' protons of the derivative and the

methyleneiminodiacetic acid groups to the 4' and 5' positions of the derivative. Thus the methyleneiminodiacetic acid derivative molecules are highly symmetrical, with one methyleneiminodiacetic acid group present in each orcinol ring and in positions 4' and 5'. All six replaceable hydrogen atoms (four carboxylic acid and two phenol entities) of the methyleneiminodiacetic acid derivative exchange rapidly with the solvent, trifluoroacetic acid. Thus, alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid are 1', 8' -dihydroxy-3', 6'-dimethyl-4', 5'-bis[N, N'-di(carboxymethyl)-aminomethyl]fluoran, 3', 8'-dihydroxy-1', 6'-dimethyl-4', 5'-bis[N, N'-di (carboxymethyl) aminomethyl] fluoran and 3', 6'-dihydroxy-1', 8'-dimethyl-4', 5'-bis [N, N'-di(carboxymethyl)aminomethyl] fluoran.

d. Elemental analysis Alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid were analyzed for percentage carbon, hydrogen and nitrogen. Oxygen was determined by difference. The results are summarized in Table 17.

The percentage carbon, hydrogen, nitrogen and oxygen determined experimentally are consistent with the respective theoretical percentages for anhydrous alpha- and beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid and for the monohydrate of gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid.

e. Residue on ignition The residue on ignition of 0.5 grams each of alpha-, beta- and gamma-dimethyldihydroxyfluoran-methyleneiminodiacetic acid was found to be less than 0.1 mg. The derivatives were therefore void of non-volatile metal impurities.

Table 17. The experimental percentage carbon, hydrogen, nitrogen and oxygen of alpha-, beta- and gamma-dimethyldihydroxy-fluoranmethyleneiminodiacetic acid and the theoretical percentage of $C_{32}H_{30}O_{13}N_2$ M. W. 650.593 and $C_{32}H_{30}O_{13}N_2 \cdot H_2O$ M. W. 668.608

Compound	Percentage			
	Carbon	Hydrogen	Nitrogen	Oxygen
alpha-dimethyldihydroxy-fluoranmethyleneimino-diacetic acid	58.98	4.89	4.23	31.90
beta-dimethyldihydroxy-fluoranmethyleneimino-diacetic acid	57.79	5.30	4.92	31.99
gamma-dimethyldihydroxy-fluoranmethyleneimino-diacetic acid	56.39	4.82	4.24	34.55
theoretical $C_{32}H_{30}O_{13}N_2$ M. W. 650.593	59.08	4.65	4.31	31.96
theoretical $C_{32}H_{30}O_{13}N_2 \cdot H_2O$ M. W. 668.608	57.49	4.82	4.19	33.50

f. Fluorescence Alpha- and beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid are non-fluorescent. Gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid is fluorescent at pH $\geq$ 2.1. Gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid is a potential selective metallofluorochromic indicator for calcium in alkaline solution. At pH 11, there is a marked increase in fluorescence on the addition of $CaCl_2$ to a solution of gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, a very slight increase in fluorescence on the addition of $MgCl_2$ and no effect on fluorescence on the addition of $AlCl_3$.

g. Solubility in non-aqueous solvents The solubility of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid was qualitatively determined in a number of solvents.

Of the solvents tested, alpha-dimethyldihydroxyfluoranmethyleneiminodiacetic acid was slightly less soluble than beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid in a given solvent; and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid was slightly more soluble than beta-dimethyldihydroxyfluoranmenthyleneiminodiacetic acid.

The solubility of beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid in a number of solvents is summarized in Table 18.

In general, the derivatives were highly insoluble, both cold and hot, in the many solvents tested. The dissolution rate, both hot and cold, was very slow. Each solvent and solid was allowed to stand for four hours at each dissolution attempt. The derivatives were somewhat

Table 18. Solubility of beta-dimethyldihydroxyfluoranmethyleneimino-diacetic acid in various solvents

Solvent	Solubility[a] Cold	Hot
Acids		
Formic	SS	S
Glacial acetic	VSS	SS
Acetic anhydride	I	I
3 M Hydrochloric	I	SS
conc. Hydrochloric	S	-
9 M Sulfuric	S	S
Trifluoroacetic	SS	S
Bases		
1 M Sodium hydroxide	S	S
Pyridine	VSS	SS
Dimethylformamide	SS	S
Aniline	I	VSS
conc. Ammonia	SS	-
Ethylenediamine	S	S
Sulfur compounds		
Dimethyl sulfoxide	SS	S
Sulfolane	I	VSS
Ethers		
Diethyl ether	VSS	-
1, 4-Dioxane	I	-
Alcohols		
Ethyl	I	VSS
Methyl	I	VSS
n-Butyl	I	VSS
t-Butyl	I	VSS
Aldehydes and ketones		
Acetone	I	VSS
Methyl iso-butyl ketone	I	I
Acetaldehyde	I	I
Benzaldehyde	I	I

[a] S = Soluble,
SS = Slightly soluble,
VSS = Very slightly soluble,
I = Insoluble.

Table 18. (Continued)

Solvents	Solubility	
	Cold	Hot
Aromatic solvents		
Benzene	I	I
Toluene	I	I
Nitrobenzene	I	I
Benzonitrile	I	I
Miscellaneous solvents		
Water	I	I
Chloroform	I	I
Acetonitrile	I	I
Ethylene dichloride	I	I
Methylene dichloride	I	I
Ethyl acetate	I	I

soluble in formic acid, trifluoroacetic acid, dimethyl sulfoxide, alkaline aqueous solution, ethylenediamine and pyridine.

h. Titration in non-aqueous solvents Alpha- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid were dissolved in ethylenediamine and titrated potentiometrically with tetraethylammonium hydroxide in ethylenediamine. Beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid was dissolved in pyridine and titrated potentiometrically with tetraethylammonium hydroxide in pyridine. The data was plotted as mV versus volume titrant. One end point was observed, (Figures 32, 33, and 34) corresponding to four moles of base added per mole of derivative.

The equivalent weight of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid was 161.40, 163.54 and 171.21. The theoretical equivalent weight of anhydrous and monohydrate derivative is 162.65 and 167.15. (The M. W. of the anhydrous derivative is 650.593, of the monohydrate 668.608.) This corresponds to a purity of 99.23 per cent and 99.45 per cent of the anhydrous alpha- and beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid 97.63 per cent of the monohydrate gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid.

Beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid was soluble in pyridine. Pyridine has a K_b of 1.71×10^{-9} (ref. Rubber Handbook, 1963) and a dielectric constant of 12.3 at 25° (ref. Lange, Handbook of Chemistry, Tenth Edition). Alpha-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, the weakest acid of the three derivatives required a stronger base, ethylenediamine, as a solvent. Ethylenediamine has a K_b of 8.5×10^{-5} (ref. Rubber Handbook, 1963) and a dielectric constant of 14.2 at 25° (ref. Lange, Handbook of Chemistry,

Figure 32. Non-aqueous potentiometric titration of alpha-dimethyldihydroxyfluoranmethyleneiminodiacetic acid in ethylenediamine with tetraethylammonium hydroxide

0.1922 g. acid
0.1426 N tetraethylammonium hydroxide

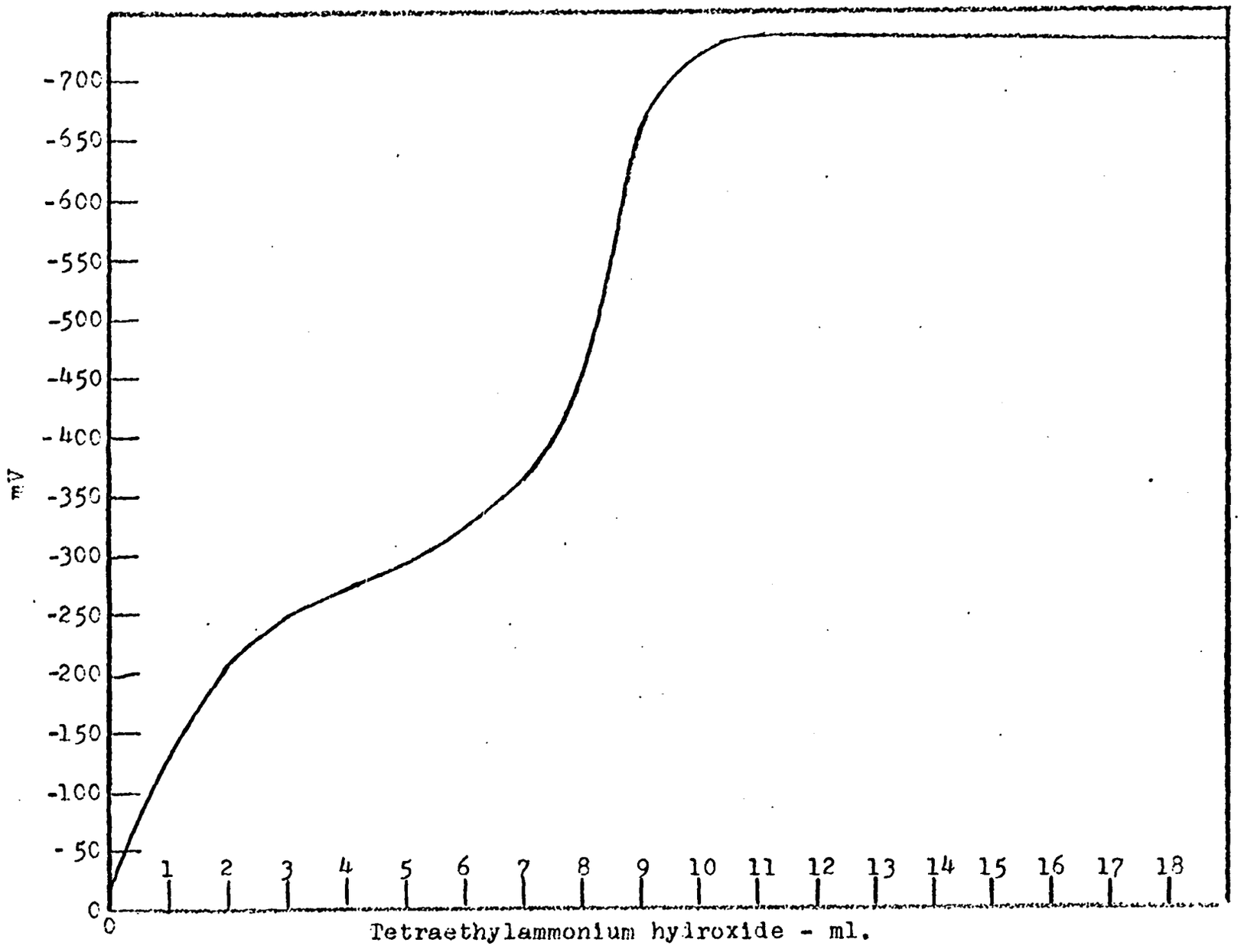

-700
-650
-600
-550
-500
-450
-400
-350
-300
-250
-200
-150
-100
-50
0
mV
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
Tetraethylammonium hydroxide - ml.

Figure 33. Non-aqueous potentiometric titration of beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid in pyridine with tetraethylammonium hydroxide

0.2468 g. acid
0.1240 N tetraethylammonium hydroxide

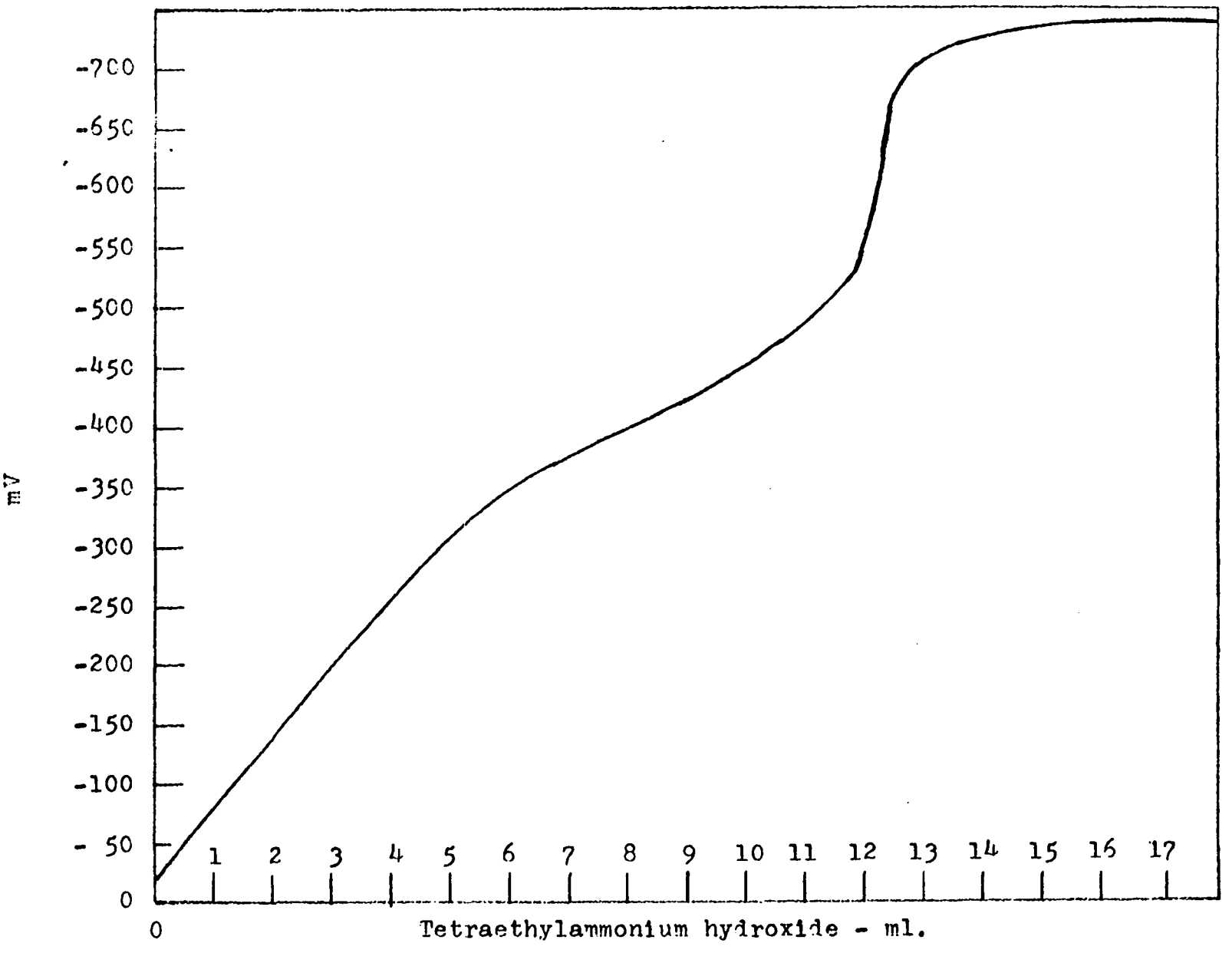
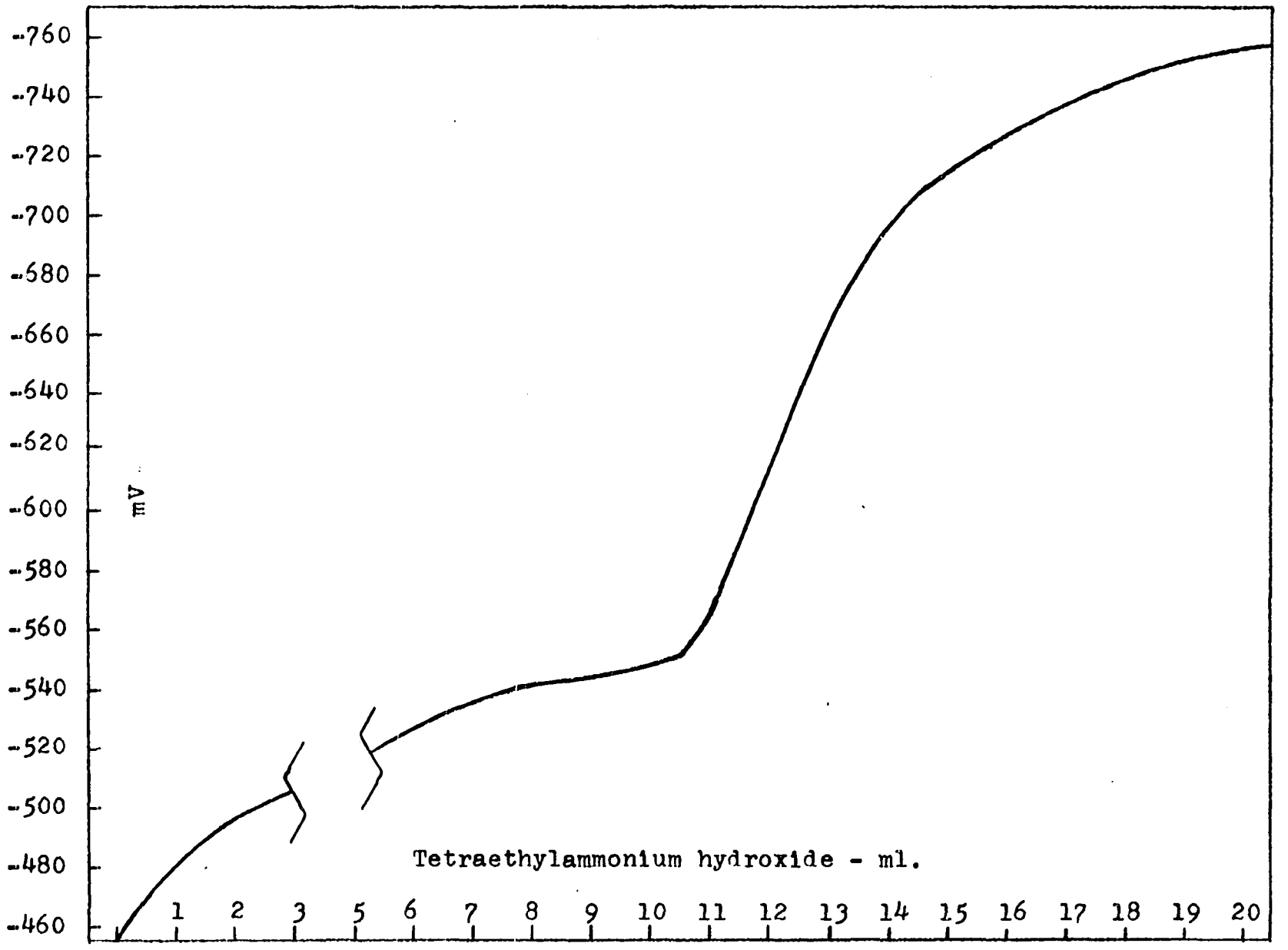

-700
-650
-600
-550
-500
-450
-400
-350
-300
-250
-200
-150
-100
- 50
0
mV
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
0
Tetraethylammonium hydroxide - ml.

Figure 34. Non-aqueous potentiometric titration of gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid in ethylenediamine with tetraethylammonium hydroxide

0.2191 g. acid
0.1063 N tetraethylammonium hydroxide


-760
-740
-720
-700
-680
-660
-640
-620
-600
-580
-560
-540
-520
-500
-480
-460
mV
Tetraethylammonium hydroxide - ml.
1 2 3 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Tenth Edition). As a matter of convenience and speed, ethylenediamine was chosen as the solvent for the titration of gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid.

The choice of a specific non-aqueous solvent was governed by that solvent's basicity, dielectric constant, and solubility of solute. The more strongly basic a solvent is, the more completely will a given acid be ionized in that solvent. Thus basic solvents "enhance" the acid properties of weak acids. Solvents having a high dielectric constant facilitate charge separation and promote ionization. The solvent chosen must have a dielectric constant greater than 5 or the high resistance of the circuit renders potentiometric methods unsuitable.

The acid functions of the derivatives in order of decreasing acid strength are the two carboxylic acid groups, the two phenolic groups and the two ammonium groups. The four strongest acid groups were titrated, that is, the two carboxylic acid and the two phenolic groups.

i. Acid dissociation constants by potentiometric titration

The second, third and fourth acid dissociation constants of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid were determined by indirect potentiometric titration. A solution of the derivative in a known excess of sodium hydroxide was back-titrated potentiometrically with standard hydrochloric acid solution. The titration curves obtained are shown in Figures 22, 23, 24 and 25.

The first acid dissociation constant was better determined by solubility measurements. The derivatives are highly insoluble in acidic solution; the derivatives precipitate out prior to complete back-titration with standard hydrochloric acid. The derivatives were too

insoluble for direct potentiometric titration with standard sodium hydroxide.

Two breaks were observed in each titration curve. The first break was due to the titration of excess sodium hydroxide with hydrochloric acid and corresponded to the calculated volume of hydrochloric acid required.

The second break corresponded to two moles of hydrochloric acid per mole of derivative and was due to the titration of two phenolic groups.

No third break was observed. However, the derivatives began to precipitate before the end of the titration was reached. This corresponded to the titration of the two carboxylic acid groups.

The pK_4 and pK_3 were obtained directly from the titration curve. The first break corresponding to the titration of excess sodium hydroxide with hydrochloric acid designated 400 per cent titration. The second break was designated as 200 per cent titration. At the midpoint of a titration, that is, at 50 per cent titration, $pH = pK_a$, where pK_a is by definition the negative logarithm of the acid dissociation constant . Thus $pH = pK_4$ at 350 per cent titration and $pH = pK_3$ at 250 per cent titration.

In like manner, the pK_2 value was obtained directly from the titration curve. At 150 per cent titration, $pH = pK_2$.

The value of pK_1 obtained directly from the titration data at 50 per cent titration was in poor agreement with that obtained from solubility measurements. The pK_1 obtained from solubility measurements was considered the better value. Precipitation of the derivative ensued at about 60 per cent titration. Thus the premise that $pH = pK_a$ at

50 per cent titration does not hold since in solution $[HA] \neq [A^-]$.

The results are summarized in Table 19.

If the premise that pH = pK_4 at 350 per cent titration, pH = pK_3 at 250 per cent titration, pH = pK_2 at 150 per cent titration is true, then the addition of a larger amount of standard alkali to a solution of the derivative followed by back-titration with standard hydrochloric acid should yield the same pK values at the respective percentage of titration. This was observed to be the case. The results are summarized in Table 20.

The presence of the one break of 200 per cent titration and the absence of inflection in the curve is indicative of uniform distribution of the pairs of numerical values of the acid dissociation constants. That is, the ratio of K_1 to K_2 and K_3 to K_4 is probably less than 100. Thus considerable overlap occurs in the titration as two successive steps and only two steps are involved at any given pH.

Each derivative may be represented as H_6A and the six dissociation steps defined by the successive acid dissociation constants, K_1, K_2, K_3, K_4, K_5 and K_6 by the equations:

$$H_6A = H^+ + H_5A^- \qquad K_1 = \frac{[H^+]\,[H_5A^-]}{[H_6A]} \qquad (14)$$

$$H_5A^- = H^+ + H_4A^{-2} \qquad K_2 = \frac{[H^+]\,[H_4A^{-2}]}{[H_5A^-]} \qquad (15)$$

$$H_4A^{-2} = H^+ + H_3A^{-3} \qquad K_3 = \frac{[H^+]\,[H_3A^{-3}]}{[H_4A^{-2}]} \qquad (16)$$

$$H_3A^{-3} = H^+ + H_2A^{-4} \qquad K_4 = \frac{[H^+]\,[H_3A^{-4}]}{[H_3A^{-3}]} \qquad (17)$$

Table 19. Acid dissociation constants of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid

Compound		pK_a
alpha-dimethyldihydroxyfluoran-methyleneiminodiacetic acid	pK_1^c	2.98
	pK_1^t	3.00
	pK_1^s	3.11
	pK_2	5.95
	pK_3	8.92
	pK_4	9.83
beta-dimethyldihydroxyfluoran-methyleneiminodiacetic acid	pK_1^c	2.44
	pK_1^t	2.74
	pK_1^s	3.44
	pK_2	4.30
	pK_3	7.33
	pK_4	8.81
gamma-dimethyldihydroxyfluoran-methyleneiminodiacetic acid	pK_1^c	2.38
	pK_1^t	2.81
	pK_1^s	3.43
	pK_2	3.74
	pK_3	5.75
	pK_4	6.73

where pK^c is the pK value calculated by use of the pK_2 value;
pK^t is the pK value determined from titration data;
pK^s is the pK value determined from solubility data.

Table 20. Acid dissociation constants of beta-dimethyldihydroxyfluoran-methyleneiminodiacetic acid upon addition of 25.00 ml. and 50.00 ml. standard sodium hydroxide solution and back-titration with hydrochloric acid

pK_a	25.00 ml. standard sodium hydroxide	50.00 ml. standard sodium hydroxide
pK_1	2.74	3.00
pK_2	4.30	4.41
pK_3	7.33	7.42
pK_4	8.81	8.87

$$H_2A^{-4} = H^+ + HA^{-5} \qquad K_5 = \frac{[H^+][HA^{-5}]}{[H_2A^{-4}]} \qquad (18)$$

$$HA^{-5} = H^+ + A^{-6} \qquad K_6 = \frac{[H^+][A^{-6}]}{[HA^{-5}]} \qquad (19)$$

Considering the fourth dissociation step as a monobasic acid, the midpoint pH (at 350 per cent titration) may be taken as a value for the negative logarithm of the acid dissociation, $pK_4 = pH_{(350\ per\ cent\ titration)} = 9.83$, 8.81 and 6.73 for alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid.

Considering the second dissociation step as a monobasic acid, the midpoint $pH_{(at\ 150\ per\ cent\ titration)}$ equals pK_2, 5.95, 4.30 and 3.74 for alpha-, beta- and gamma-dimethyldihydroxyfluoranmethylene-iminodiacetic acid.

A more elaborate way of doing the same thing is to use the observed pH at various stages of the titration and Equation (20) as obtained by rearranging Equation (17).

$$K_4 = \frac{[H^+][H_2A^{-4}]}{[H_3A^{-3}]} \qquad (17)$$

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

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

$$pH = pK4 + \log \frac{[H_2A^{-4}]}{[H_3A^{-3}]} \qquad (20)$$

For $[H_3A^{-3}]$, the untitrated acid, the successive values of a, a = 0.30, 0.40, . . . 0.70 were used, for $[H_2A^{-4}]$, the salt formed, 1.00 - a. The values obtained for alpha-dimethyldihydroxyfluoranmethylene-iminodiacetic acid were:

a	pH	$+\log\left[\frac{1.00-a}{a}\right]$	pK_4
0.30	9.30	+0.37	8.93
0.40	9.07	+0.18	8.89
0.50	8.81	0.00	8.81
0.60	8.64	-0.18	8.82
0.70	8.46	-0.37	8.83

Average: $pK_4 = 8.85$

A plot of pH vs $\log\left[\frac{1.00-a}{a}\right]$ gave a straight line having the theoretical slope of 1.0. The pK_4 value obtained directly from the titration curve at $pH_{(350\ per\ cent\ titration)} = pK_4$ was 8.81.

The values obtained for gamma-dimethyldihydroxyfluoran-methyleneiminodiacetic acid were:

a	pH	$+\log\left[\frac{1.00-a}{a}\right]$	pK_4
0.30	7.10	+0.37	6.73
0.40	6.84	+0.18	6.66
0.50	6.73	0.00	6.73
0.60	6.56	-0.18	6.74
0.70	6.34	-0.37	6.71

Average: $pK_4 = 6.71$

A plot of pH as $\log\left[\frac{1.00-a}{a}\right]$ gave a straight line having the theoretical slope of 1.0. The pK_4 value obtained directly from the titration curve at at $pH_{(350\ per\ cent\ titration)} = pK_4$ was 6.73.

The successive dissociation constant pK_3 was determined by considering the successive steps in pairs. For a given dibasic acid, the ionization reactions and the two acid dissociation constants are:

$$H_2A = H^+ + HA^- \qquad K_1 = \frac{[H^+][HA^-]}{[H_2A]} \qquad (21)$$

$$HA^- = H^+ + A^{-2} \qquad K_2 = \frac{[H^+][A^{-2}]}{[HA^-]} \qquad (22)$$

Multiplication of Equation (9) by (10) gives

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

$$[H^+] = \sqrt{\frac{K_1K_2 [H_2A]}{[A^{-2}]}} \qquad (23)$$

As applied to the determination of the third dissociation constant of the derivative

$$[H^+] = \sqrt{\frac{K_3K_4 [H_2A^{-4}]}{[H_4A^{-2}]}} \qquad (24)$$

At 300 per cent titration, theoretically only H_3A^{-3} should be present. Actually, some H_2A^{-4} has formed and some H_4A^{-2} remains untitrated, the amounts of the two being equal:

$$[H_4A^{-2}] = [H_2A^{-4}]$$

Equation (12) then becomes

$$[H^+] = \sqrt{K_3K_4}$$

or $1/2\ pK_3 = pH\ (300\ per\ cent\ titration) - 1/2\ pK_4$ (25)

For alpha-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, $pH = 9.42$ (300 per cent titration) and $pK_4 = 9.81$, gives

$$1/2\ pK_3 = 9.42 - 1/2\ (9.81)$$

$$pK_3 = 9.04$$

The pK_3 value determined directly from the titration curve at pH (350 per cent titration) $= pK_3$ was 8.92.

For beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, $pH = 8.00$ (300 per cent titration) and $pK_4 = 8.85$, gives

$$1/2\ pK_3 = 8.00 - 1/2\ (8.85)$$

$$pK_3 = 7.16$$

The pK_3 value determined directly from the titration curve at

$pH_{(250\text{ per cent titration})} = pK_3$ was 7.33.

For gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, pH at $_{(300\text{ per cent titration})} = 6.17$ and $pK_4 = 6.71$, gives

$$1/2\ pK_3 = 6.17 - 1/2\,(6.71)$$

$$pK_3 = 5.64$$

The pK_3 value determined directly from the titration curve at

$pH_{(250\text{ per cent titration})} = pK_3$ was 5.75.

The value for pK_2 was obtained in a manner similar to pK_4, since an end point at $_{(200\text{ per cent titration})}$ was observed.

$$K_2 = \frac{[H^+][H_4A^{-2}]}{[H_5A^-]} \qquad (26)$$

$$[H^+] = \frac{K_2\,[H_5A^-]}{[H_4A^{-2}]}$$

$$-\log [H^+] = -\log K_2 - \log \frac{[H_5A^-]}{[H_4A^{-2}]}$$

$$pH = pK_2 + \log \frac{[H_4A^{-2}]}{[H_5A^-]} \qquad (27)$$

For $[H_5A^-]$, the untitrated and the successive values of a, a = 0.30 0.40, . . . 0.70, were used, for $[H_4A^{-2}]$, the salt formed, 1.00 -a.

The values obtained for alpha-dimethyldihydroxyfluoranmethylene-iminodiacetic acid were:

a	pH	$+\log\left[\frac{1.00-a}{a}\right]$	pK_2
0.30	6.44	+0.37	6.07
0.40	6.21	+0.18	6.03
0.50	5.95	0.00	5.95
0.60	5.68	-0.18	5.86
0.70	5.56	-0.37	5.93

Average: $pK_2 = 5.97$

A plot of pH vs. log $\left[\frac{1.00-a}{a}\right]$ gave a straight line having the theoretical slope of 1.0. The pK_2 value obtained directly from the titration curve at $pH_{(150 \text{ per cent titration})} = pK_2$ was 5.95.

The values obtained for beta-dimethyldihydroxyfluoranmethylene-iminodiacetic acid were:

a	pH	$+ \log \left[\frac{1.00-a}{a}\right]$	pK_2
0.30	6.44	+0.37	6.07
0.40	6.21	+0.18	6.03
0.50	5.95	0.00	5.95
0.60	5.68	-0.18	5.86
0.70	5.56	-0.37	5.93

Average: $pK_2 = 5.97$

A plot of pH vs. log $\left[\frac{1.00-a}{a}\right]$ gave a straight line having the theoretical slope of 1.0. The pK_2 value obtained directly from the titration curve at $pH_{(150 \text{ per cent titration})} = pK_2$ was 5.95.

The values obtained for beta-dimethyldihydroxyfluoranmethylene-iminodiacetic acid were:

a	pH	$+ \log \left[\frac{1.00-a}{a}\right]$	pK_2
0.30	4.69	+0.37	4.32
0.40	4.57	+0.18	4.39
0.50	4.30	0.00	4.30
0.60	4.09	-0.18	4.27
0.70	3.87	-0.37	4.24

Average: $pK_2 = 4.30$

A plot of pH vs. log $\left[\frac{1.00-a}{a}\right]$ gave a straight line having the theoretical slope of 1.0. The pK_2 value obtained directly from the titration curve at $pH_{(150 \text{ per cent titration})} = pK_2$ was 4.30.

The values obtained from gamma-dimethyldihydroxyfluoran-methyleneiminodiacetic acid were:

a	pH	$+\log\left[\frac{1.00-a}{a}\right]$	pK_2
0.30	4.14	+0.37	3.77
0.40	3.98	+0.18	3.80
0.50	3.74	0.00	3.74
0.60	3.59	-0.18	3.77
0.70	3.44	-0.37	3.81

Average: $pK_2 = 3.78$

A plot of pH vs. $\log\left[\frac{1.00-a}{a}\right]$ gave a straight line having the theoretical slope of 1.0. The pK_2 value obtained directly from the titration curve at $pH_{(150 \text{ per cent titration})} = pK_2$ was 3.74.

The successive dissociation constant pK_1 can be determined by considering the successive steps in pairs. By analogy to the determination of pK_3 by Equation (25).

$$1/2\ pK_1 = pH_{(100 \text{ per cent titration})} - 1/2\ pK_2$$

For alpha-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, $pH_{(100 \text{ per cent titration})} = 4.47$ and $pK_2 = 5.95$, gives

$$1/2\ pK_1 = 4.47 = 1/2\ (5.95)$$

$$pK_1 = 2.98$$

The pK_1 value obtained directly from the titration data was 3.00 and from solubility measurements was 3.11.

For beta-dimethyldihydroxyfluoranmethyleneiminodiacetic acid, $pH_{(100 \text{ per cent titration})} = 3.37$ and $pK_2 = 4.30$, gives

$$1/2\ pK_1 = 3.37 - 1/2\ (4.30)$$

$$pK_1 = 2.44$$

The pK_1 value obtained directly from the titration data was 2.74 and from solubility measurements was 3.44.

For gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic

acid, $pH_{(100\ per\ cent\ titration)} = 3.06$ and $pK_2 = 3.74$, gives

$1/2\ pK_1 = 3.06 - 1/2\ (3.74)$

$pK_1 = 2.38$

The pK_1 value obtained directly from the titration data was 2.81 and from solubility measurements was 3.43.

In conclusion, the acid dissociation constants of each of the Mannich condensation products have been determined and assigned to specific groups in the molecules.

Compound	$pK_1(C_1)$	$pK_2(C_2)$	$pK_3(P_1)$	$pK_4(P_2)$
alpha-dimethyldihydroxyfluoran-methyleneiminodiacetic acid	3.11	5.95	8.92	9.83
beta-dimethyldihydroxyfluoran-methyleneiminodiacetic acid	3.44	4.30	7.33	8.81
gamma-dimethyldihydroxyfluoran-methyleneiminodiacetic acid	3.43	3.74	5.75	6.73

(C) representing a carboxyl group and (P) a phenolic group.

The phenolic protons in the derivatives are stronger acids than in the parent compounds owing to the presence of the positive charge on the neighboring nitrogen atom (Zwitter ion).

j. Acid dissociation constants by solubility measurements

The determination of the acid dissociation constants of the derivatives was by both potentiometric and solubility methods. The potentiometric titrations of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethylene-iminodiacetic acid (Figures 22-25) show two end-points, one at 200 per cent titration and one at 400 per cent titration. However, the derivatives did not dissolve completely until just before 100 per cent titration. Thus, it was not possible to calculate the first acid dissociation constant

directly from the titration curve.

The first acid dissociation constant of the carboxylic proton of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid was determined by the method of Krebs and Speakman (9). This method is based on the solubility of an acid as a function of pH. The data obtained are summarized in Table 10.

For a given monobasic acid, HA, the theory can be summarized as follows:

$$HA = H^+ + A^- \tag{28}$$

$$K_a = \frac{[H^+][A^-]}{[HA]} \tag{29}$$

The acid is present in solution in the two forms HA and A^-. The relative amount of HA and A^- present is a function of pH. The total material in solution, the solubility S_o, is the sum of the neutral molecule and of the anion in solution.

$$S_o = [HA] + [A^-] \tag{30}$$

It is assumed that the solubility of the unionized acid is constant over the pH range. The solubility of the neutral molecule, HA, is the intrinsic solubility, S_i. Such that,

$$S_i = [HA] \tag{31}$$

and

$$S_o = S_i + [A^-] \tag{32}$$

from the equilibrium expression,

$$[A^-] = \frac{K_a [HA]}{[H^+]} \tag{33}$$

such that

$$S_o = S_i + \frac{K_a S_i}{[H^+]} \tag{34}$$

At high hydrogen ion concentration, S_i approaches S_o. The activities of

the neutral molecule and the anion are assumed to be one.

Rearrangement of Equation (34) gives

$$S_o = S_i \left[1 + \text{antilog}\,(pH - pK_a) \right] \qquad (35)$$

Rearrangement of Equation (35) gives

$$pK_a = pH - \log\left[\frac{S_o}{S_i} - 1\right] \qquad (36)$$

Prior to use of Equation(36), S_i must be determined. S_i can be determined by plotting S_o versus $1/[H^+]$. According to Equation (34) extrapolation of $1/[H^+]$ to 0 gives S_i (Figures 26, 27, 28). The acid dissociation constant was then calculated using Equation (36) and by plotting $\log [S_o/S_i - 1]$ versus pH. In the latter case, the pK_a is the pH at which the straight line intersects the abscissa. Both methods yielded essentially identical results. The results are summarized in Table 21.

Table 21. Acid dissociation constants of the carboxylic acid group proton of alpha-, beta- and gamma-dimethyldihydroxyfluoranmethyleneiminodiacetic acid determined from solubility measurements

Compound	S_o (mg./100 ml.)	pH	S (mg./100 ml.)	pK (Equation 36)	pK (graph)
alpha-dimethyldihydroxyfluoran-methyleneiminodiacetic acid	0.930	2.13	1.031	3.09	
		2.56	1.197	3.10	
		2.67	1.258	3.12	
		2.95	1.577	3.11	3.10
				3.11 (Average)	
beta-dimethyldihydroxyfluoran-methyleneiminodiacetic acid	4.662	2.31	5.014	3.43	
		2.55	5.256	3.45	
		2.80	5.738	3.44	
		3.05	6.623	3.43	3.43
				3.44 (Average)	
gamma-dimethyldihydroxyfluoran-methyleneiminodiacetic acid	17.10	2.48	19.10	3.41	
		2.90	21.73	3.47	
		3.22	27.91	3.42	
		3.47	37.15	3.40	3.43
				3.43 (Average)	

IV. SUMMARY

Three new metallofluorochromic indicators, derived from three dimethyldihydroxyfluorans by the introduction of a chelating group, have been prepared and studied.

Three previously known dimethyldihydroxyfluorans, alpha-, beta- and gamma-dimethyldihydroxyfluoran, have been prepared, separated and purified by a new method, and characterized. The purity, composition, structure and properties of each of these compounds have been established, the various techniques used being mass spectroscopy, infrared spectroscopy, nuclear magnetic resonance spectroscopy, fluorescence, elemental analysis, residue on ignition, solubility in non-aqueous solvents, titration in non-aqueous solvents, determination of equivalent weight by neutralization and determination of dissociation constants.

The methyleneiminodiacetic acid derivatives of each of the three dimethyldihydroxyfluorans have been prepared, purified, and studied as metallofluorochromic indicators. The methyleneiminodiacetic acid groups have been introduced into the parent fluoran by condensation with formaldehyde and iminodiacetic acid (Mannich reaction). The composition and structure of the derived compounds have been established by mass spectroscopy, infrared spectroscopy, nuclear magnetic resonance spectroscopy, elemental analysis, residue on ignition, solubility in non-aqueous solvents, fluorescence, titration in non-aqueous solvents, determination of equivalent weight by neutralization and determination of acid dissociation constants.

Following the synthesis by condensation of phthalic anhydride and orcinol in fuming sulfuric acid, the three dimethyldihydroxyfluorans have been separated by extraction from ether into water buffered at various values of pH from 7 to 13. This separation is based on differences in the strengths of the three isomers as acids, the acidity decreasing in the order gamma-, beta- and alpha-dimethyldihydroxyfluoran.

The original structure assignments of the parent fluorans by Orndorff and Allen (12) proved to be correct. Their alpha-, beta- and gamma-dimethyldihydroxyfluoran are 1', 8'-dihydroxy-3', 6'-dimethylfluoran, 3', 8'-dihydroxy-1', 6'-dimethylfluoran and 3', 6'-dihydroxy-1', 8'-dimethylfluoran.

The mass spectra, infrared spectra and NMR spectra of the parent fluorans are consistent with the assigned structures. In the NMR spectra, the peaks observed were assigned to the protons of the phthalate ring, the protons of the orcinol ring and the protons of the two methyl groups on the basis of position in the spectrum and integration.

Of the parent fluorans, only the gamma isomer (3', 6'-dihydroxy-1', 8'-dimethylfluoran) is fluorescent.

The equivalent weight and purity of the parent fluorans have been determined by potentiometric non-aqueous titration in ethanol and pyridine with standard sodium hydroxide; two replaceable hydrogen atoms per molecule have been observed.

The first acid dissociation constants of the parent fluorans have been determined by spectroscopic and solubility methods.

The mass spectra and infrared spectra of the methylenemino-

diacetic acid derivatives of the parent fluorans are consistent with the structures that have been assigned. The presence of both a free carboxyl and a carboxylate anion was observed in the infrared spectra.

Analysis of the NMR spectra of the derivatives indicates that the molecule is highly symmetrical and with one methyleneiminodiacetic acid group present in each orcinol ring, that is, at positions 4' and 5'.

The fluorescence of the gamma derivative (3',6'-dihydroxy-1',8'-dimethyl-4',5'-bis /N,N'-di(carboxymethyl)aminomethyl/ fluoran) at pH 11 has been found to be enhanced by the addition of calcium and magnesium.

The equivalent weight and purity of the derivatives have been determined by potentiometric non-aqueous titrations in pyridine and ethylenediamine with standard tetraethylammonium hydroxide; four replaceable hydrogen atoms per molecule were observed.

The acid dissociation constants of the derivatives have been determined by potentiometric, spectroscopic and solubility methods and assignments made of the various acid functions to specific groups in the molecules.

The gamma derivative (3',6'-dihydroxy-1',8'-dimethyl-4',5'-bis /N,N'-di(carboxymethyl)aminomethyl/ fluoran) has been found to function as a metallofluorochromic indicator for calcium and magnesium in alkaline solution. The alpha- and beta- derivatives (1',8'-dihydroxy-3',6'-dimethyl-4',5'-bis /N,N'-di(carboxymethyl)aminomethyl/ fluoran and 3',8'-dihydroxy-1',6'-dimethyl-4',5'-bis /N,N'-di(carboxymethyl) aminomethyl/ fluoran) and the alpha- and beta- parent fluorans (1',8'-

dihydroxy-3', 6'-dimethylfluoran and 3', 8'-dihydroxy-1', 6'-dimethyl-fluoran) do not fluoresce and do not become fluorescent on the addition of calcium or magnesium.

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