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SUBSTITUTED COUMARINS AS METALLOFLUOROCHROMIC INDICATORS

by

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Approved:

Signature was redacted for privacy.
In Charge of Major Work

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Head of Major Department

Signature was redacted for privacy.
Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1967

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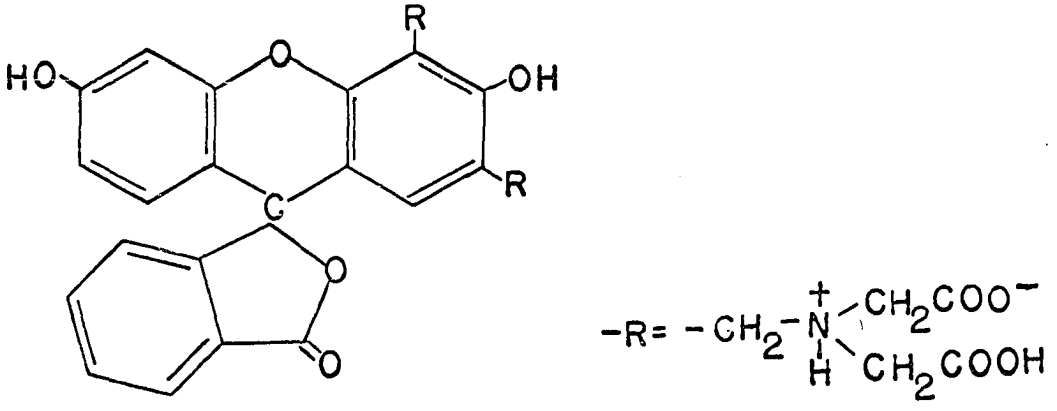
I. INTRODUCTION

Metallochromic and metallofluorochromic indicators are organic compounds which are colored or which fluoresce and which react with the ions of certain metallic elements in such a way as to change this color or fluorescence. The change in fluorescence may or may not be accompanied by a change in color. Such indicators are important in chemical analysis because the change in color or fluorescence can be brought about by exceedingly small amounts of the metal. Thus, the metallofluorochromic indicator, Calcein, in a highly alkaline solution does not fluoresce at all but becomes highly fluorescent when treated with but a few tenths of a microgram, that is 10^{-7} grams, of calcium. Use is made of this in the determination of calcium in blood, a matter of considerable importance in medical practice and a problem in which the amount of sample permitted the analyst may be distinctly limited.

It has been the history of metallochromic and metallofluorochromic indicators that they were almost always first used to mark the end-point in the titration of a metal with ethylenediaminetetraacetic acid (EDTA) and later used for the direct spectrophotometric or fluorometric determination of the metal. Thus, Calcein was first used as an indicator in titration of calcium and only later found use in direct fluorometric determination of calcium.

Calcein (I), the metallofluorochromic indicator just mentioned, has the structure



I

It was first prepared and its use described by Diehl and Ellingboe (3). It found widespread use almost immediately as an indicator in the titration of calcium with EDTA and in the last few years has been adapted to the direct fluorometric determination of calcium in the presence of magnesium. At pH 12 and above, the indicator itself is not fluorescent and is yellow-brown in color; in the presence of calcium, it exhibits a strong yellow-green fluorescence. At values of pH between 4 and 10 solutions of Calcein alone, that is, in the absence of calcium, exhibit a strong fluorescence, this fluorescence dropping off sharply at pH values above 10. The restoration of this fluorescence by calcium at high pH is

called the "indicator reversal" effect and is quite in contrast to the action of the ions of copper and other transition metals which quench the fluorescence. Such quenching is the normal effect of metal ions on fluorescent organic molecules which have the property of forming non-dissociated compounds with the transition metals.

Magnesium, the chemistry of which greatly resembles calcium, also brings about the indicator reversal effect on Calcein but because magnesium forms a soluble but non-dissociated magnesium hydroxide and at high concentrations insoluble magnesium hydroxide at high pH, this effect is not observed at pH 12 and higher. Advantage is taken of this in the titration and in the fluorometric determination of calcium in the presence of magnesium.

The fluorescence of fluorescein, the parent molecule of Calcein, is pH dependent, that is, fluorescein is a fluorescent acid-base indicator. The introduction into the fluorescein molecule of two methyleneiminodiacetic acid groups (each a half of the ethylenediaminetetraacetic acid molecule) gives to the molecule the property of uniting with metals, that is, makes Calcein a fluorochromic indicator. The introduction of these methyleneiminodiacetic acid groups is accomplished by means of the Mannich reaction, the condensation of fluorescein with formaldehyde and iminodiacetic acid.

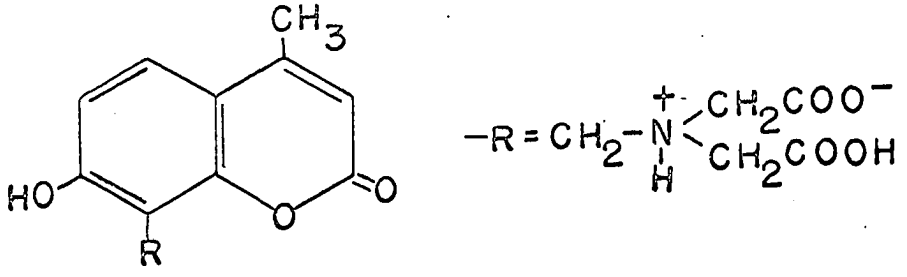
Numerous other fluorescent acid-base indicators exist,

notably 4-methylumbelliferone, a phenolic coumarin. In 1960, D. H. Wilkins (12) reported the synthesis of a methyleneiminodiacetic acid derivative of 4-methylumbelliferone. The compound exhibited a brilliant fluorescence which depended on the presence or absence of various metals and was given the name Calcein Blue. Some advantages are claimed for Calcein Blue over Calcein. In alkaline solution the fluorescence is excited by light of wavelength 365 mu which is closer to the principal line of the mercury spectrum than that of Calcein, 488 mu, and this is important when using fluorometers with mercury vapor lamps as light sources.

Wilkins (13) also prepared another derivative of 4-methylumbelliferone, this one by the Mannich condensation with formaldehyde and sarcosine (N-methylglycine). He named this compound Methyl Calcein Blue and used it as an indicator in the titration of various metal ions with hydroxyethylethylenediaminetriacetic acid (HEDTA).

Unfortunately, Wilkins did little more with these compounds than to describe methods for their use. He employed Calcein Blue in the direct determination of calcium, barium and strontium (12) and in the indirect determination of nickel and chromium in mixtures without prior separations (12). He also developed a method for the determination of aluminum, nickel and manganese using HEDTA as the titrating agent and Methyl Calcein Blue as the indicator (13). Wilkins' work was

expanded by Eggers (4) who investigated the absorption and fluorescence spectra of Calcein Blue with fifteen metal ions. Additional papers have appeared describing methods for the determination of metals using Calcein Blue as the indicator (5,6). The composition, structure and properties of Calcein Blue was the subject of the thesis submitted by the present author for the Master of Science degree (8). In that study, a satisfactory method for the synthesis of Calcein Blue was devised, the compound was obtained in pure form, the composition established and the structure determined. One methyleneiminodiacetic acid group had been introduced into the molecule and at position eight.



II

The acid dissociation constants were determined spectrophotometrically and studies were made of the absorption and fluorescence spectra.

In the present work a more detailed study has been made of Calcein Blue and of five additional compounds similar in structure to Calcein Blue. The effect of removing the methyl group at position 4 on the umbelliferone molecule has been investigated and the effect of a second hydroxy group at position 6 was studied. The effect of altering the iminodiacetic acid group has also been studied.

II. EXPERIMENTAL WORK

A. Synthesis

1. Apparatus and reagents

Measurements of pH were made with a Corning Model 10 pH meter equipped with a Beckman No. 40495 high alkalinity glass electrode and a Beckman asbestos fiber type saturated calomel electrode.

Electrophoresis studies were made with a Model R-Series D Beckman Paper Electrophoresis Cell (Durrum Type).

4-Methylumbelliferone was prepared according to the procedure outlined in Organic Syntheses (7). M.p.: 190-192°, reported 186-188°.

Umbelliferone was prepared according to the procedure outlined in Organic Reactions (1). M.p.: 234-235°, reported 227-228°.

Hydroxyhydroquinonetriacetate was prepared according to the method outlined in Organic Syntheses (7). M.p.: 100-102°, reported 96-97°.

4-Methylesculetin was prepared according to the method outlined in Organic Syntheses (7). M.p.: 279-291°, reported 272-274°.

Disodium iminodiacetate monohydrate was obtained from the Geigy Chemical Company and was not further purified.

Glycine was obtained from Eastman Organic Chemicals and

was not further purified.

Sarcosine was obtained from Mann Research Laboratories, Inc. and was not further purified.

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

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

Buffers used to standardize the pH meter were Mallinckrodt buffers of pH 4, 7, and 10.

All reagent chemicals were of reagent grade quality.

2. Mannich condensation of umbelliferone, formaldehyde and iminodiacetic acid

To 120 ml. of glacial acetic acid at 70° was added 0.045 mole (6.80 g.) of disodium iminodiacetate monohydrate with stirring. To this mixture was added 0.03 mole (4.9 g.) of umbelliferone. This was followed by the dropwise addition of 0.045 mole (4.44 ml.) of 37 per cent formaldehyde. The reaction was allowed to proceed at 65° to 70° for eight hours with constant stirring. The yellow crystalline material which separated was filtered and washed with deionized water and recrystallized by dissolving it in the minimum amount of potassium hydroxide. The solution was filtered to remove insoluble impurities and the pH of the filtrate was adjusted to 3.1 by the dropwise addition of dilute hydrochloric acid (one part acid to five parts water). The precipitate was

filtered, washed with deionized water and recrystallized two more times in the same manner. The product was obtained as fine, yellow crystals which did not melt at temperatures below 300°. Neutralization equivalent found: 309.2. Analysis (by Galbraith Laboratories, Inc.): found C 54.57, H 4.35, N 4.41; calculated for $C_{14}H_{13}NO_7$, molecular weight 307.25, C 54.72, H 4.27, N 4.56.

3. Mannich condensation of 4-methylesculetin, formaldehyde and iminodiacetic acid

To 120 ml. of glacial acetic acid at 70° was added 0.045 mole (8.78 g.) of disodium iminodiacetate monohydrate with stirring. To this mixture was added 0.03 mole (5.76 g.) of 4-methylesculetin. This was followed by the dropwise addition of 0.045 mole (4.44 ml.) of 37 per cent formaldehyde. The reaction was allowed to proceed for eight hours with constant stirring. The grey crystalline material which separated was filtered, washed with deionized water and recrystallized by dissolving it in the minimum amount of potassium hydroxide. The solution was filtered to remove insoluble impurities and the pH of the filtrate was adjusted to 3.1 by the dropwise addition of dilute hydrochloric acid (one part acid to five parts water). The precipitate was filtered, washed with deionized water, and recrystallized two more times in the same manner. The product was obtained as fine grey crystals which did not melt at temperatures below 300°. Neutralization

equivalent found: 349.1. Analysis (by Galbraith Laboratories, Inc.): found C 51.71, H 4.65, N 3.98; calculated for $C_{15}H_{16}NO_{8.5}$, molecular weight 346.24, C 52.00, H 4.66, N 4.04.

4. Mannich condensation of 4-methylumbelliferone, formaldehyde and glycine

To 50 ml. of water was added 0.06 mole (10.57 g.) of 4-methylumbelliferone, 0.09 mole (6.75 g.) of glycine and 0.09 mole (8.88 ml.) of 37 per cent formaldehyde solution. The reaction was allowed to proceed at 70° to 75° for eight hours with constant stirring. The yellow crystalline material which separated was filtered, washed with deionized water and acetone and recrystallized by dissolving it in the minimum amount of potassium hydroxide. The solution was filtered to remove insoluble impurities and the pH of the filtrate was adjusted to 4.7 by the dropwise addition of dilute hydrochloric acid (one part acid to five parts water). The precipitate was filtered, washed with deionized water and acetone, and recrystallized once more in the same manner. The product was slurried twice in acetone and filtered. The product was obtained as yellow crystals which did not melt at temperatures below 300°. Paper electrophoresis yielded one band indicating a pure product. Neutralization equivalent found: 264.9. Analysis (by Galbraith Laboratories, Inc.): found C 59.10, H 5.04, N 5.44; calculated for $C_{13}H_{13}NO_5$, molecular weight 263.1, C 59.30, H 4.98, N 5.32.

5. Mannich condensation of umbelliferone, formaldehyde and glycine

To 150 ml. of water was added 0.06 mole (9.73 g.) of umbelliferone, 0.09 mole (6.75 g.) of glycine and 0.09 mole (8.88 ml.) of 37 percent formaldehyde solution. The reaction was allowed to proceed at 70° to 75° for eight hours with constant stirring. The yellow crystalline material which separated was filtered, washed with deionized water and acetone, and recrystallized by dissolving it in the minimum amount of potassium hydroxide. The solution was filtered to remove insoluble impurities and the pH of the filtrate was adjusted to 4.75 by the dropwise addition of dilute hydrochloric acid (one part acid to five parts water). The precipitate was filtered, washed with deionized water and acetone, and recrystallized once more in the same manner. The product was then slurried twice in acetone and filtered. The product was obtained as yellow crystals which did not melt at temperatures below 300°. Paper electrophoresis yielded one band indicating a pure product. Neutralization equivalent found: 256.45. Water present (Karl Fischer titration): 7.41 g. Analysis (by Galbraith Laboratories, Inc.): found C 55.54, H 4.61, N 5.74; calculated for $C_{12}H_{12}NO_{5.5}$, molecular weight 258.3, C 55.75, H 4.68, N 5.44.

6. Mannich condensation of 4-methylumbelliferone, formaldehyde and sarcosine

A mixture of 0.12 mole (21.44 g.) of 4-methylumbelliferone, 0.18 mole (16.03 g.) of sarcosine and 0.18 mole (17.76 ml.) of 37 per cent formaldehyde solution in 200 ml. of water was allowed to react at 90° to 95° until the solution became clear and a spongy material formed. The reaction was allowed to proceed for another half hour. The clear yellow liquid was poured off and allowed to stand for 12 hours. The light yellow material which precipitated was filtered, dissolved in potassium hydroxide, and filtered to remove insoluble impurities. The pH of the filtrate was adjusted to 4.5 by the dropwise addition of dilute hydrochloric acid (one part acid to five parts water). The precipitate was filtered, and washed with deionized water and acetone. The product was slurried three times in acetone and filtered. The yellow crystals did not melt at temperatures below 300°. Neutralization equivalent found: 303.6. Analysis (by Galbraith Laboratories, Inc.): found C 55.26, H 5.96, N 4.60; calculated for $C_{14}H_{18}NO_{6.5}$, molecular weight 304.26, C 55.53, H 5.96, N 4.42.

B. Neutralization Equivalent

1. Apparatus and reagents

Measurements of pH were made with a Corning Model 10 pH meter equipped with a Beckman No. 40495 high alkalinity glass

electrode and a Beckman asbestos fiber type saturated calomel electrode.

Buffers used to standardize the pH meter were Mallinckrodt buffers of pH 4, 7, and 10.

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.

2. Potentiometric titration

The neutralization equivalents of umbelliferone-8-methyleneiminodiacetic acid, 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O, 4-methylumbelliferone-8-methylenelycine, umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O were obtained by adding known amounts of the desired compound to 50 ml. of 0.1 M potassium chloride and titrating with 0.1 N sodium hydroxide made up in 0.1 M potassium chloride. The titrations curves obtained are shown in Figures 1, 2, 3, 4, and 5 respectively.

C. Structure

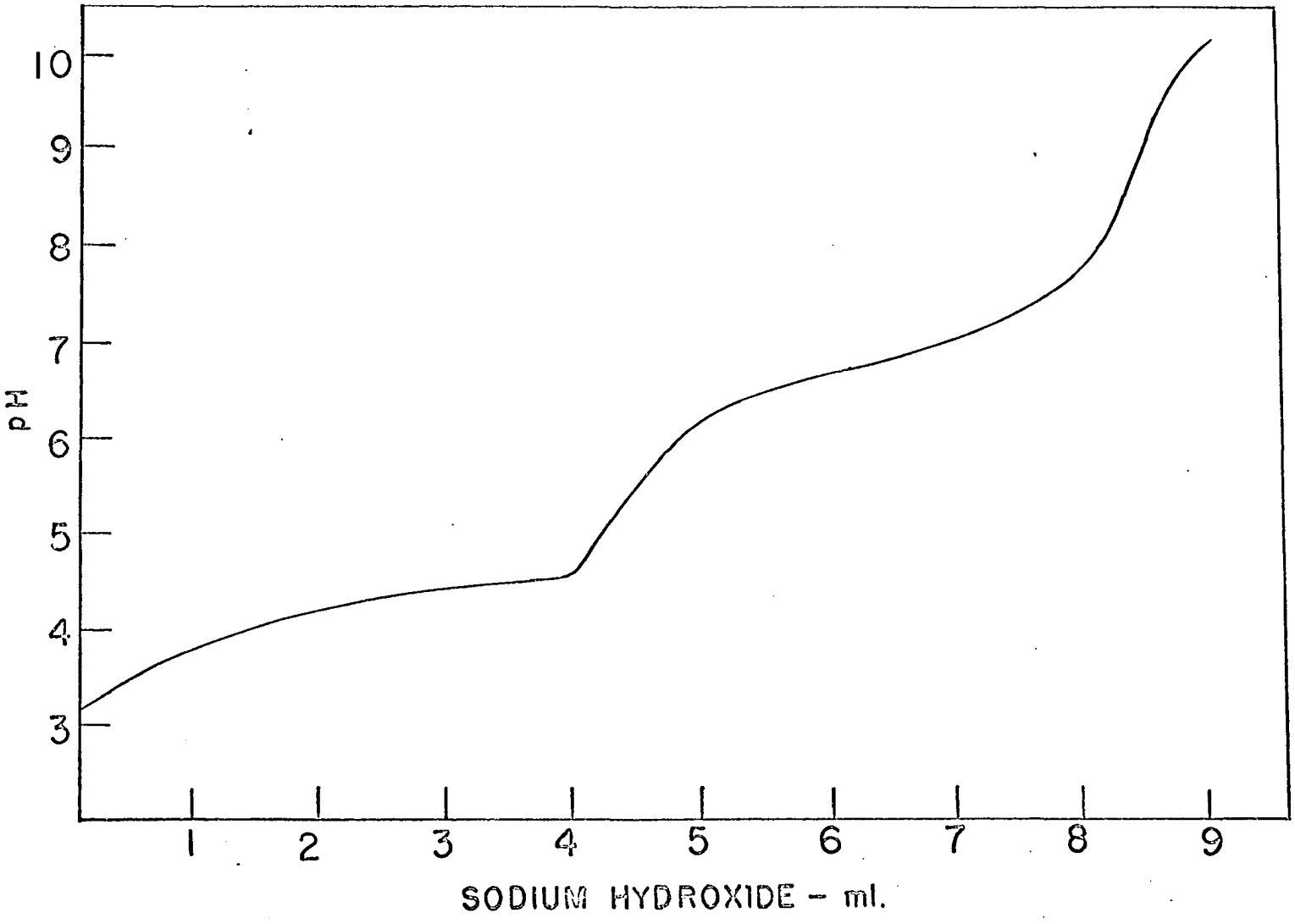
1. Apparatus

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

2. Structure

Nuclear magnetic resonance spectra of umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O

Figure 1. Titration of umbelliferone-8-methyleneiminodiacetic acid with sodium hydroxide
Solvent: 0.1 M potassium chloride; sodium hydroxide: 0.0984 N in 0.1 M potassium chloride; sample: 0.1320 g.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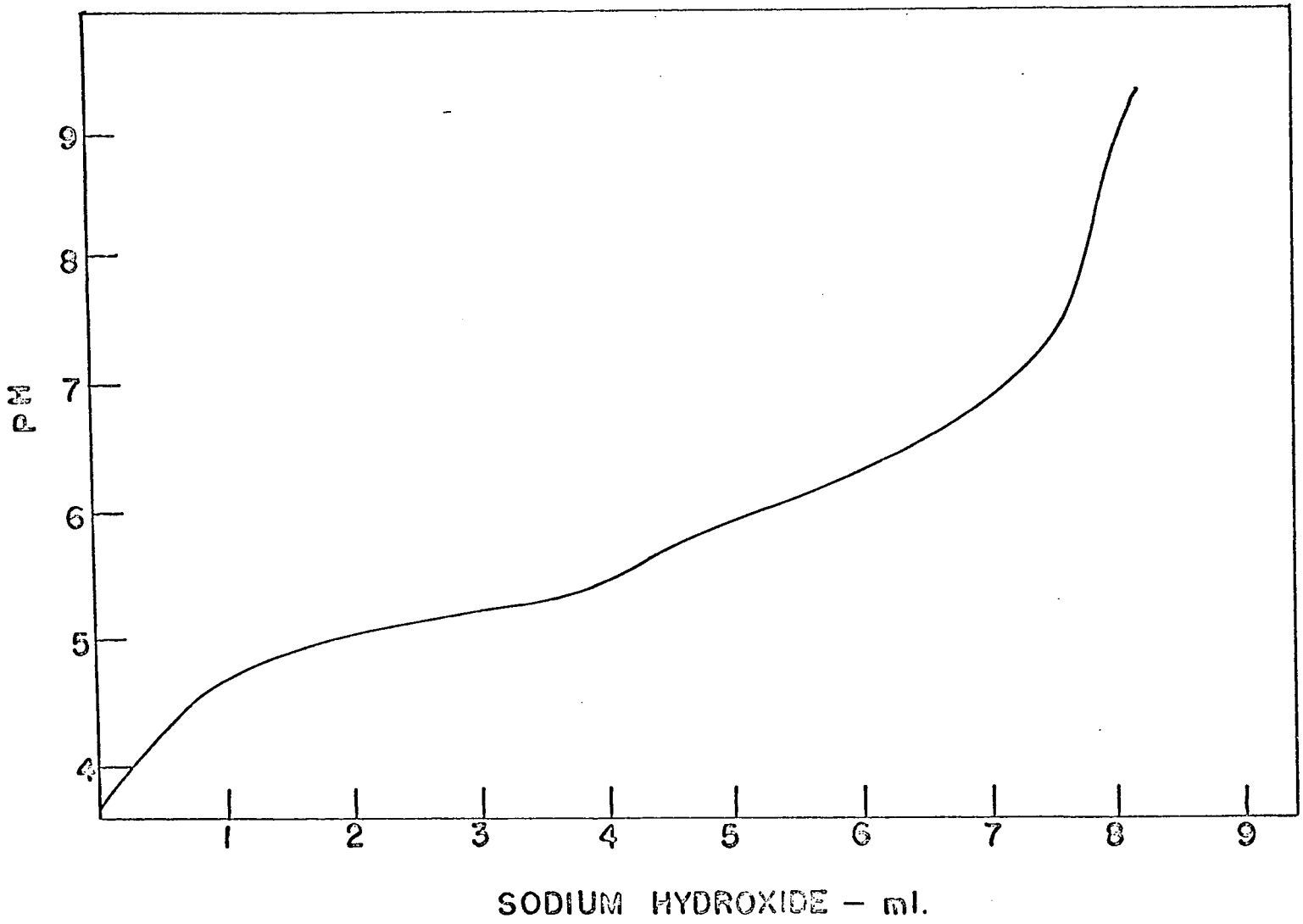
SODIUM HYDROXIDE – ml.

Figure 2. Titration of 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O with sodium hydroxide
Solvent: 0.1 M potassium chloride; sodium hydroxide: 0.0984 N in 0.1 M potassium chloride; sample: 0.1462 g.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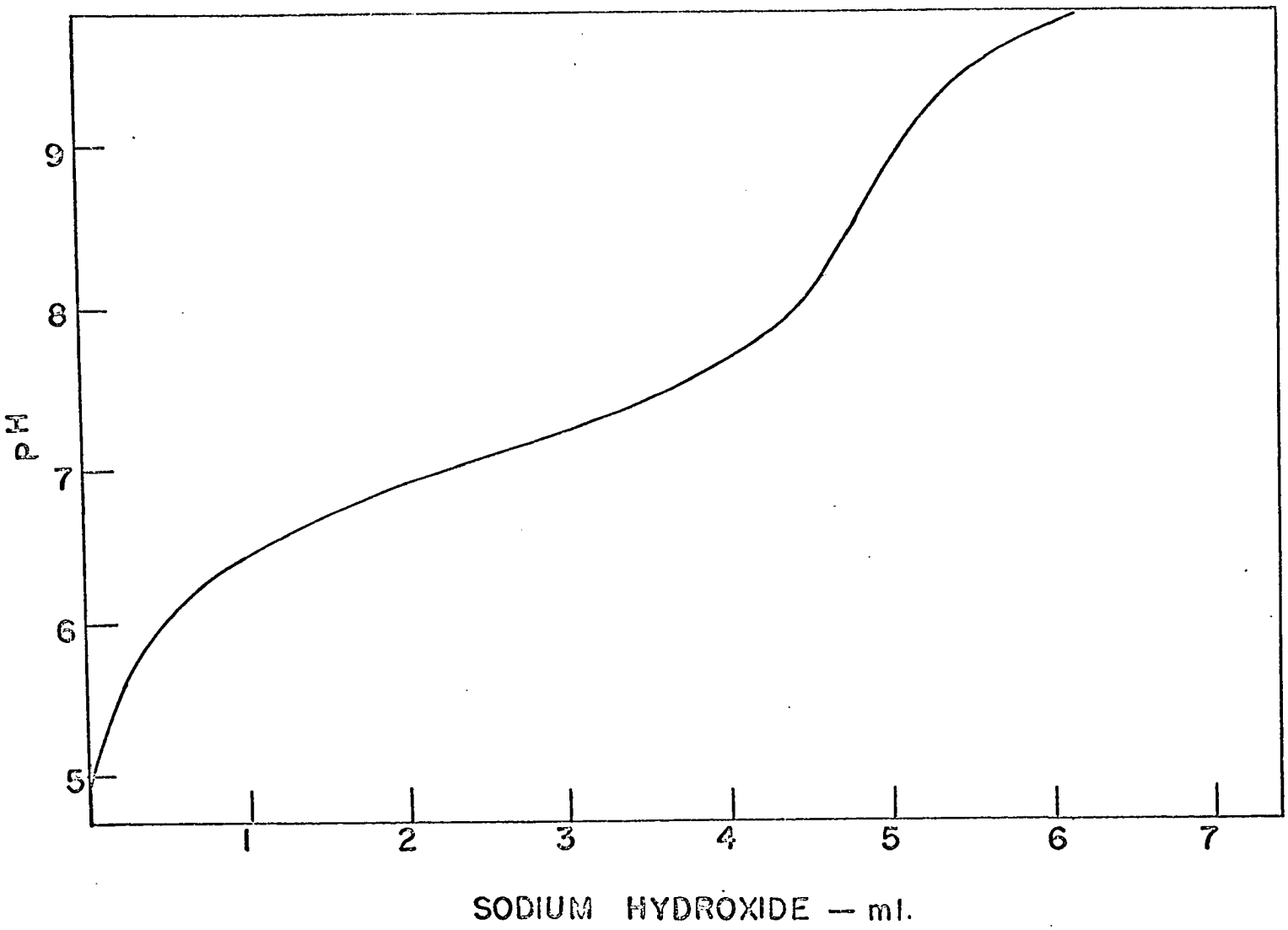
9
SODIUM HYDROXIDE – ml.

Figure 3. Titration of 4-methylumbelliferone-8-methyleneglycine with sodium hydroxide
Solvent: 0.1 M potassium chloride; sodium hydroxide: 0.1066 N in 0.1 M potassium chloride; sample: 0.1462 g.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pH
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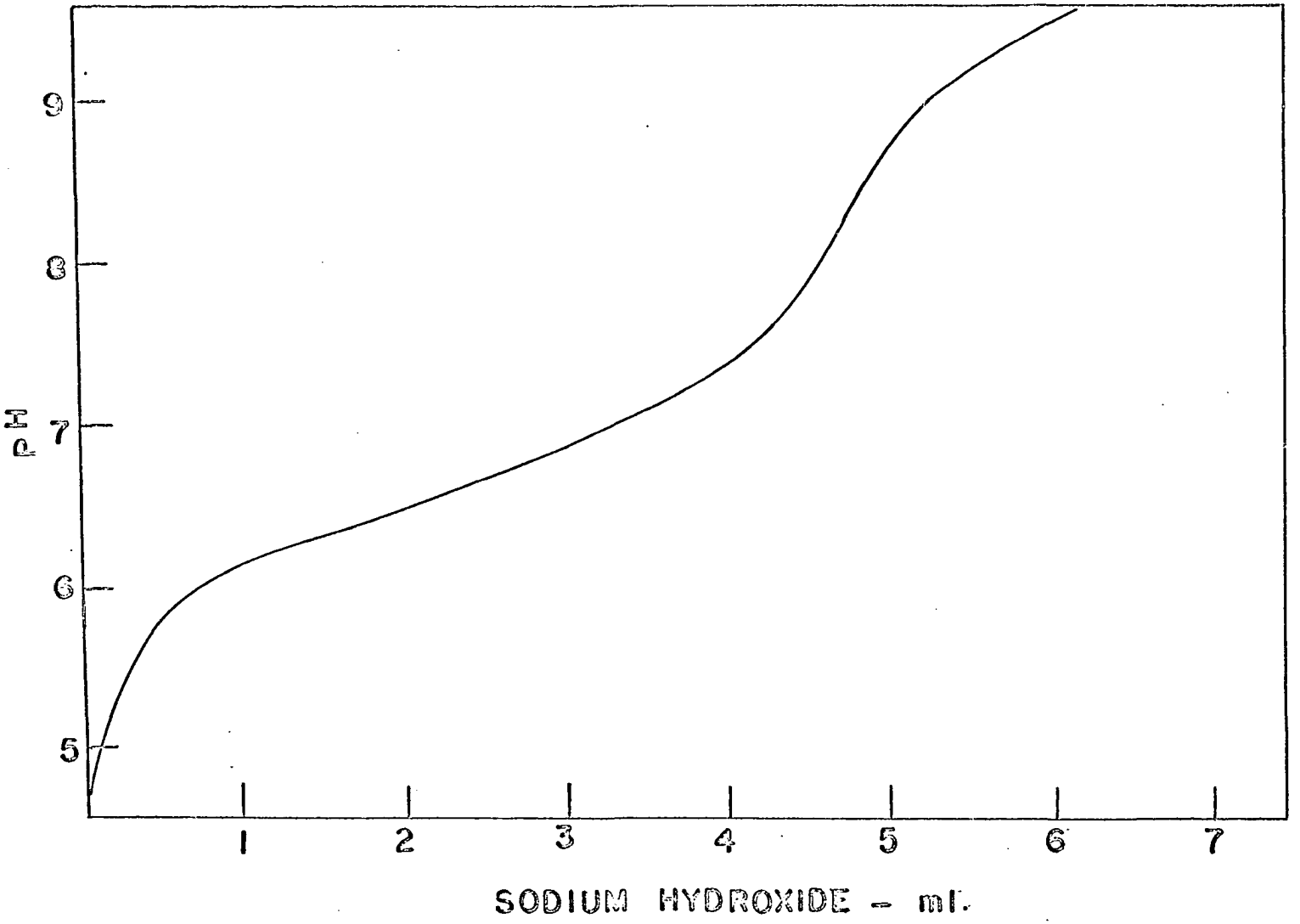
SODIUM HYDROXIDE — ml.

Figure 4. Titration of umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O with sodium hydroxide
Solvent: 0.1 M potassium chloride; sodium hydroxide: 0.1066 N in 0.1 M potassium chloride; sample: 0.1407 g.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pH
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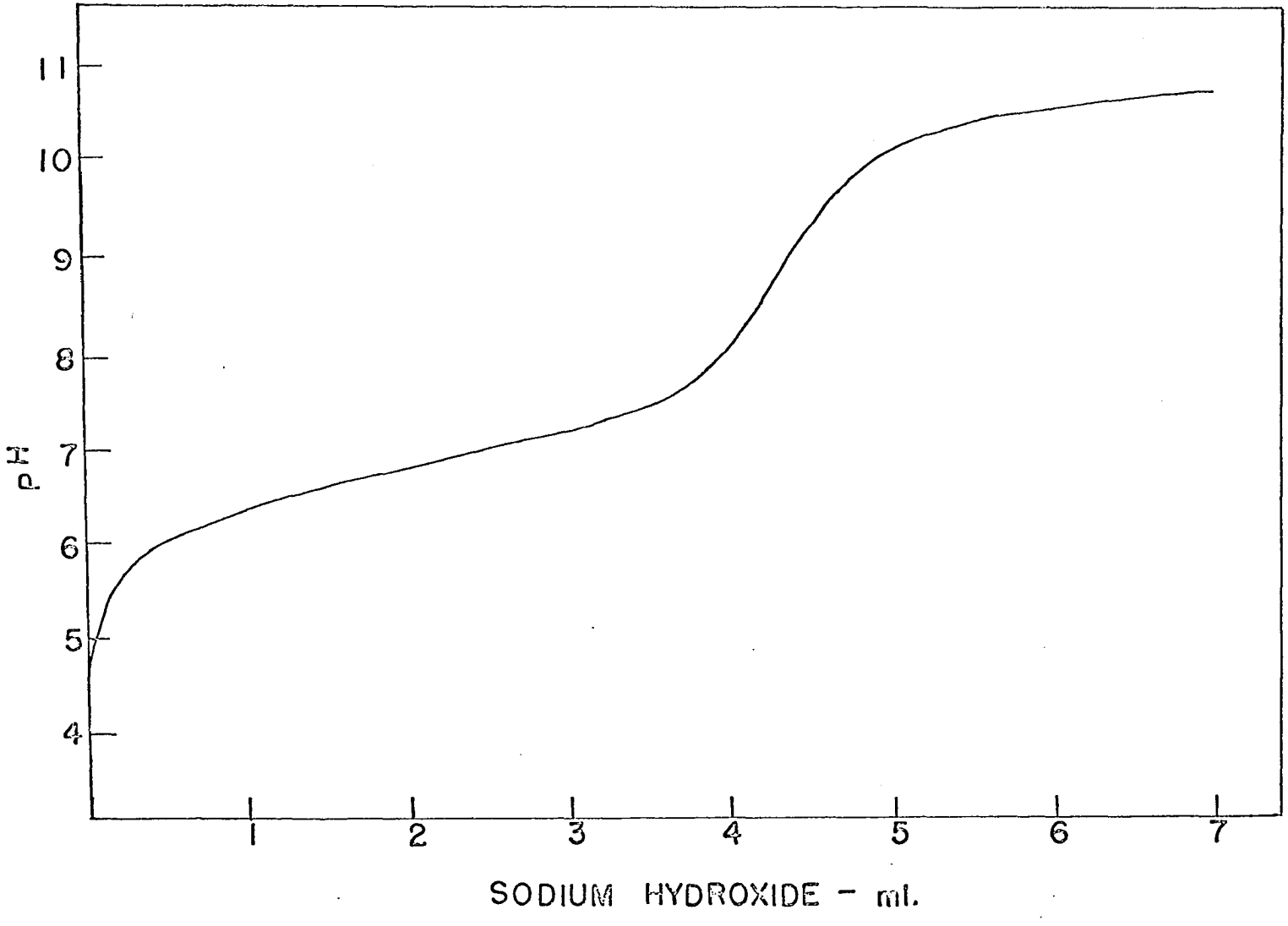
SODIUM HYDROXIDE - ml.

Figure 5. Titration of 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O with sodium hydroxide.
Solvent: 0.1 M potassium chloride; sodium hydroxide: 0.1050 N in 0.1 M potassium chloride; sample: 0.1638 g.


11
10
9
8
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pH
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3
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7
SODIUM HYDROXIDE - ml.

and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O were obtained in deuterium oxide-potassium carbonate media using tetramethylsilane as the standard.

The nuclear magnetic resonance spectrum of 4-methylesculetinmethyleneiminodiacetic acid was obtained in dimethylsulfoxide using tetramethylsilane as the standard.

D. Fluorescence Study

1. Apparatus and reagents

Fluorescence spectra were obtained using an Aminco-Bowan Spectrofluorometer. The slit widths used were in order: excitation monochromator; 1/8, 1/16 and 1/8 in., emission monochromator; 1/8, 1/16, 1/8 and 1/16 in. A 1 cm. quartz cell was used. Spectra were recorded on a Moseley XY recorder.

A G. K. Turner Associates Model 10 fluorometer equipped with a flow-through cell was used to monitor fluorometric titrations.

Measurements of pH were made with a Corning Model 10 pH meter equipped with a Beckman No. 40495 high alkalinity glass electrode and a Beckman asbestos fiber type saturated calomel electrode.

A Micro Metric Model SB 2 microburet and a Model S5Y syringe were used in delivering appropriate volumes of reagent stock solutions for fluorometric work.

Stock solutions of 4-methylumbelliferone, umbelliferone, 4-methylesculetin, 4-methylumbelliferone-8-methyleneiminodi-

acetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O were prepared within a few hours of use and were all 3.11×10^{-3} M. Small quantities of potassium hydroxide were used to facilitate dissolution.

Stock solutions of calcium chloride dihydrate, cupric chloride dihydrate and aluminum chloride were prepared for studies of the effect of metal ions on fluorescence and were all 3.11×10^{-3} M.

2. Effect of pH on fluorescence

The fluorescence excitation and emission spectra of 4-methylumbelliferone, umbelliferone, 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-metylumbelliferone-8-methylenesarcosine·1½ H_2O were obtained at one half pH unit intervals ranging from pH 1.5 to pH 13.0. Solutions on which the spectra were obtained were prepared by mixing 15 ul. of the desired indicator stock solution, 0.25 ml. of 0.01 M EDTA to sequester any metal ions present, 10 ml. of buffer solution, and diluting to 25 ml. with 0.1 M potassium chloride. The pH of each of the solutions was checked after the spectra were obtained. Fluorescence excitation and emission spectra of 4-methylumbelliferone and umbelliferone at pH

4 and 10 are shown in Figures 6 and 7. Fluorescence excitation and emission spectra of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O at pH 4 and 9 are shown in Figures 8, 9, 10, 11 and 12. The relative fluorescence of each of the buffered solutions was plotted against the pH of that solution for each compound. The graphs are shown in Figures 13, 14, 15, 16, 17, 18, and 19.

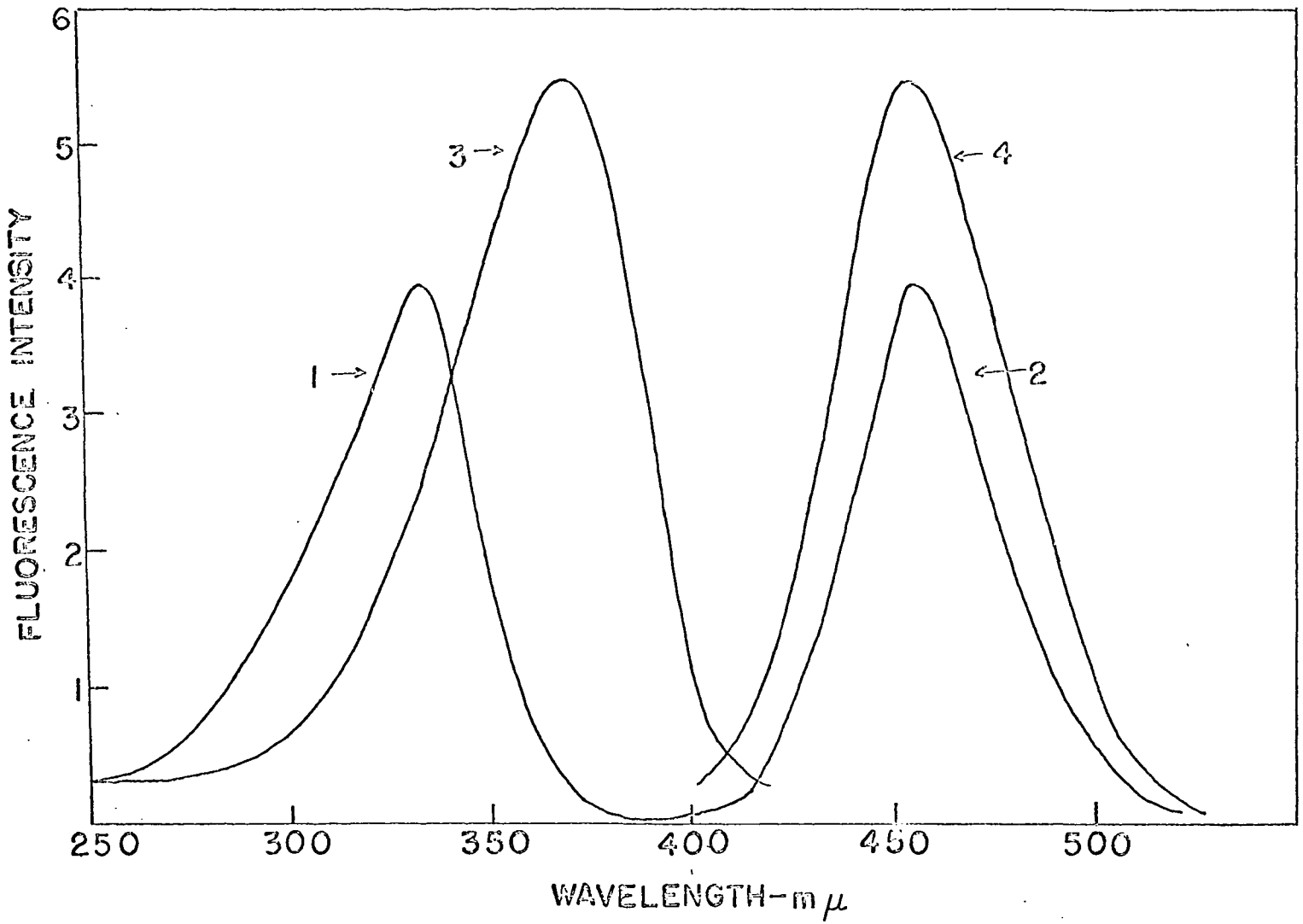
Fluorescence excitation and emission spectra of 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O at pH 9 are shown in Figures 20 and 21. The relative fluorescence of the compounds at pH values varying from pH 2.5 to 11.0 was obtained by titrating solutions containing 7 ul. of the indicator stock solution and 0.75 ml. of 0.1 M EDTA to sequester any metal ions present in 75 ml. of 0.1 M potassium chloride with 0.2 M potassium hydroxide. Graphs showing the variation in fluorescence with changes in pH are shown in Figures 22 and 23.

3. Effect of copper on fluorescence

The effect of copper on the fluorescence of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O was studied

Figure 6. Fluorescence spectra of 4-methylumbelliferone at pH 4 and 10

1. Excitation, fluorescence monochromator set at 455 mu, pH 4
2. Fluorescence, excitation monochromator set at 328 mu, pH 4
3. Excitation, fluorescence monochromator set at 455 mu, pH 10
4. Fluorescence, excitation monochromator set at 365 mu, pH 10


FLUORESCENCE INTENSITY
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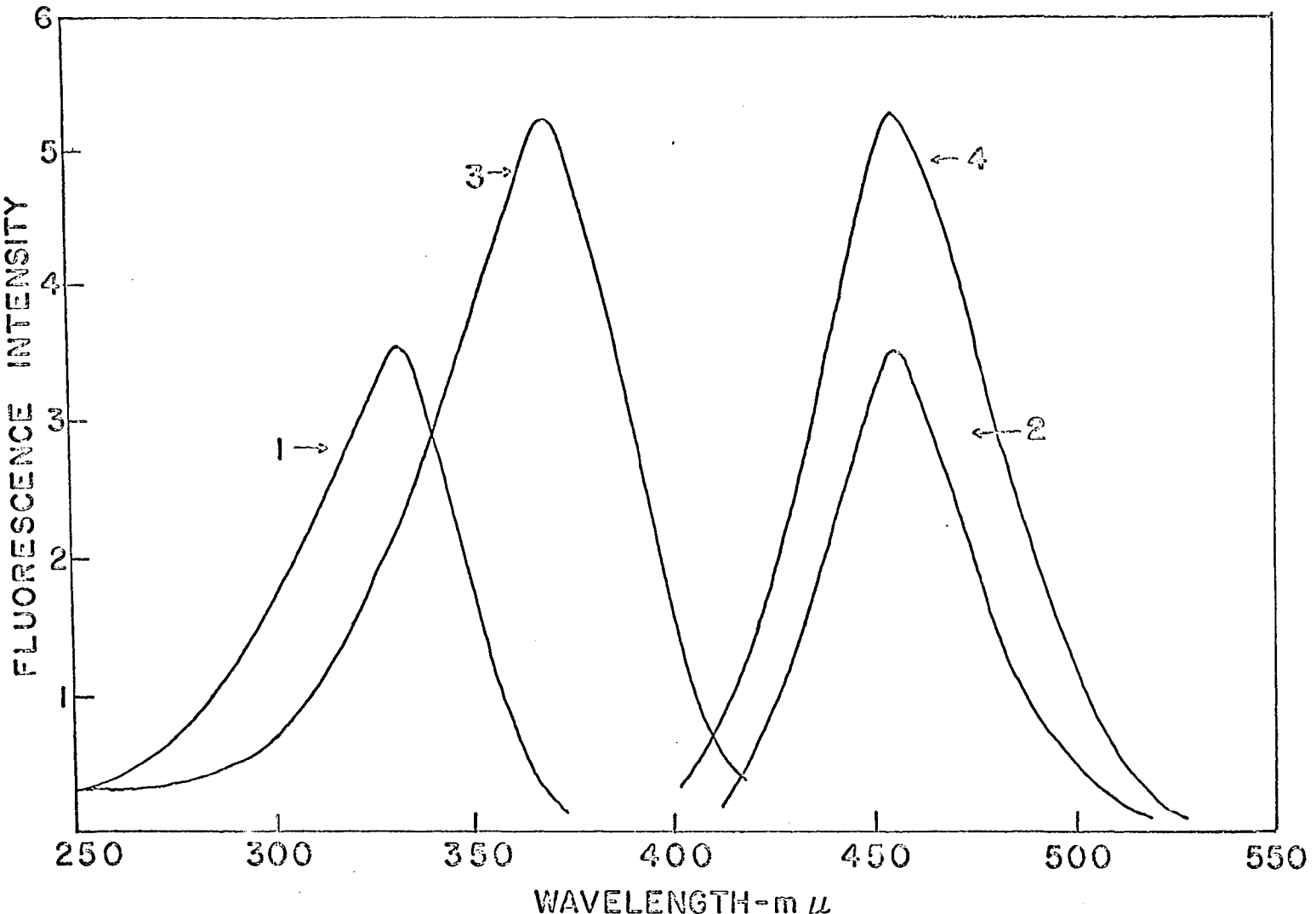
1 →
2 ←
3 →
4 ←
250
300
350
400
450
500
WAVELENGTH-mμ

Figure 7. Fluorescence spectra of umbelliferone at pH 4 and 10

1. Excitation, fluorescence monochromator set at 460 mμ, pH 4
2. Fluorescence, excitation monochromator set at 330 mμ, pH 4
3. Excitation, fluorescence monochromator set at 460 mμ, pH 10
4. Fluorescence, excitation monochromator set at 370 mμ, pH 10


FLUORESCENCE INTENSITY
6
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3→
4←
250
300
350
400
450
500
550
WAVELENGTH-mμ

Figure 8. Fluorescence spectra of 4-methylumbelliferone-8-methyleneiminodiacetic acid

1. Excitation, fluorescence monochromator set at 455 mu, pH 4
2. Fluorescence, excitation monochromator set at 330 mu, pH 4
3. Excitation, fluorescence monochromator set at 455 mu, pH 9
4. Fluorescence, excitation monochromator set at 360 mu, pH 9

FLUORESCENCE INTENSITY
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3
4
5
6
250
300
350
400
450
500
550
WAVELENGTH - mμ
1
3
2
4

Figure 9. Fluorescence spectra of umbelliferone-8-methyleneiminodiacetic acid

1. Excitation, fluorescence monochromator set at 455 mu, pH 4
2. Fluorescence, excitation monochromator set at 328 mu, pH 4
3. Excitation, fluorescence monochromator set at 455 mu, pH 9
4. Fluorescence, excitation monochromator set at 370 mu, pH 9

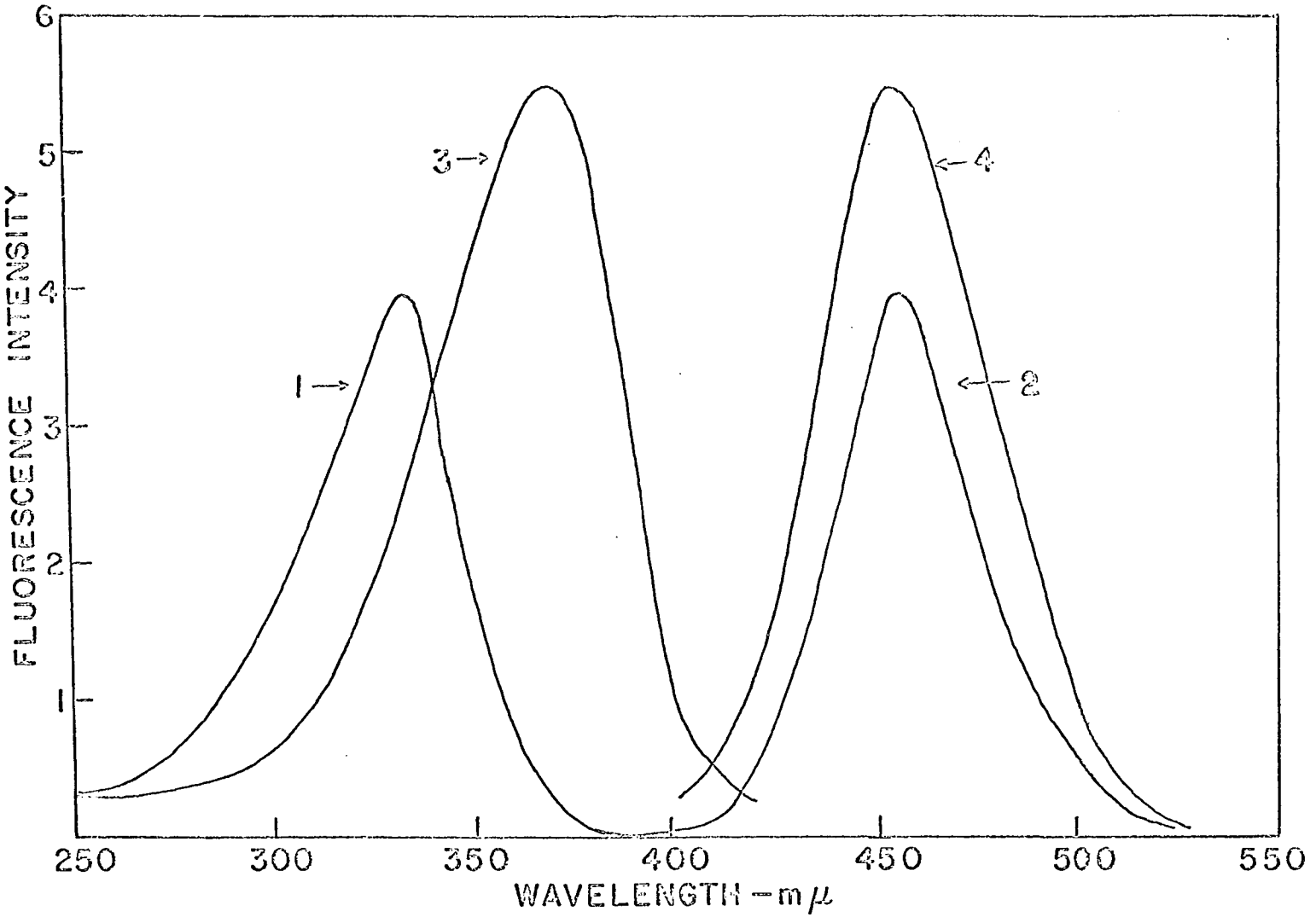
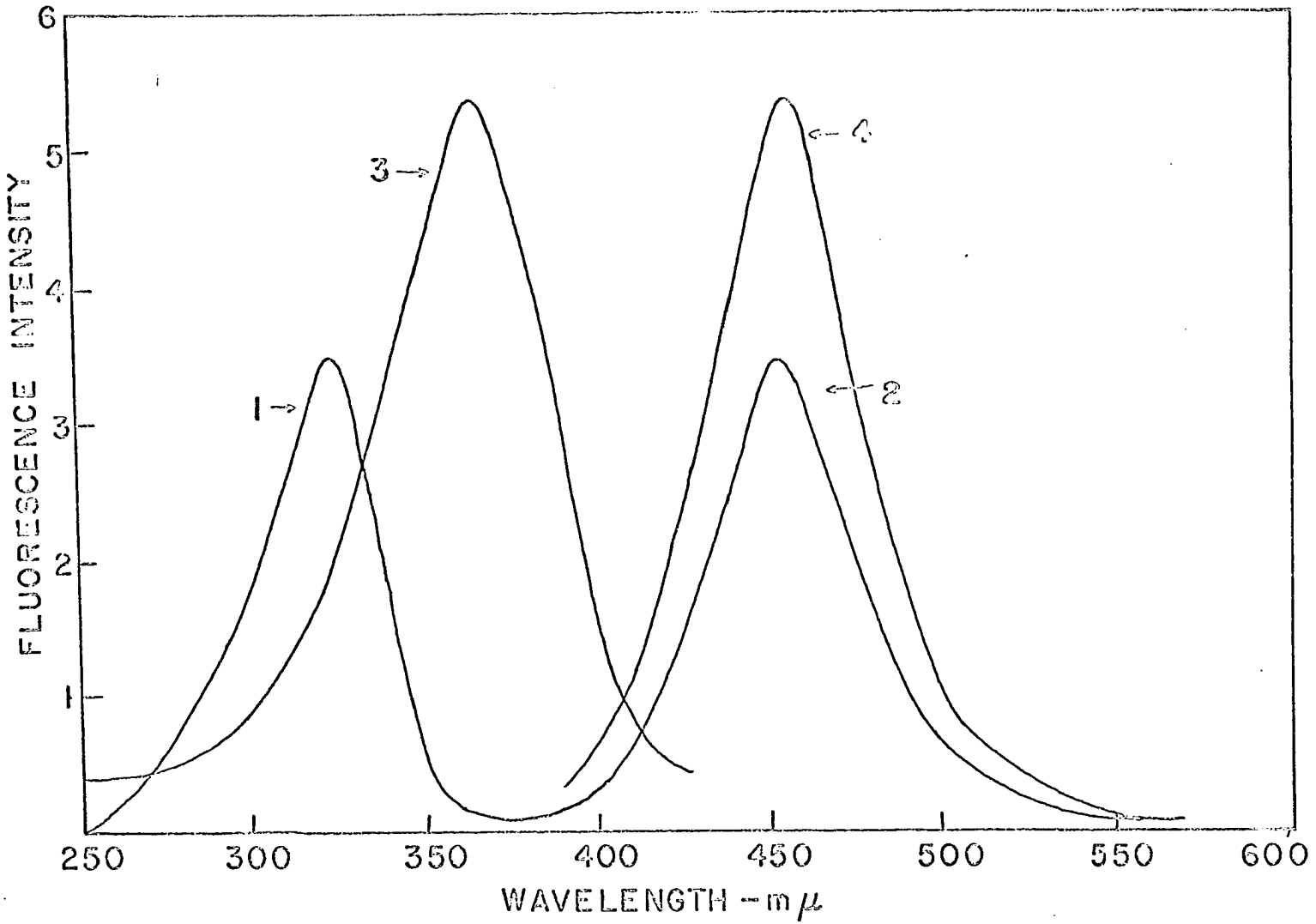

FLUORESCENCE INTENSITY
WAVELENGTH – mμ
1
2
3
4
6
5
4
3
2
1
250
300
350
400
450
500
550

Figure 10. Fluorescence spectra of 4-methylumbelliferone-8-methylene[illegible]e

1. Excitation, fluorescence monochromator set at 450 mu, pH 4
2. Fluorescence, excitation monochromator set at 328 mu, pH 4
3. Excitation, fluorescence monochromator set at 450 mu, pH 9
4. Fluorescence, excitation monochromator set at 365 mu, pH 9


FLUORESCENCE INTENSITY
6
5
4
3
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450
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550
600
WAVELENGTH – mμ

Figure 11. Fluorescence spectra of umbelliferone-8-methyleneglycine·½ H_2O

1. Excitation, fluorescence monochromator set at 455 mu, pH 4
2. Fluorescence, excitation monochromator set at 330 mu, pH 4
3. Excitation, fluorescence monochromator set at 455 mu, pH 9
4. Fluorescence, excitation monochromator set at 370 mu, pH 9

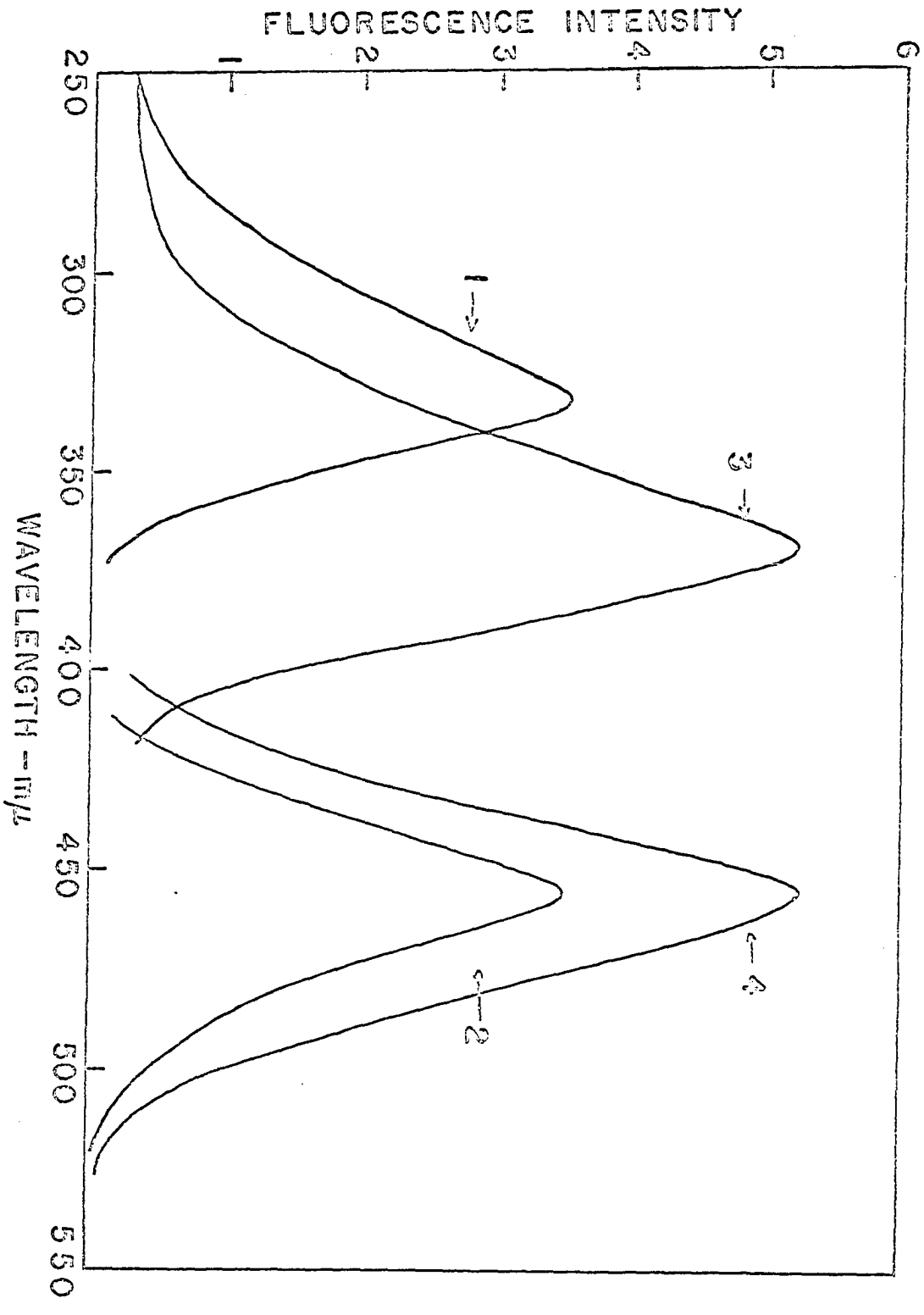

FLUORESCENCE INTENSITY
1
2
3
4
5
6
250
300
350
400
450
500
550
WAVELENGTH - mμ
1
2
3

Figure 12. Fluorescence spectra of 4-methylumbelliferone-8-methylene-sarcosine·1½ H_2O

1. Excitation, fluorescence monochromator set at 448 mu, pH 4
2. Fluorescence, excitation monochromator set at 328 mu, pH 4
3. Excitation, fluorescence monochromator set at 448 mu, pH 9
4. Fluorescence, excitation monochromator set at 360 mu, pH 9

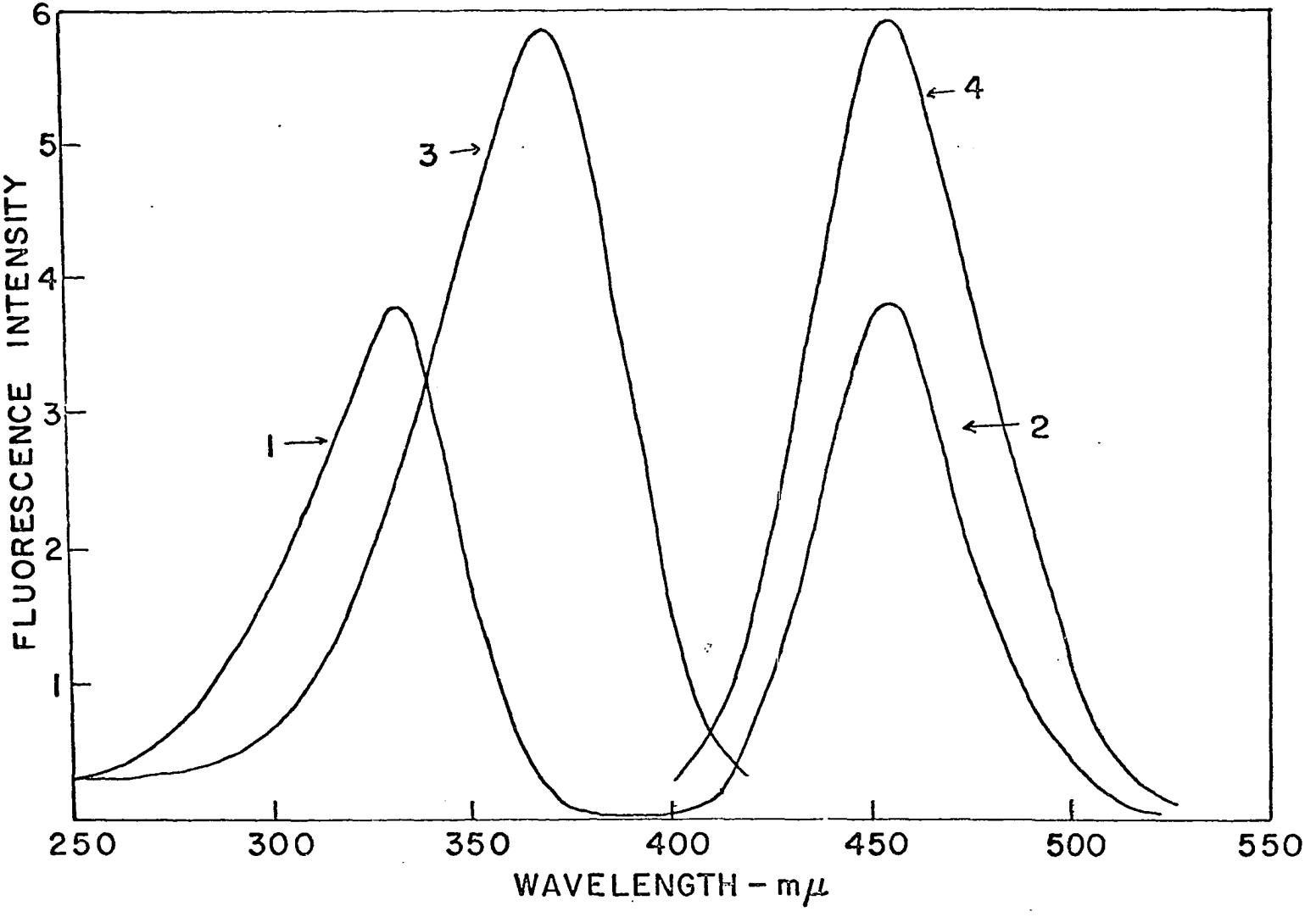

FLUORESCENCE INTENSITY
6
5
4
3
2
1
250
300
350
400
450
500
550
WAVELENGTH – mμ
1
2
3
4

Figure 13. Variation in intensity of fluorescence of 4-methylumbelliferone with pH at two wavelengths of exciting light

1. Excitation monochromator set at 328 mu
2. Excitation monochromator set at 365 mu

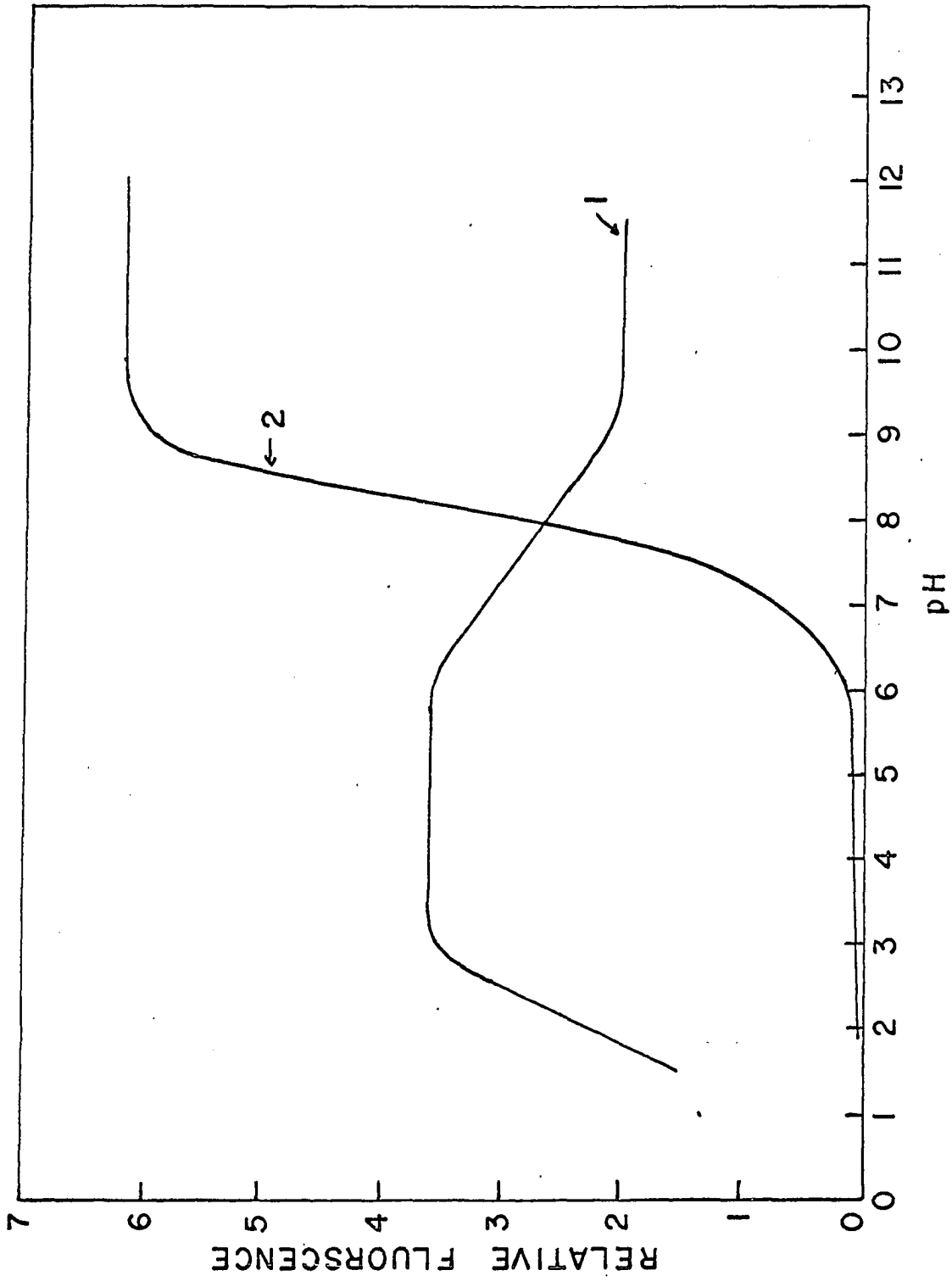

RELATIVE FLUORSCENCE
0
1
2
3
4
5
6
7
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2

Figure 14. Variation in intensity of fluorescence of umbelliferone with pH at two wavelengths of exciting light

1. Excitation monochromator set at 330 mu
2. Excitation monochromator set at 365 mu

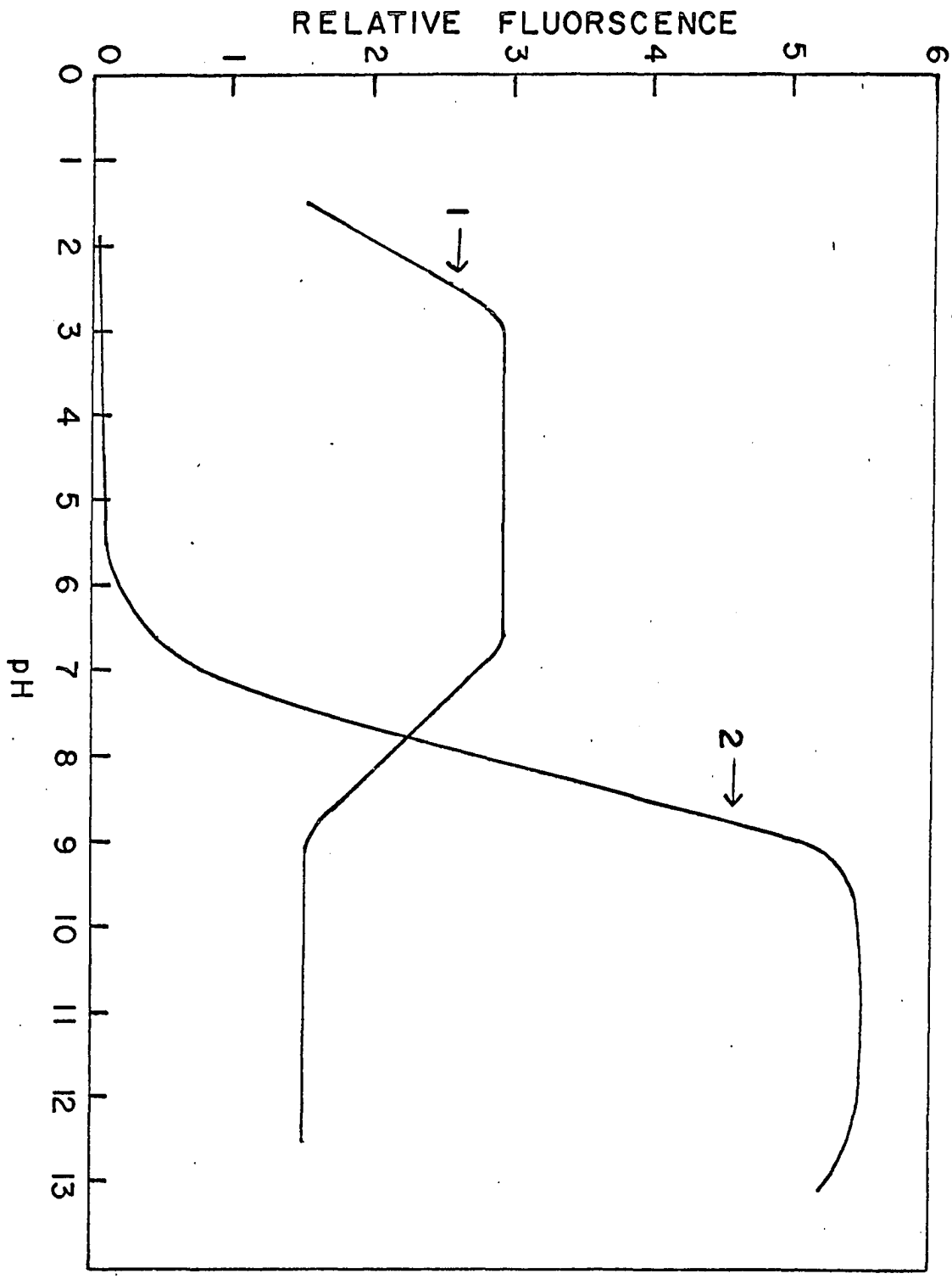

RELATIVE FLUORSCENCE
0
1
2
3
4
5
6
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2

Figure 15. Variation in intensity of fluorescence of 4-methylumbelliferone-8-methyleneiminodiacetic acid, its calcium derivative and its copper derivative with pH.

1. Free indicator - excitation monochromator set at 330 mu
2. Free indicator - excitation monochromator set at 360 mu
3. Calcium derivative - excitation monochromator set at 360 mu
4. Copper derivative - excitation monochromator set at 330 mu below pH 6.5 and at 360 mu above pH 6.5

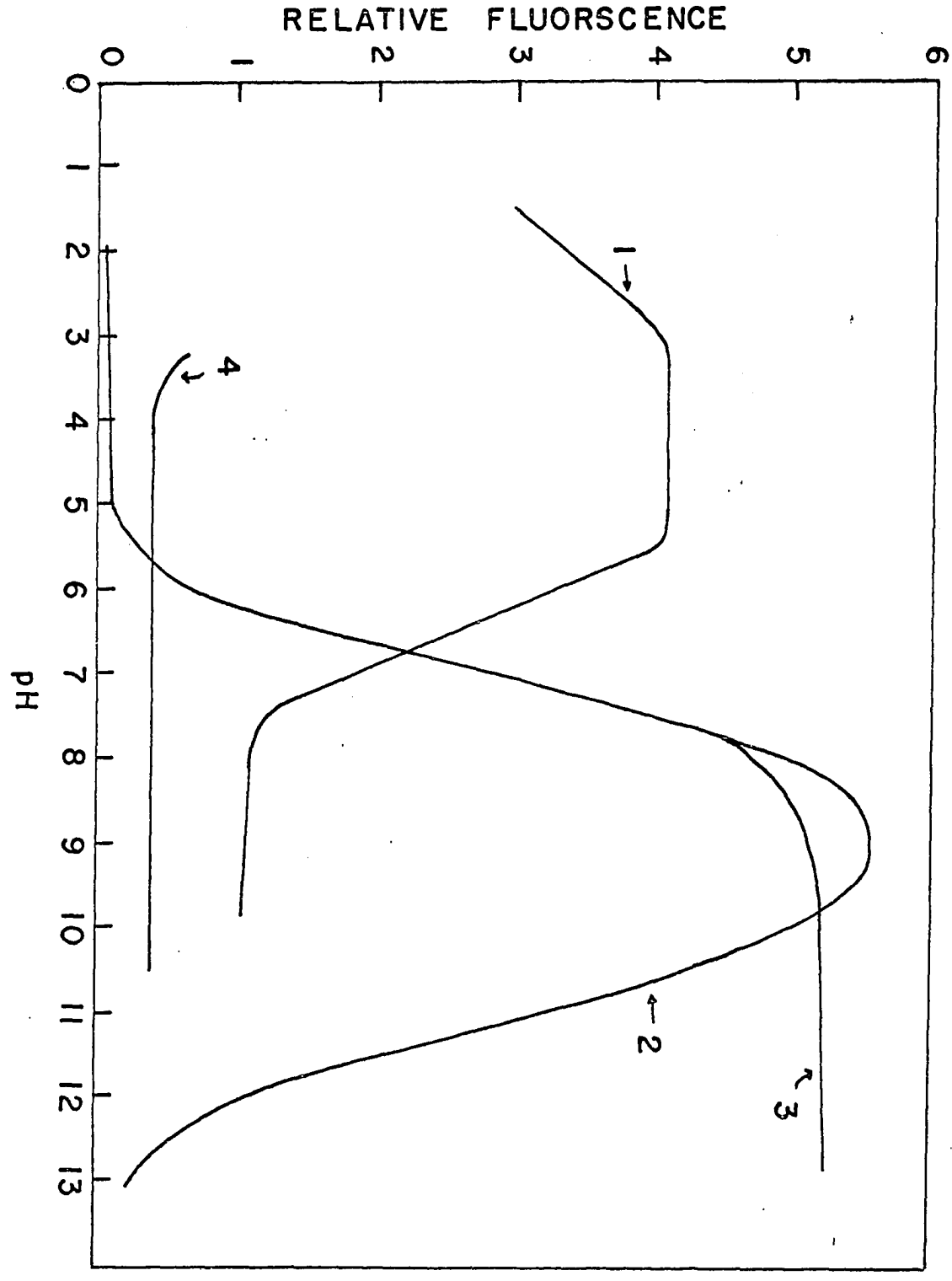

RELATIVE FLUORSCENCE
0
1
2
3
4
5
6
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2
3
4

Figure 16. Variation in intensity of fluorescence of umbelliferone-8-methyleneiminodiacetic acid, its calcium derivative and its copper derivative with pH

1. Free indicator - excitation monochromator set at 328 mu
2. Free indicator - excitation monochromator set at 370 mu
3. Calcium derivative - excitation monochromator set at 370 mu
4. Copper derivative - excitation monochromator set at 328 mu below pH 6.5 and at 360 mu above pH 6.5

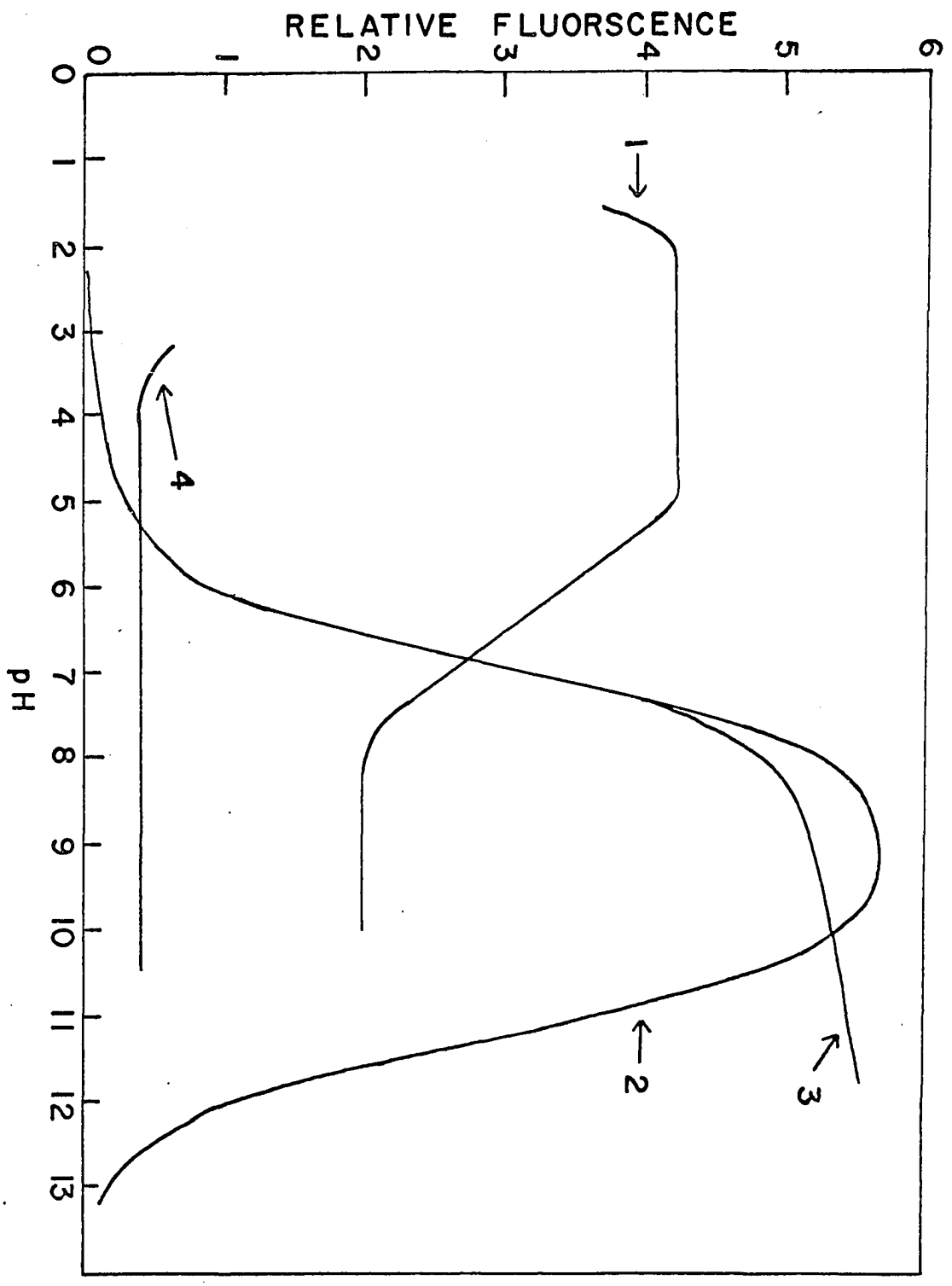

RELATIVE FLUORSCENCE
0
1
2
3
4
5
6
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2
3
4

Figure 17. Variation in intensity of fluorescence of 4-methylumbelliferone-8-methyleneglycine and its copper derivative with pH

1. Free indicator - excitation monochromator set at 328 mu
2. Free indicator - excitation monochromator set at 370 mu
3. Calcium derivative - excitation monochromator set at 370 mu
4. Copper derivative - excitation monochromator set at 328 mu below pH 6 and at 370 mu above pH 6

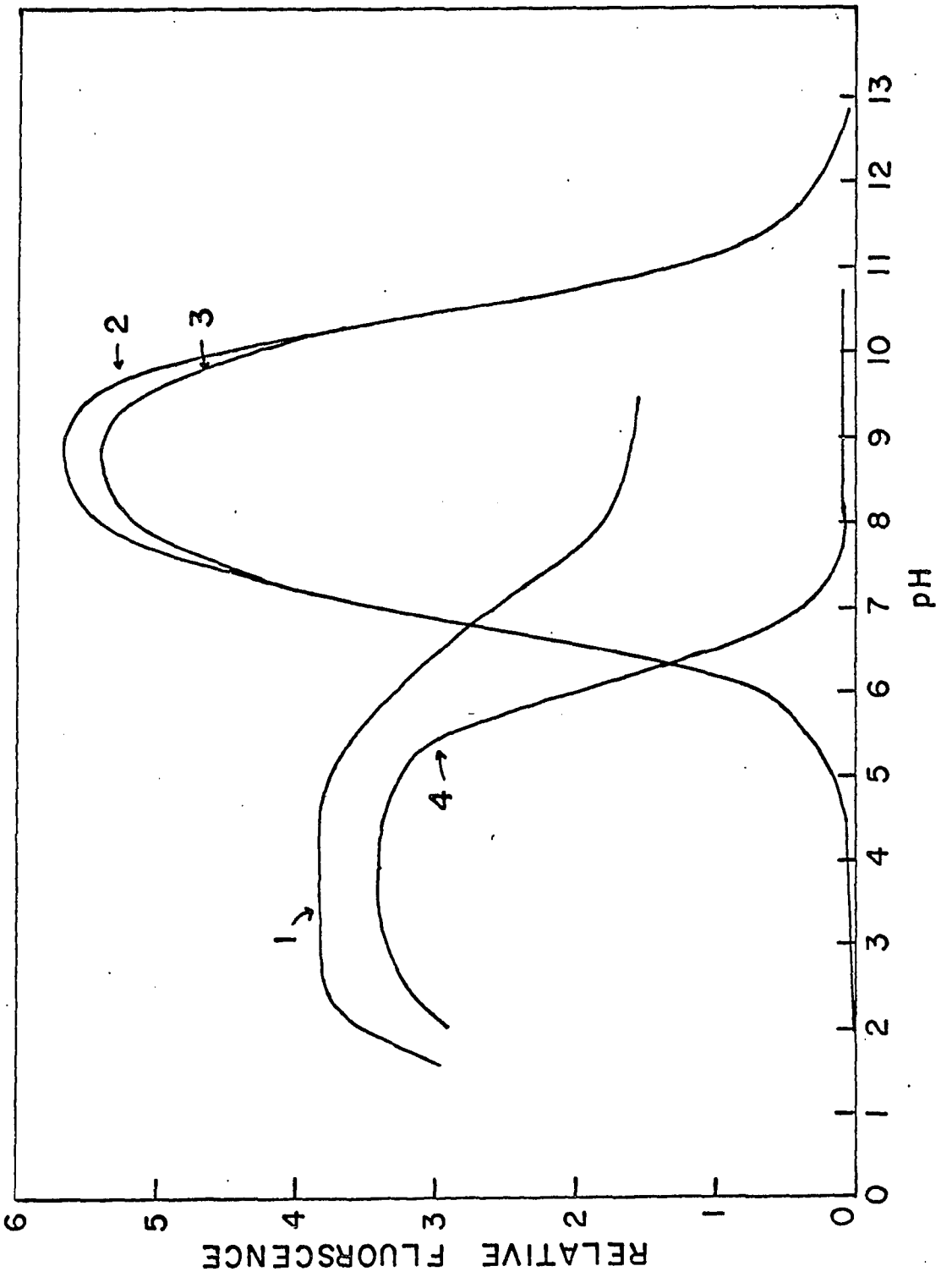

RELATIVE FLUORSCENCE
0
1
2
3
4
5
6
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2
3
4

Figure 18. Variation in intensity of fluorescence of umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O and its copper derivative with pH

1. Free indicator - excitation monochromator set at 330 mu
2. Free indicator - excitation monochromator set at 370 mu
3. Calcium derivative - excitation monochromator set at 370 mu
4. Copper derivative - excitation monochromator set at 330 mu below pH 6 and at 370 mu above pH 6

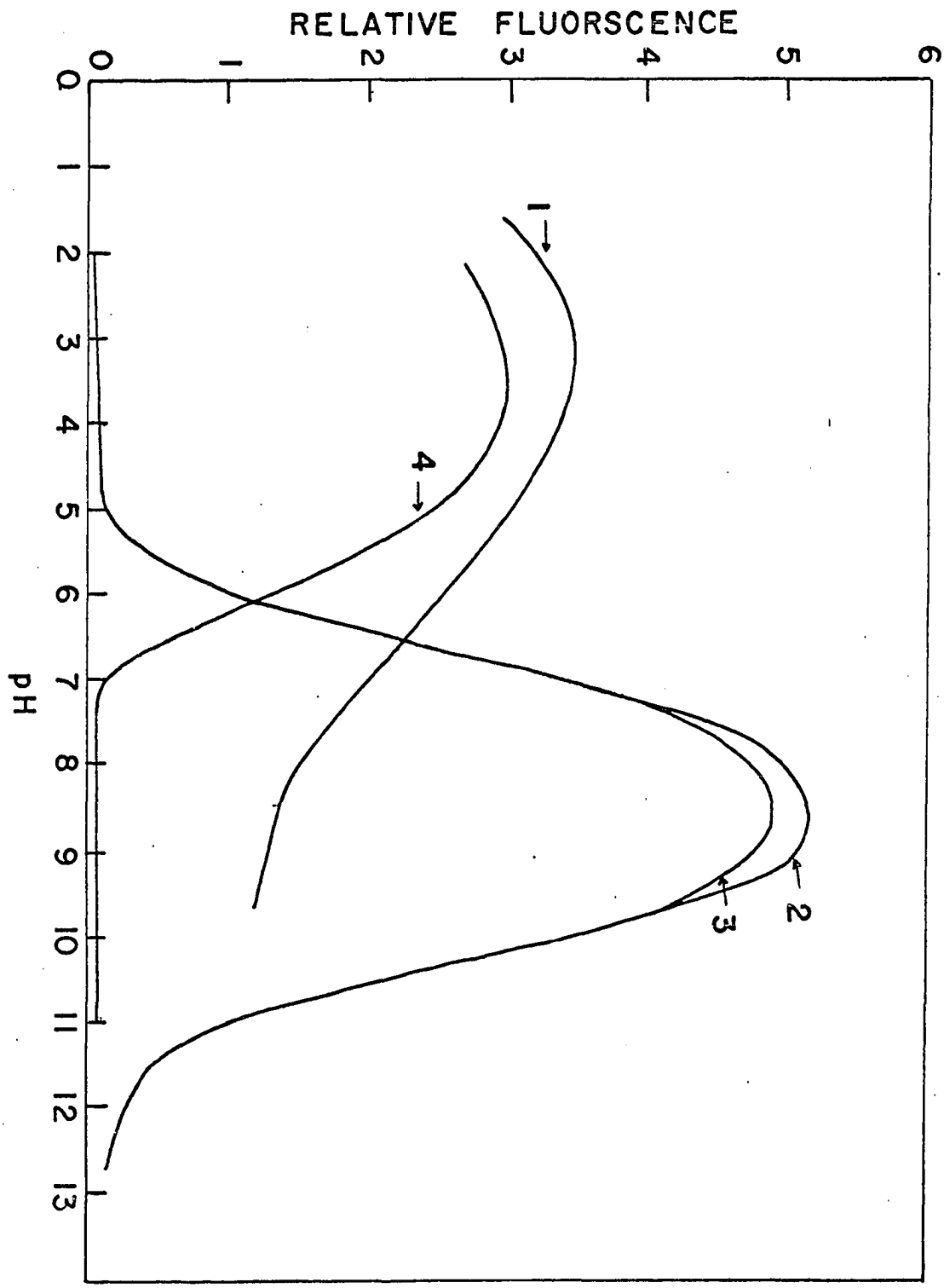

RELATIVE FLUORSCENCE
0
1
2
3
4
5
6
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2
3
4

Figure 19. Variation in intensity of fluorescence of 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O with pH

1. Free indicator - excitation monochromator set at 328 mu
2. Free indicator - excitation monochromator set at 365 mu
3. Calcium derivative - excitation monochromator set at 365 mu
4. Copper derivative - excitation monochromator set at 328 mu below pH 6 and at 365 mu above pH 6

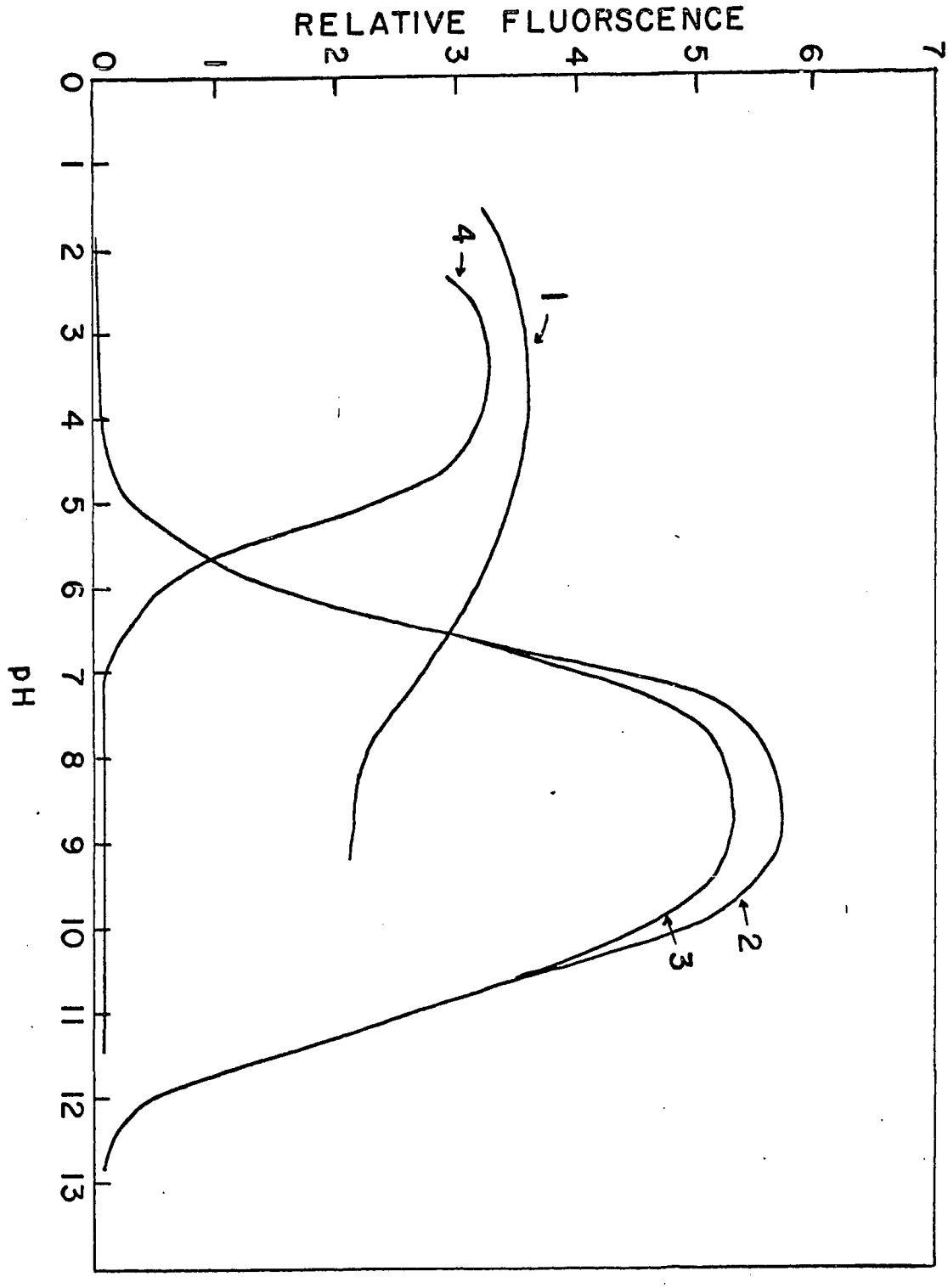

RELATIVE FLUORSCENCE
0
1
2
3
4
5
6
7
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2
3
4

Figure 20. Fluorescence spectra of 4-methylesculetin at pH 9

1. Excitation, fluorescence monochromator set at 460 mu
2. Fluorescence, excitation monochromator set at 370 mu

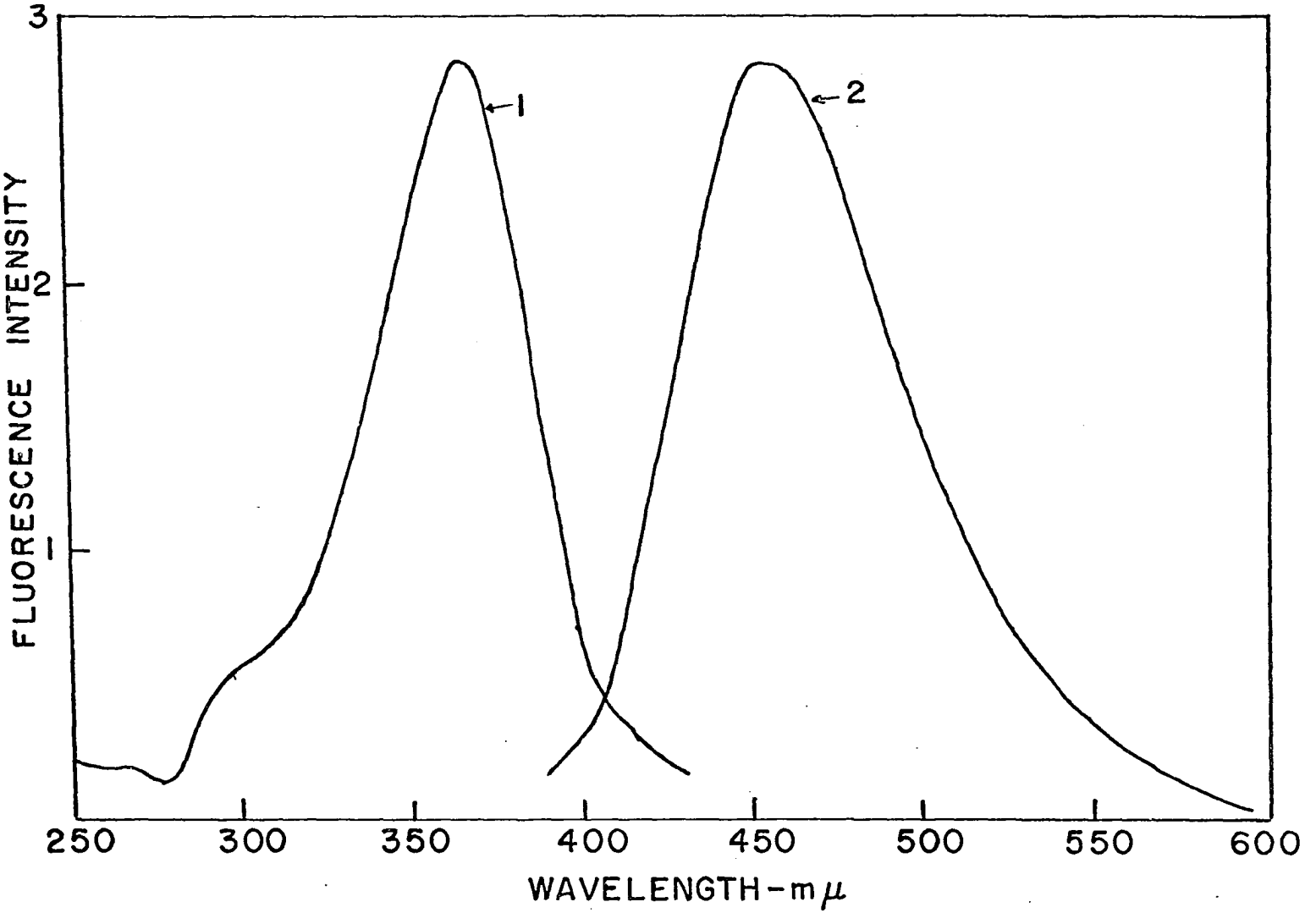

FLUORESCENCE INTENSITY
3
2
1
1
2
250
300
350
400
450
500
550
600
WAVELENGTH - mμ

Figure 21. Fluorescence spectra of 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O at pH 9

1. Excitation, fluorescence monochromator set at 459 mu
2. Fluorescence, excitation monochromator set at 369 mu

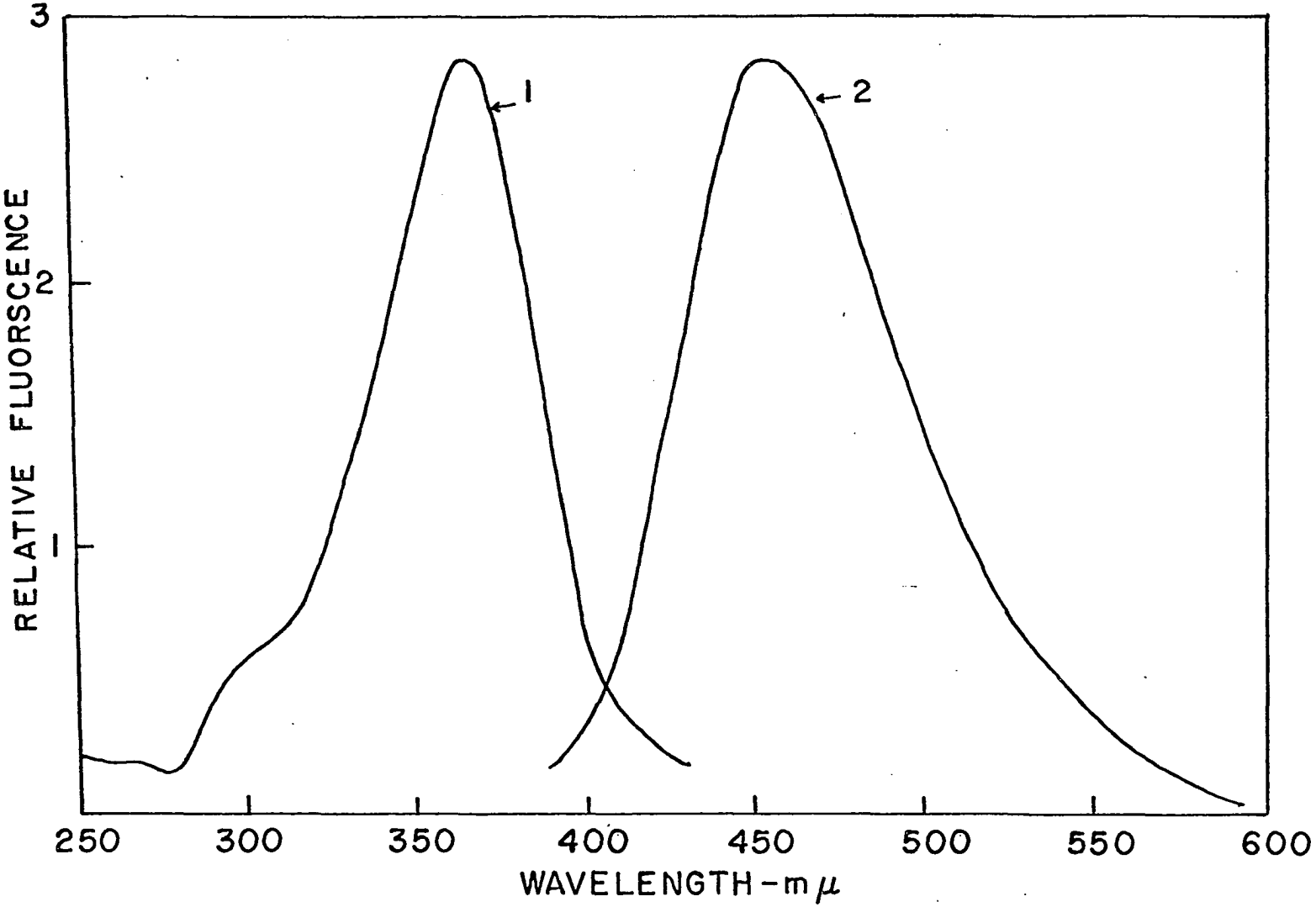

3
2
1
RELATIVE FLUORSCENCE
1
2
250
300
350
400
450
500
550
600
WAVELENGTH - mμ

Figure 22. Variation in intensity of fluorescence of 4-methylesculetin and its copper derivative with pH

1. Free indicator - excitation monochromator set at 370 mu
2. Copper derivative - excitation monochromator set at 370 mu

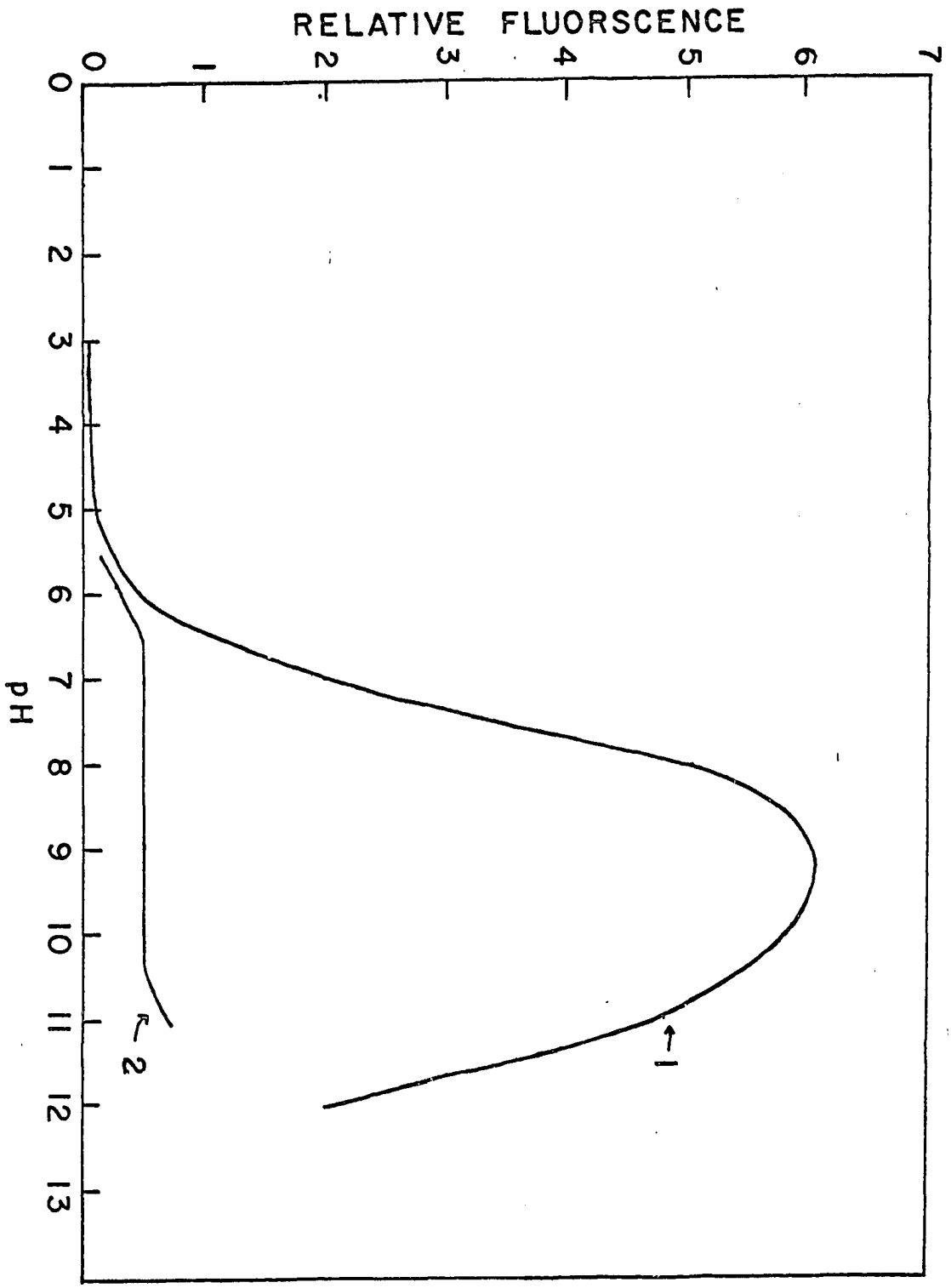
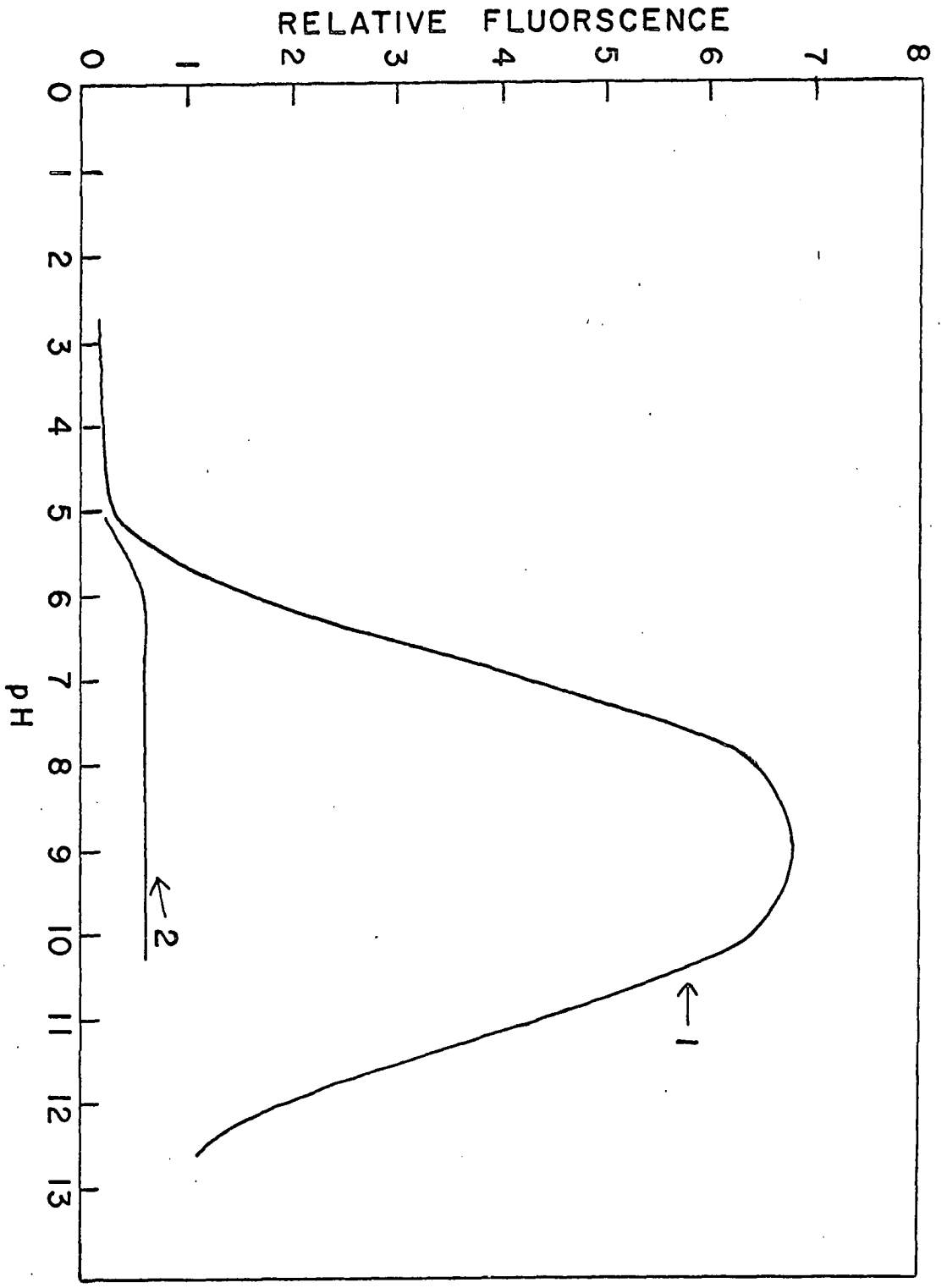

RELATIVE FLUORSCENCE
0
1
2
3
4
5
6
7
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2

Figure 23. Variation in intensity of fluorescence of 4-methylesculetinmethylene-iminodiacetic acid·$\frac{1}{2}$ H_2O and its copper derivative with pH

1. Free indicator - excitation monochromator set at 370 mu
2. Copper derivative - excitation monochromator set at 370 mu


RELATIVE FLUORSCENCE
0
1
2
3
4
5
6
7
8
pH
0
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2

by measuring the relative fluorescence of solutions whose pH values varied from 3.5 to 10.5. These solutions were prepared by mixing 15 ul. of indicator stock solution, 15 ul. of metal ion stock solution, and 10 ml. of buffer solution, and diluting to 25 ml. with 0.1 M potassium chloride. The pH of each of the solutions was checked after the spectra were obtained. The effect of copper on the relative fluorescence of the compounds is shown in Figures 15, 16, 17 and 18.

The effect of copper on the fluorescence of 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O was determined by titrating solutions containing 7 ul. of indicator stock solution and 7 ul. of copper ion stock solution in 75 ml. of 0.1 M potassium chloride with 0.2 M potassium hydroxide. The titrations were followed fluorometrically. The effect of copper ion on relative fluorescence with changes in pH is shown in Figures 22 and 23.

The combining ratios of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid·$\frac{1}{2}$ H_2O, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O with copper were determined by titrating solutions containing a known amount of indicator stock solution and 10 ml. of buffer in 75 ml. of 0.1 M potassium chloride with copper ion stock solution. The decrease in fluorescence with increase in copper ion concen-

tration was recorded. The variation in relative fluorescence with increasing copper ion concentration is shown in Figures 24, 25, 26, 27, 28, 29 and 30. Combining ratios were determined at pH values where the fluorescence of the free indicator was most intense.

4. Effect of calcium on fluorescence

The effect of calcium on the fluorescence of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, 4-methylumbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O was determined by measuring the relative fluorescence of solutions prepared by mixing 15 ul. of indicator stock solution, 15 ul. of calcium ion stock solution, and 10 ml. of buffer solution, and diluting to 25 ml. with 0.1 M potassium chloride. The pH was checked after the spectra were obtained. The effect of calcium on the fluorescence of the compounds is shown in Figures 15, 16, 17, 18 and 19.

The effect of calcium on the fluorescence of 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O was determined by measuring the fluorescence of two solutions buffered at pH 12.60, one containing free indicator and the other containing the indicator and an equimolar amount of calcium ion. The data obtained is shown in Table 1.

Figure 24. Change in fluorescence of 4-methylumbelliferone-8-methyleneimino-diacetic acid with copper ion concentration at pH 4.5

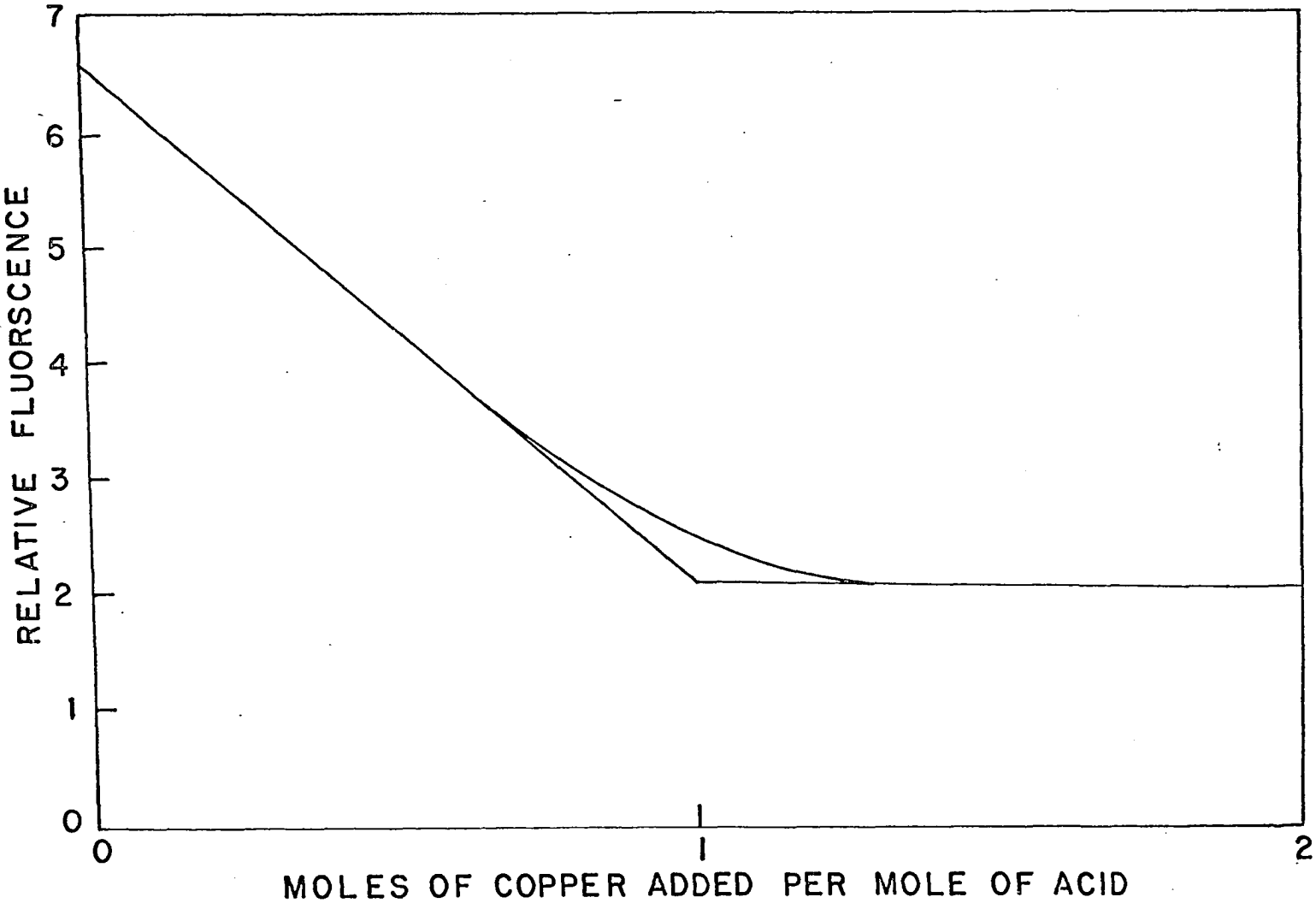

RELATIVE FLUORSCENCE
7
6
5
4
3
2
1
0
0
1
2
MOLES OF COPPER ADDED PER MOLE OF ACID

Figure 25. Change in fluorescence of umbelliferone-8-methyleneiminodiacetic acid with copper ion concentration at pH 4.5

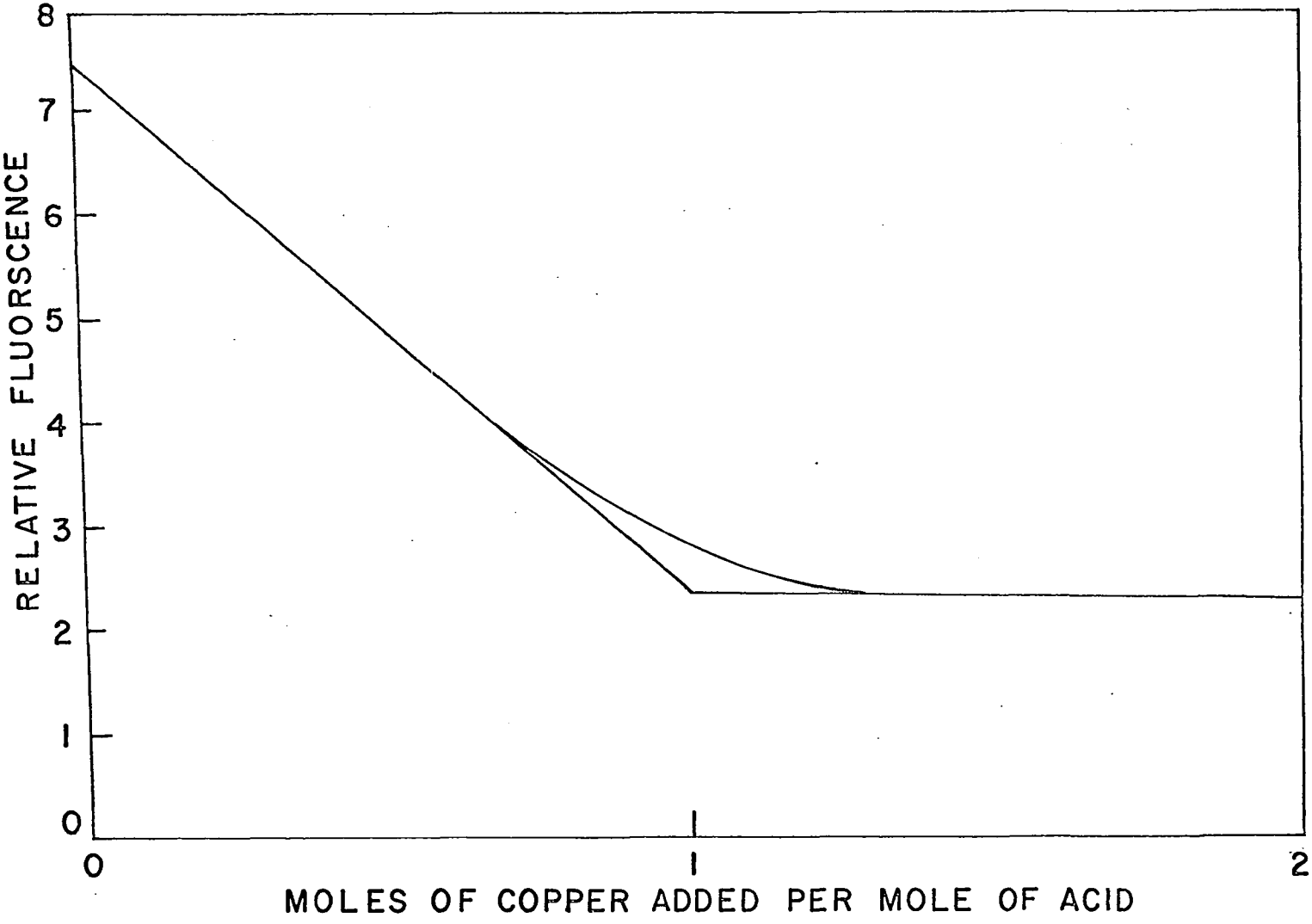

RELATIVE FLUORSCENCE
8
7
6
5
4
3
2
1
0
0
1
2
MOLES OF COPPER ADDED PER MOLE OF ACID

Figure 26. Change in fluorescence of 4-methylumbelliferone-8-methyleneglycine with copper ion concentration at pH 8.5

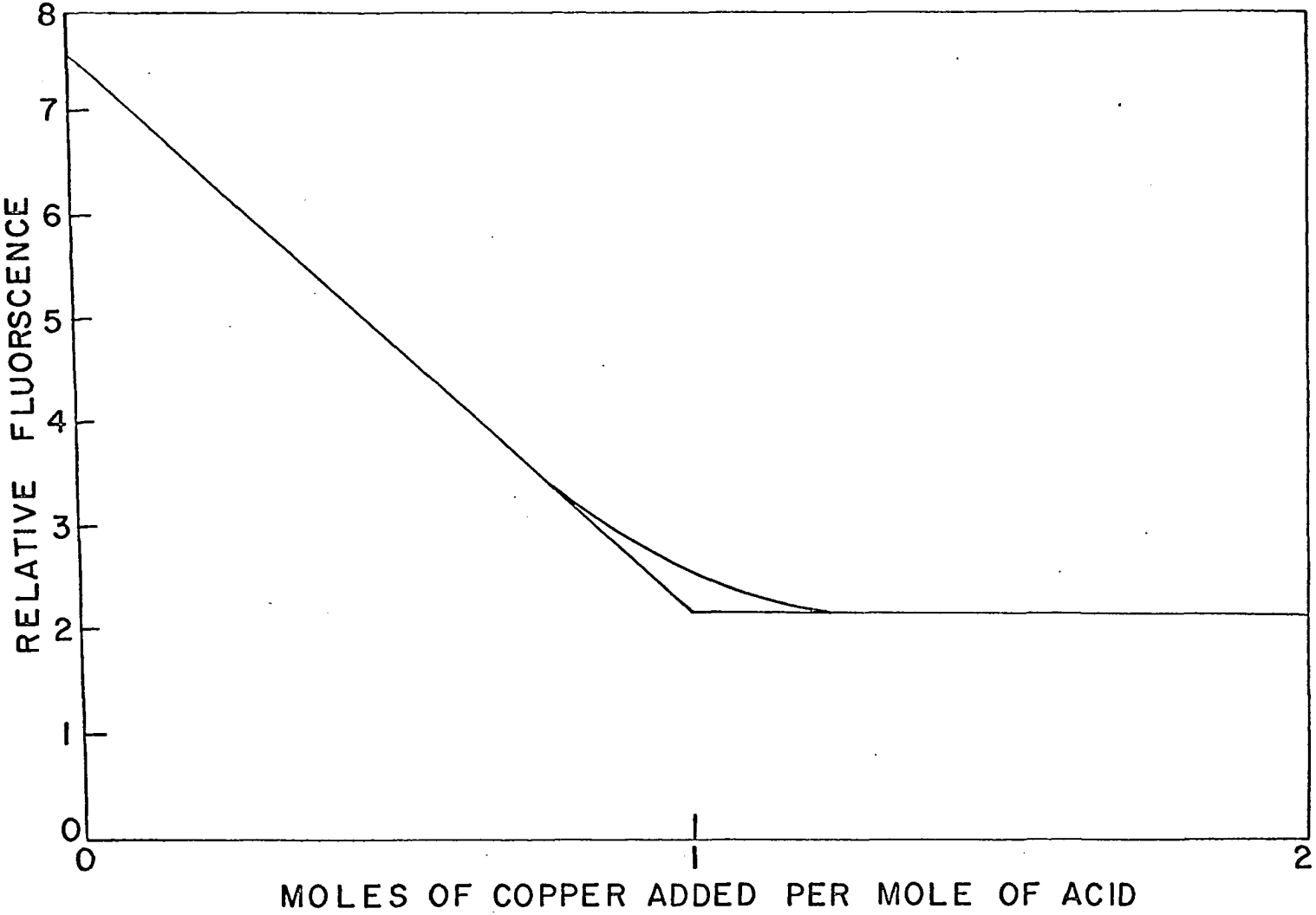

RELATIVE FLUORSCENCE
8
7
6
5
4
3
2
1
0
0
1
2
MOLES OF COPPER ADDED PER MOLE OF ACID

Figure 27. Change in fluorescence of umbelliferone-8-methyleneglycine.$\frac{1}{2}H_2O$ with copper ion concentration at pH 8.5

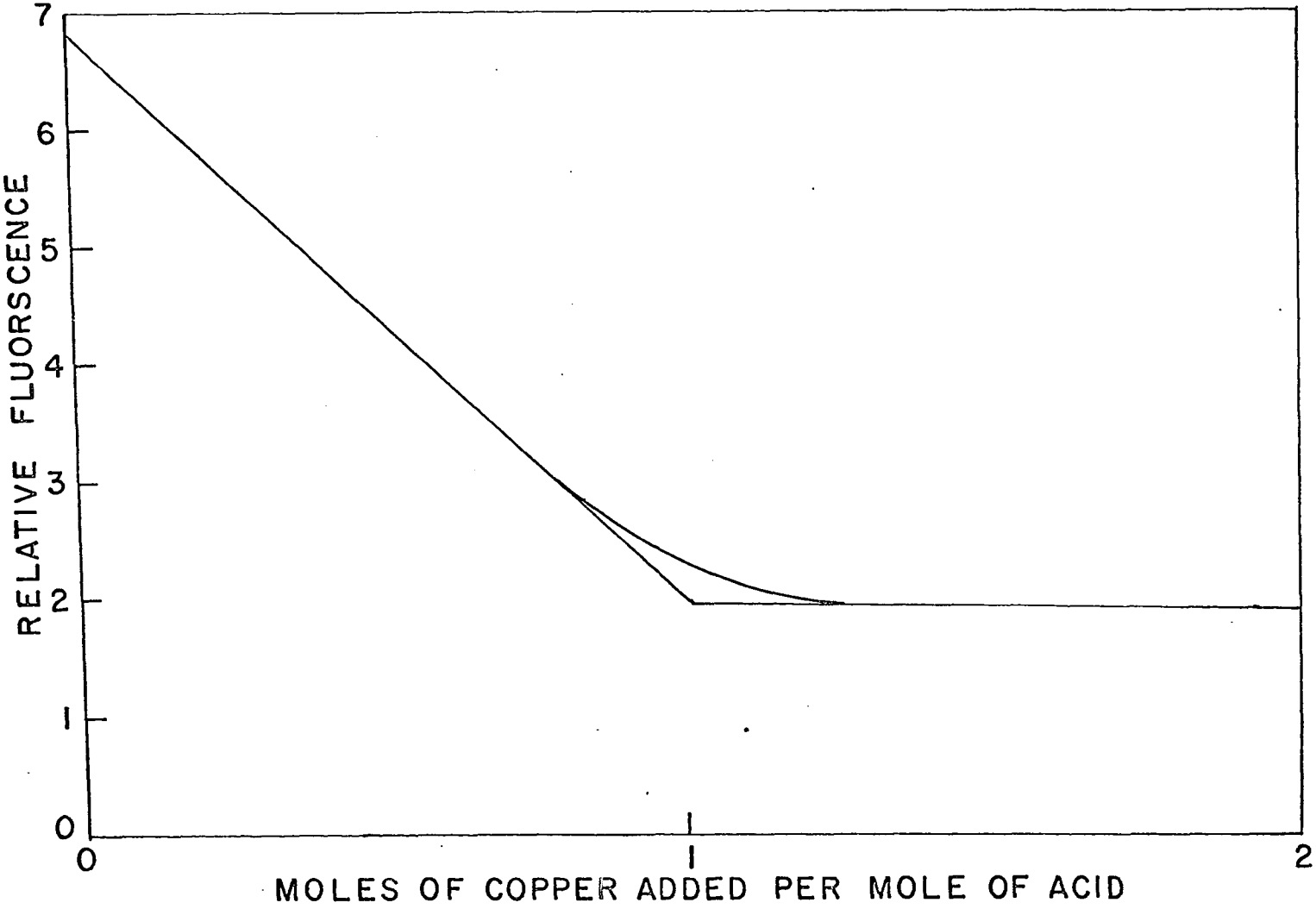

RELATIVE FLUORSCENCE
7
6
5
4
3
2
1
0
0
1
2
MOLES OF COPPER ADDED PER MOLE OF ACID

Figure 28. Change in fluorescence of 4-methylumbelliferone-8-methylene-sarcosine·$1\frac{1}{2}$ H_2O with copper ion concentration at pH 8.5

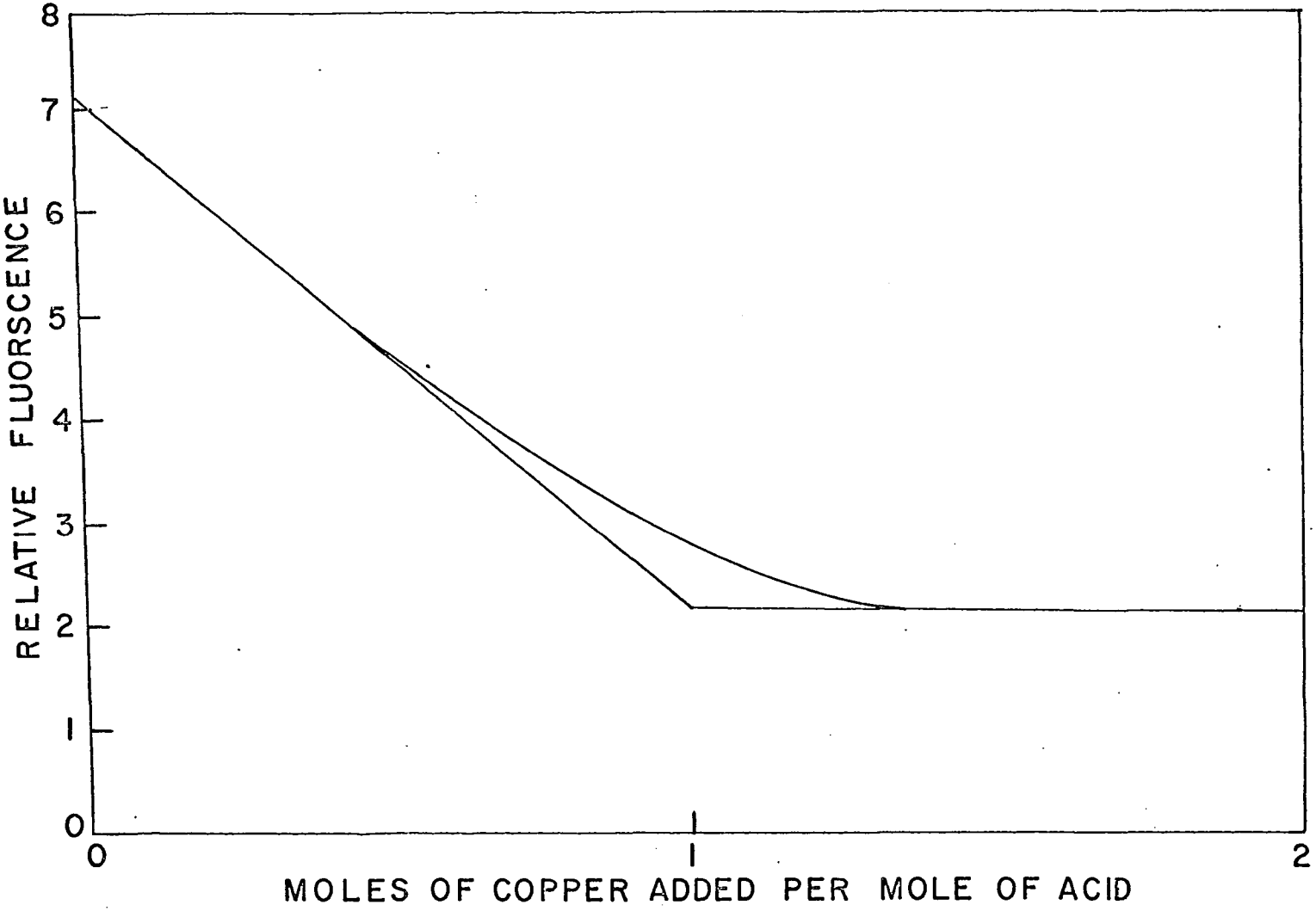

RELATIVE FLUORSCENCE
8
7
6
5
4
3
2
1
0
0
1
2
MOLES OF COPPER ADDED PER MOLE OF ACID

Figure 29. Change in fluorescence at 4-methylesculetin with copper ion concentration with copper ion concentration at pH 9.1

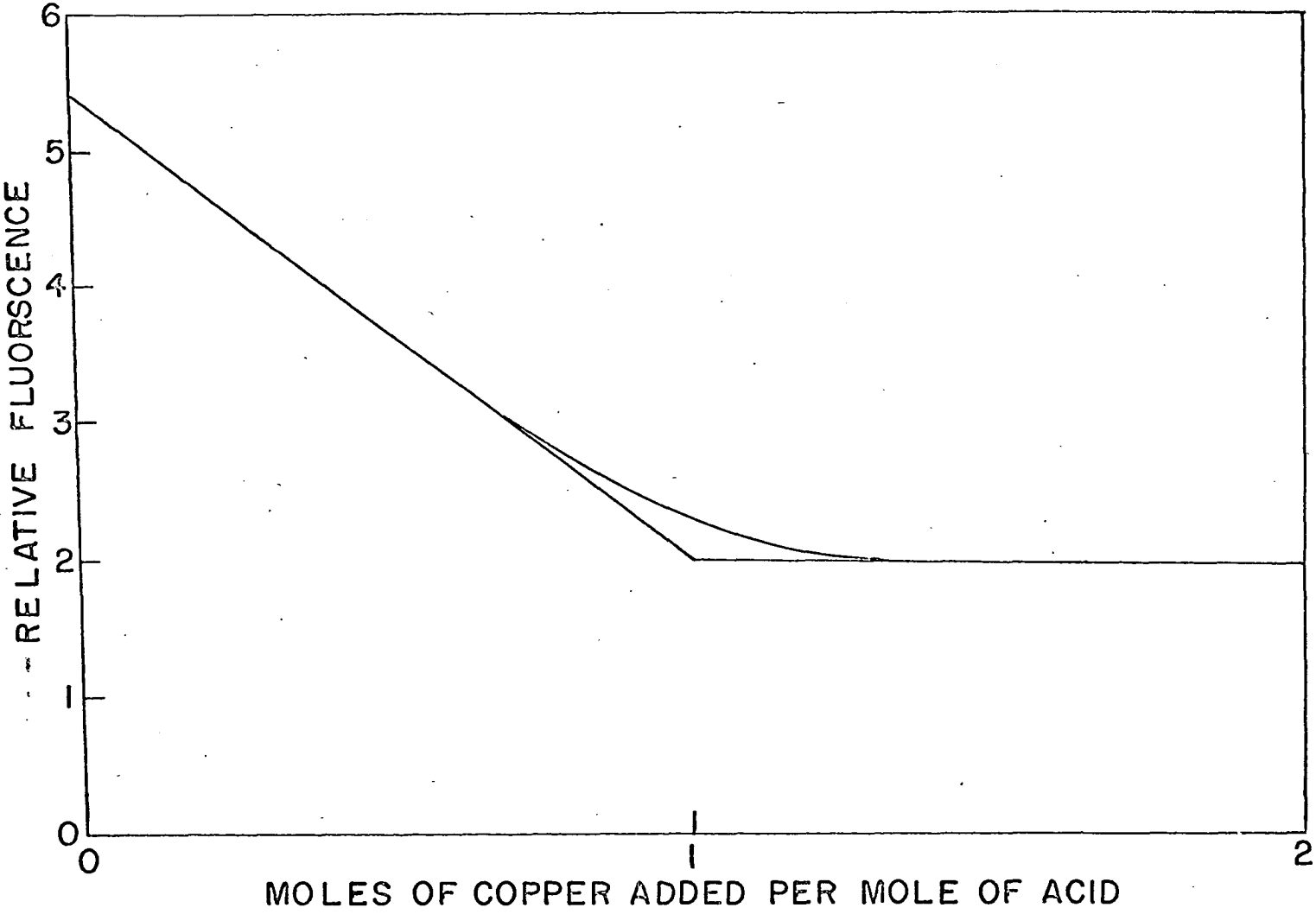

6
5
4
3
2
1
0
RELATIVE FLUORSCENCE
0
1
2
MOLES OF COPPER ADDED PER MOLE OF ACID

Figure 30. Change in fluorescence of 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O with copper ion concentration at pH 9.1

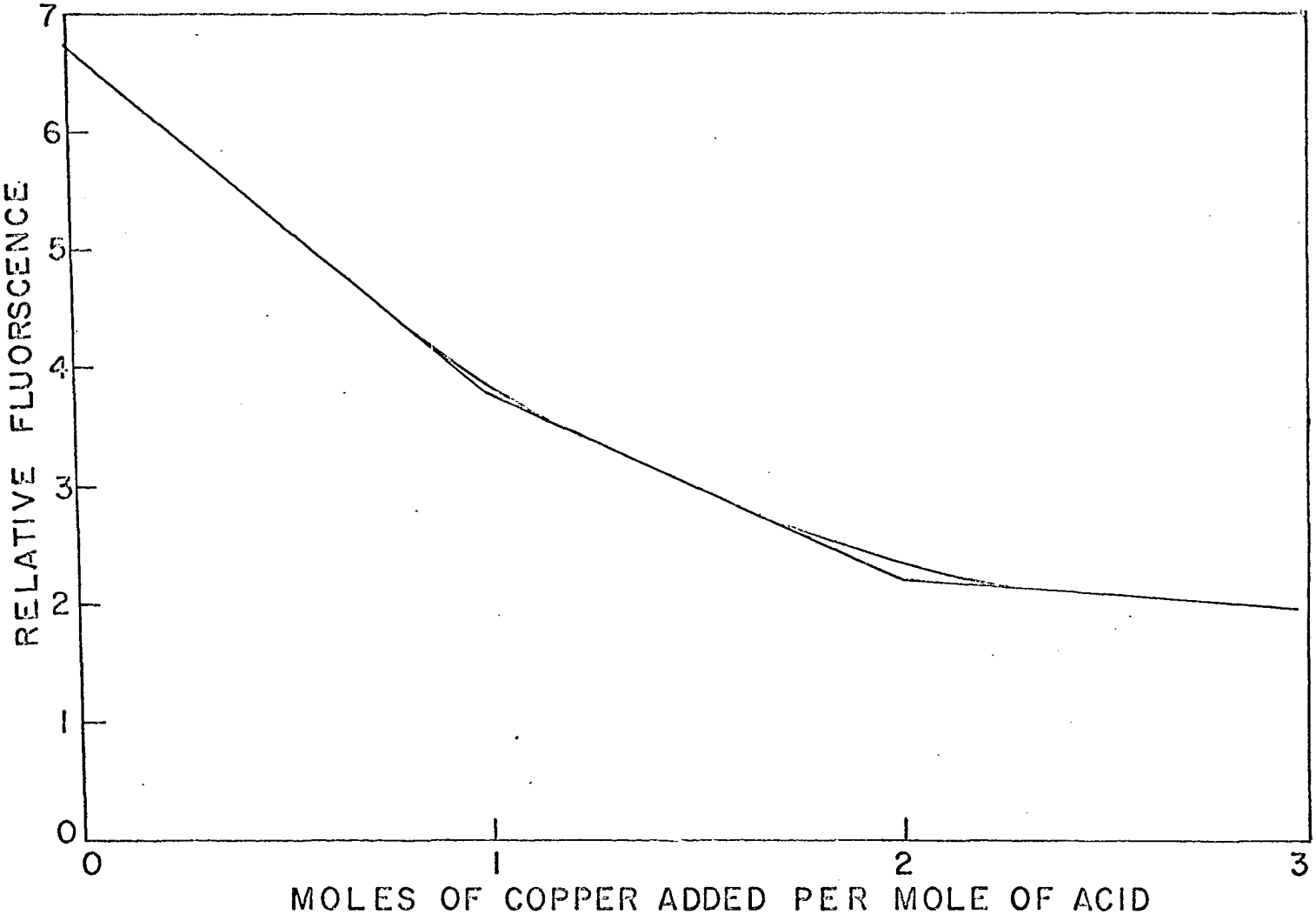

RELATIVE FLUORSCENCE
7
6
5
4
3
2
1
0
0
1
2
3
MOLES OF COPPER ADDED PER MOLE OF ACID

Table 1. The effect of calcium on the fluorescence of 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O at pH 12.60

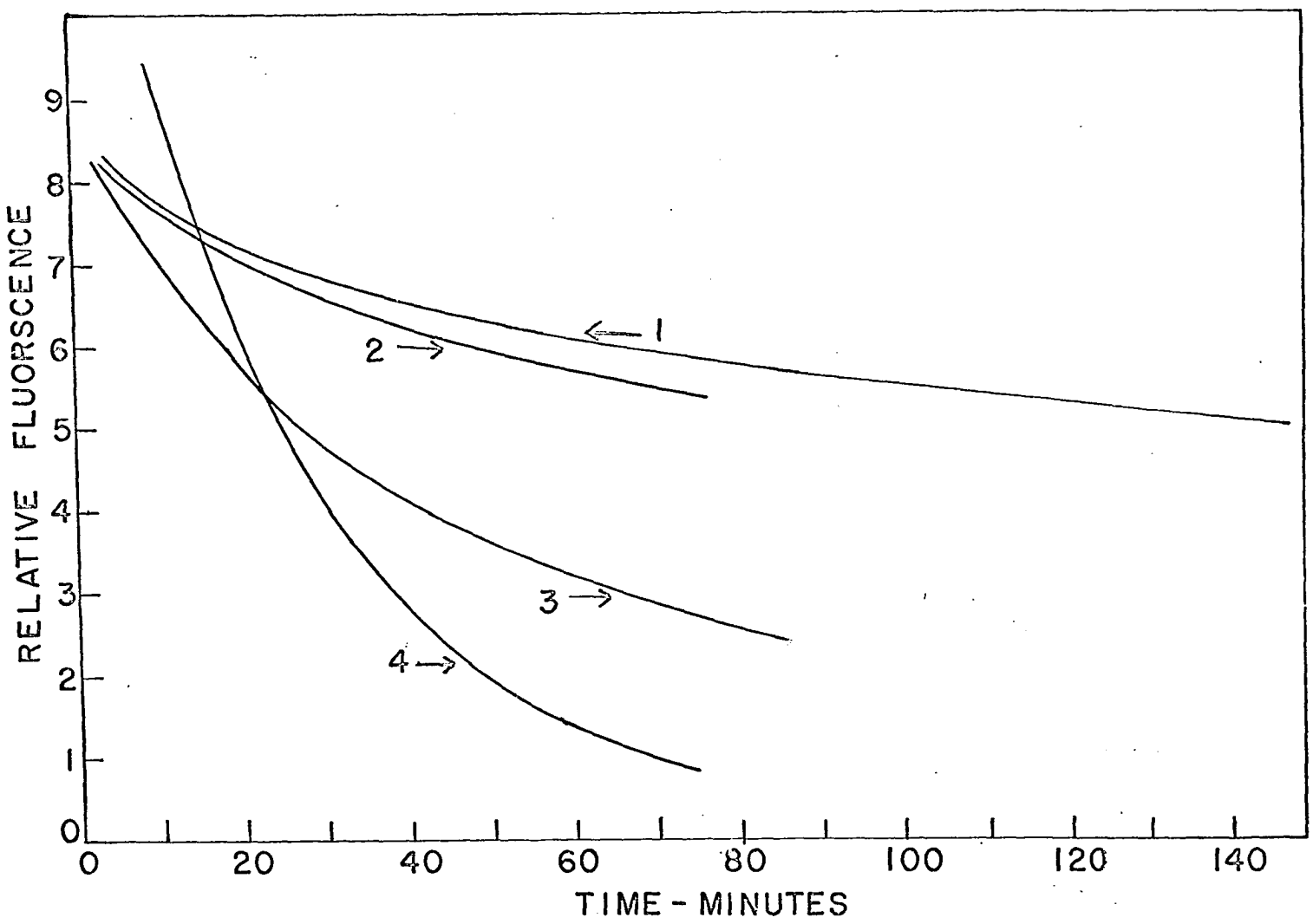
Compound	Fluorescence of 2.5 ul. of compound	Fluorescence of 2.5 ul. of compound + 2.5 ul. of calcium
4-Methylesculetin	0.90	1.75
4-Methylesculetin-methyleneiminodi-acetic acid·½ H_2O	1.00	1.60

A study of the stability of the calcium derivative of 4-methylumbelliferone-8-methyleneiminodiacetic acid was carried out by observing the change in fluorescence with time at pH 12.8, 12.9 and 13. The solutions on which these studies were made contained 65 ul. of 4-methylumbelliferone-8-methyleneiminodiacetic acid stock solution, 65 ul. of calcium ion stock solution and 10 ml. of buffer solution in 100 ml. of 0.1 M potassium chloride. The relationship between fluorescence and time is shown in Figure 31.

The stability of the calcium compound of umbelliferone-8-methyleneiminodiacetic acid at pH 12.80 was also studied. The solution on which this study was made contained 65 ul. of umbelliferone-8-methyleneiminodiacetic acid, 65 ul. of calcium ion stock solution and 10 ml. of buffer solution in 100 ml. of 0.1 M potassium chloride. The relationship between fluorescence and time is shown in Figure 31.

Figure 31. Variation in intensity of fluorescence of the calcium derivatives of 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid with time

1. Calcium-4-methylumbelliferone-8-methyleneiminodiacetic acid at pH 12.8
2. Calcium-4-methylumbelliferone-8-methyleneiminodiacetic acid at pH 12.9
3. Calcium-4-methylumbelliferone-8-methyleneiminodiacetic acid at pH 13.0
4. Calcium-umbelliferone-8-methyleneiminodiacetic acid at pH 12.8


RELATIVE FLUORSCENCE
0
1
2
3
4
5
6
7
8
9
0
20
40
60
80
100
120
140
TIME - MINUTES
1
2
3
4

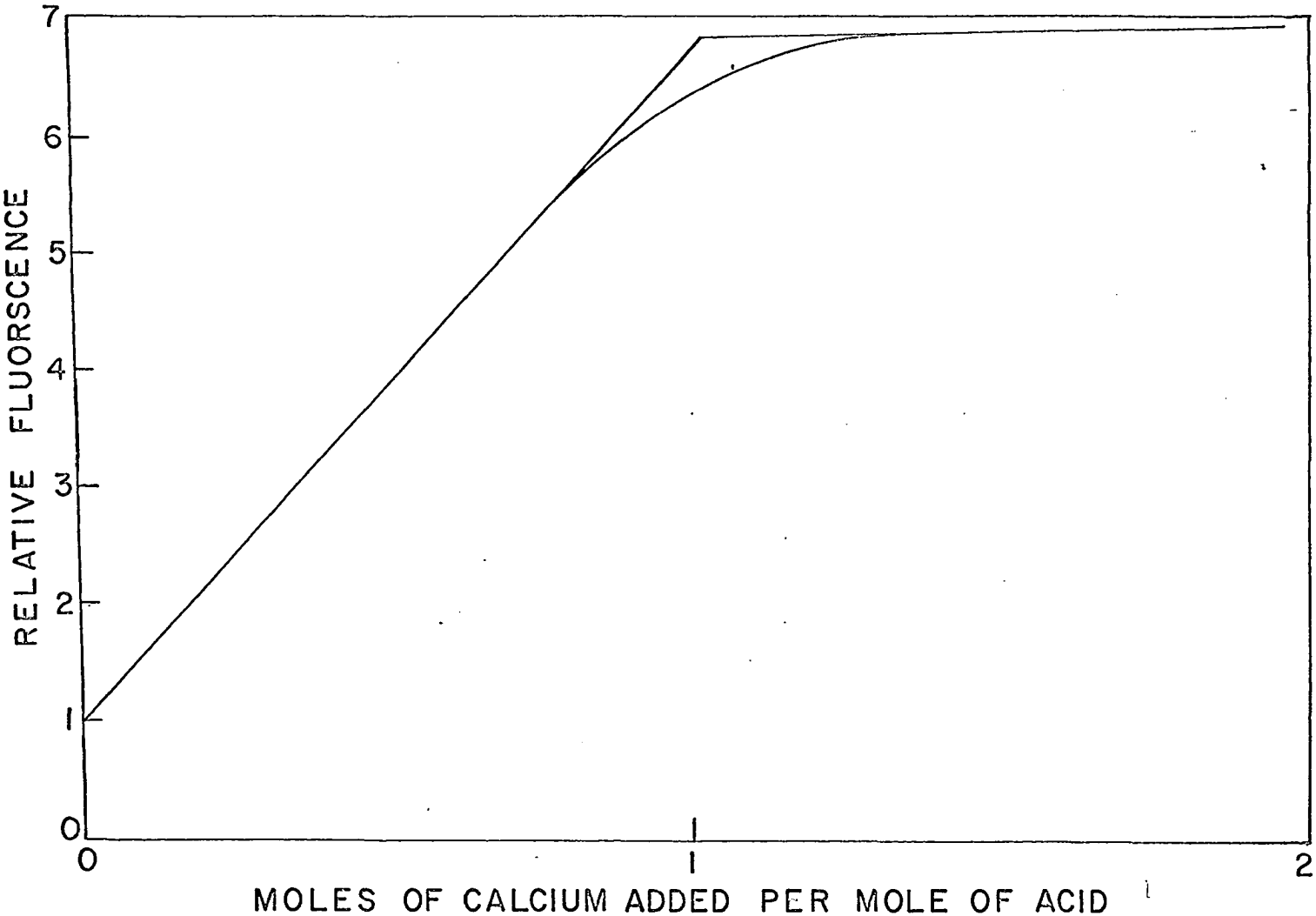
To determine if 4-methylumbelliferone-8-methyleneiminodiacetic acid could be used in a direct analytical method for calcium, solutions containing an excess of the compound in the presence of lesser amounts of calcium at pH 12.8 and 13.1 were prepared. The results of the fluorescence vs. time study are given in Table 2.

Table 2. The effect of pH on the stability of the calcium compound of 4-methylumbelliferone-8-methyleneiminodiacetic acid

pH	Compound (ul.)	Calcium (ul.)	Stable (time)
12.80	20	7	1 hour
13.10	20	7	unstable

The combining ratio of 4-methylumbelliferone-8-methyleneiminodiacetic acid with calcium was determined at pH 12.80 by observing the increase in relative fluorescence with increase in calcium ion concentration. The solution on which the determination of combining ratios was performed contained 30 ul. of compound and 10 ml. of buffer solution in 75 ml. of 0.1 M potassium chloride. Enough 0.1 M EDTA was added to just sequester the calcium present in the reagents used. The variation in relative fluorescence with calcium ion concentration is shown in Figure 32.

Figure 32. Change in fluorescence of 4-methylumbelliferone-8-methyleneimino-diacetic acid with calcium ion concentration at pH 12.0


RELATIVE FLUORSCENCE
7
6
5
4
3
2
1
0
0
1
2
MOLES OF CALCIUM ADDED PER MOLE OF ACID

5. Effect of aluminum on fluorescence

The effect of aluminum on the fluorescence of 4-methylumbelliferone-8-methyleneiminodiacetic acid was studied by observing the change in fluorescence of a solution containing 25 ul. of compound stock solution and 25 ul. of aluminum ion stock solution in 75 ml. of 0.1 M potassium chloride as it was titrated with 0.2 M potassium hydroxide. The pH range studied was 1.5 to 4.0.

E. Acid Dissociation Constants

1. Apparatus and reagents

Absorption spectra were obtained on a Cary Model 15 recording spectrophotometer.

All spectrophotometric data used in obtaining acid dissociation constants were obtained using a Beckman DU spectrophotometer.

A Micro Metric Model SB2 microburet and a Model S5Y syringe were used in delivering appropriate volumes for use in spectrophotometric work.

The buffers used in the determination of absorption spectra and in the determination of acid dissociation constants were prepared according to R. Bates (2) and were of constant ionic strength.

Stock solutions of the compounds being studied were of the same concentration as those used for the fluorescence work.

2. Determination of acid dissociation constants using solubility data

The solubility of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid and 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O at pH values before the first end point was determined by buffering solutions containing an excess of the compound at specific pH values and shaking for twelve hours. Appropriate volumes of the filtrates containing 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid were adjusted to pH 10 and diluted to 25 ml. in volumetric flasks with 0.1 M potassium chloride while appropriate volumes of the filtrates containing 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O were adjusted to pH 4.0 and diluted to 25 ml. in volumetric flasks with 0.1 M potassium chloride. The amount of compound in each solution was determined spectrophotometrically on a Beckman DU spectrophotometer at the analytical wavelength for the compound. The results are shown in Table 3. Graphs of solubility against $1/[H^+]$, shown in Figure 33, yielded values for the intrinsic solubility of each compound. From the values of solubility at known pH and from the intrinsic solubility, the acid dissociation constant of the carboxyl group was determined for each compound.

Figure 33. Change in solubility with pH

1. 4-Methylumbelliferone-8-methyleneiminodiacetic acid
2. Umbelliferone-8-methyleneiminodiacetic acid
3. 4-Methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O

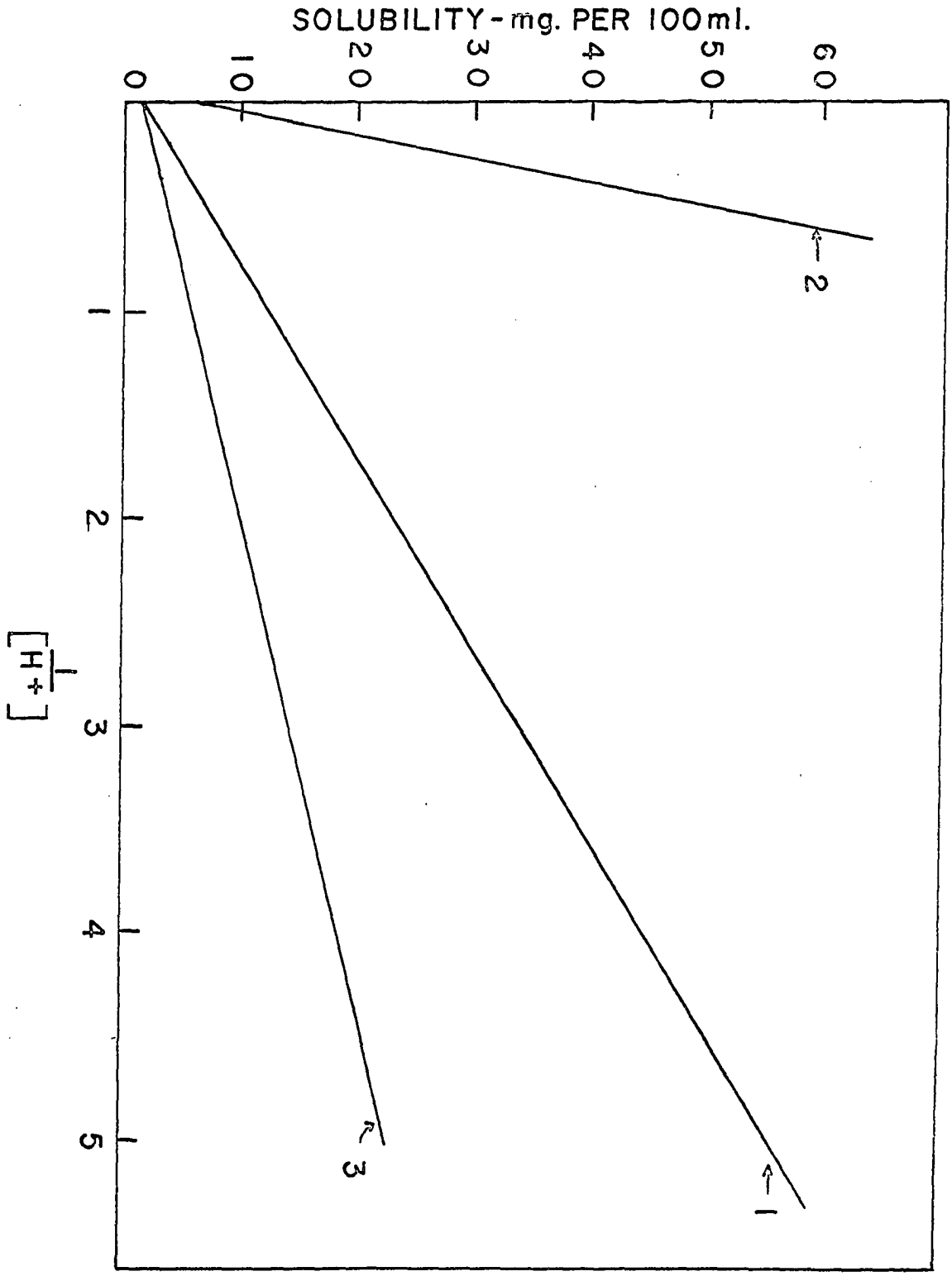

SOLUBILITY - mg. PER 100 ml.
0
10
20
30
40
50
60
1
2
3
4
5
[1/H+]
1
2
3

Table 3. Solubility of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid and 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O as a function of pH

Compound	pH	Solubility (mg./100 ml.)
4-methylumbelliferone-8-methyleneiminodiacetic acid	2.36	1.20
	2.80	1.71
	3.39	3.77
	4.45	32.37
	4.65	48.19
	4.74	59.44
umbelliferone-8-methyleneiminodiacetic acid	3.26	22.66
	3.84	65.66
	3.96	84.80
	4.06	94.33
4-methylesculetinmethyleneiminodiacetic acid	3.34	1.58
	3.76	3.55
	4.06	5.63
	4.41	12.94

3. Determination of acid dissociation constants using spectrophotometric data

The absorbance spectra of 4-methylumbelliferone, umbelliferone, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O were obtained at one half pH unit intervals ranging from pH 1.5 to 13.0. Solutions on which the spectra were run were prepared by mixing 375 ul. of the indicator stock solution, 0.25 ml. of 0.01 M EDTA to sequester any metal ions present, and 10 ml. of buffer solution, and diluting to

25 ml. with 0.1 M potassium chloride. The pH of each of the solutions was checked after the spectra were run. Absorption spectra of each compound at pH 4 and 9 are shown in Figures 35, 36, 37, 38, 39 and 40. The absorbance of each of the solutions was plotted against the pH of that solution for each compound studied. The graphs are shown in Figures 41, 42, 43, 44, 45 and 46.

The acid dissociation constant of the phenolic proton of each of the above compounds was determined in the following manner. The absorbance of a solution containing the acid form of the compound, of another solution containing the anion form of the compound and of seven additional solutions whose pH values were numerically equal to the estimated pK_a value +0.0, +0.2, +0.4, +0.6, -0.2, -0.4 and -0.6 and thus containing both forms of the compound were measured on a Beckman DU spectrophotometer. The log-ratio method was then applied to determine pK_a values.

Absorption spectra of 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O at pH 4 and 9 are shown in Figures 47 and 48. The absorbance of the compounds at pH values ranging from pH 2.5 to 11.0 was obtained by titrating solutions containing 1 ml. of the compound and 0.75 ml. of 0.1 M EDTA in 75 ml. of 0.1 M potassium chloride with 0.2 M potassium hydroxide. The titrations were followed spectrophotometrically using a Beckman DU spectrophotometer

Figure 34. Change in log [(S/S°) - 1] with pH

--- 4-Methylumbelliferone-8-methyleneiminodiacetic acid
—— Umbelliferone-8-methyleneiminodiacetic acid
---- 4-Methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O

LOG [(S/S°)-1]
-2
-1
0
+1
+2
pH
0
1
2
3
4
5
6

Figure 35. Absorption spectra of 4-methylumbelliferone

1. At pH 4
2. At pH 9

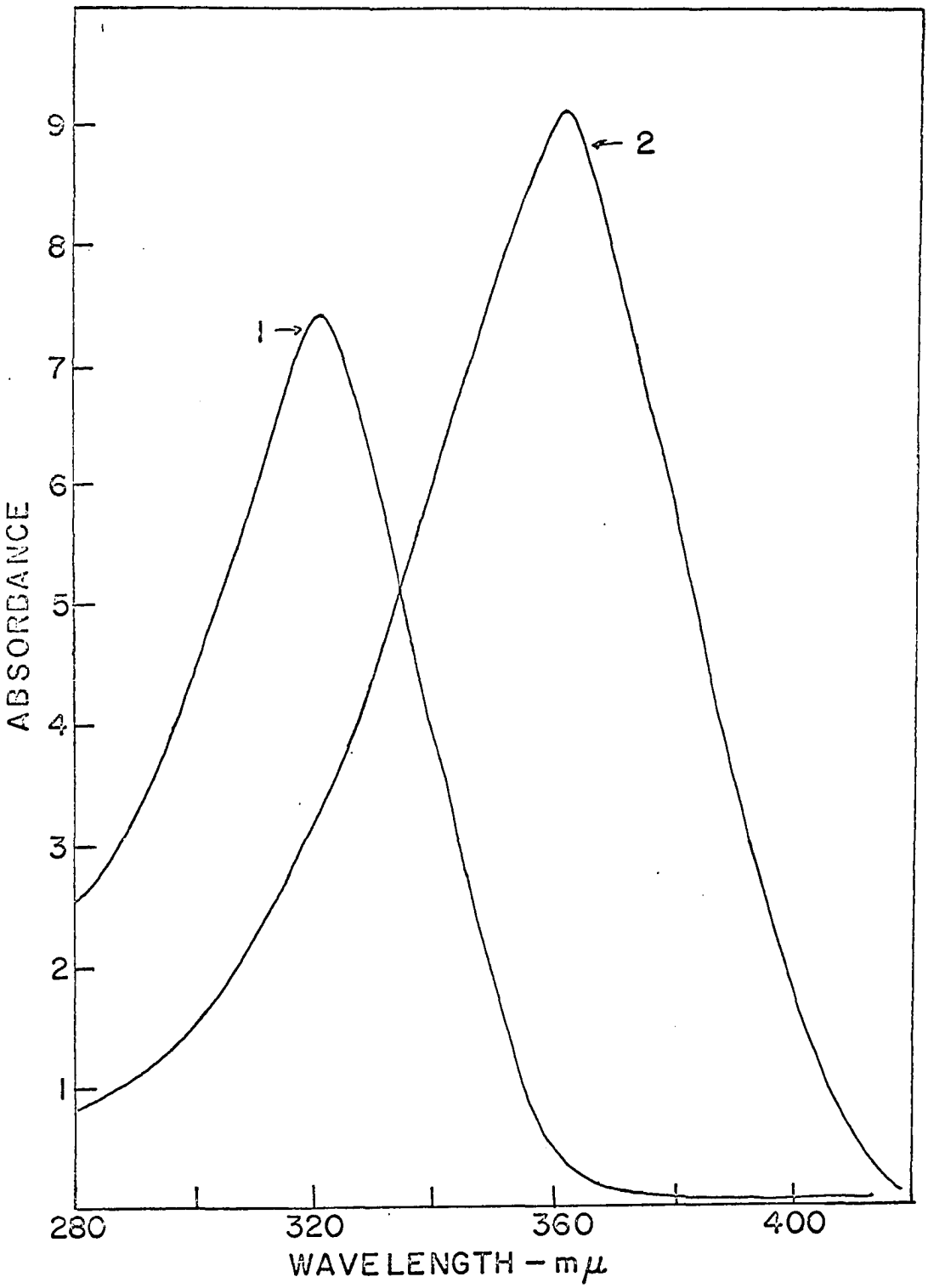

ABSORBANCE
9
8
7
6
5
4
3
2
1
1
2
280
320
360
400
WAVELENGTH – mμ

Figure 36. Absorption spectra of umbelliferone

1. At pH 4
2. At pH 9

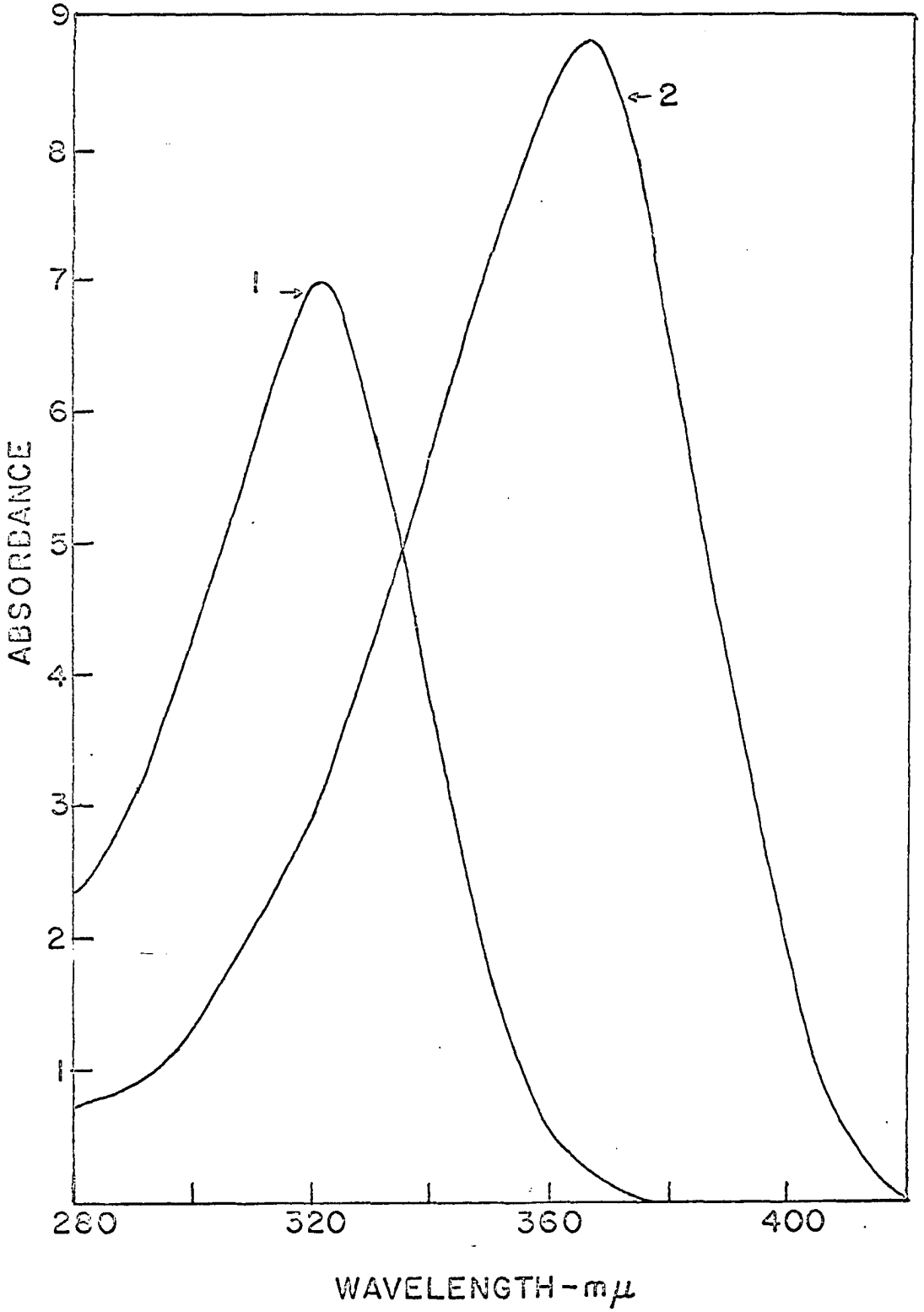

9
8
7
6
5
4
3
2
1
ABSORBANCE
1
2
280
320
360
400
WAVELENGTH - mμ

Figure 37. Absorption spectra of umbelliferone-8-methyleneiminodiacetic acid

1. At pH 4
2. At pH 9

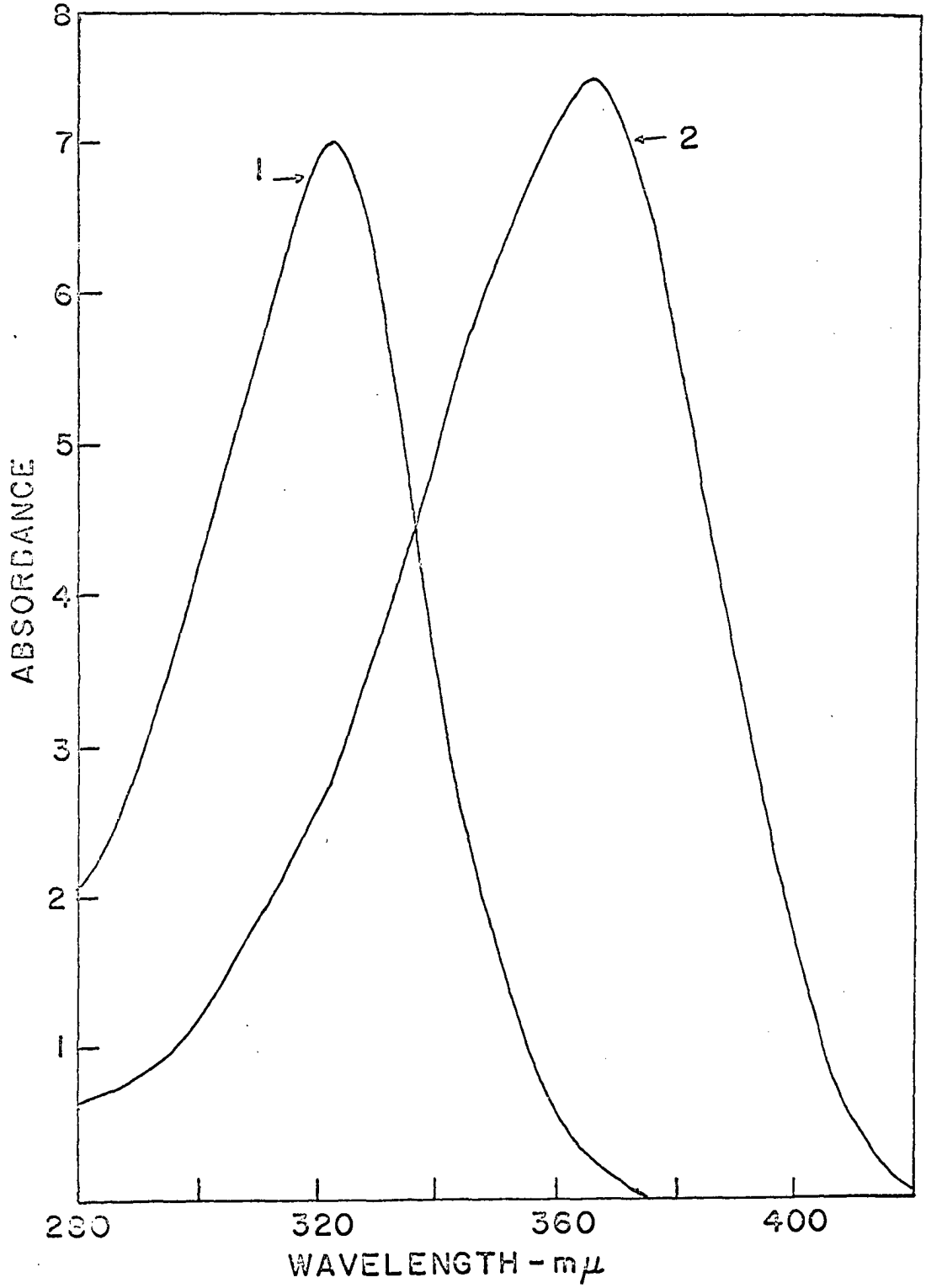

8
7
6
5
4
3
2
1
ABSORBANCE
1
2
280
320
360
400
WAVELENGTH - mμ

Figure 38. Absorption spectra of 4-methylumbelliferone-8-methyleneglycine

1. At pH 4
2. At pH 9

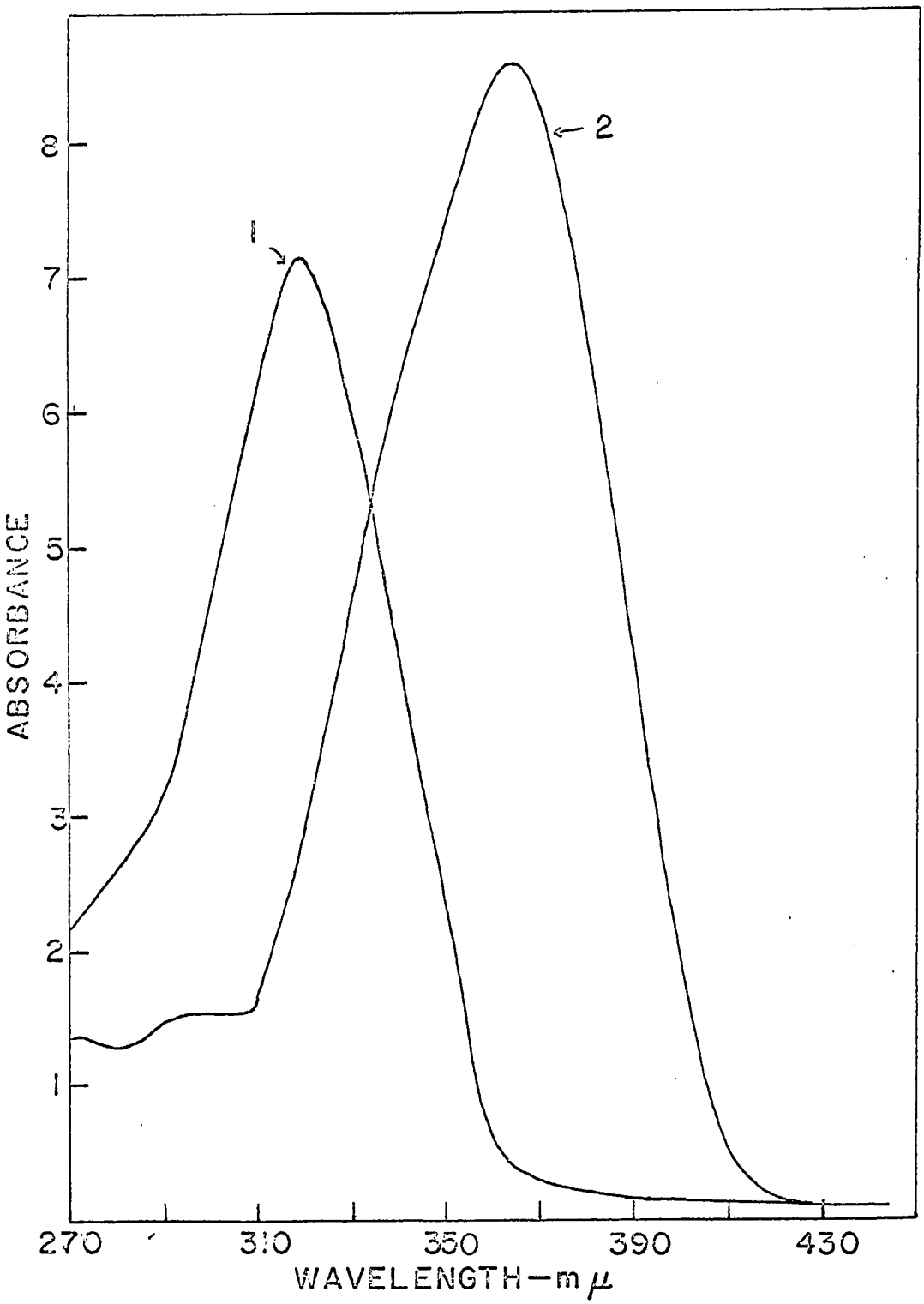

ABSORBANCE
8
7
6
5
4
3
2
1
270
310
350
390
430
WAVELENGTH—mμ
1
2

Figure 39. Absorption spectra of umbelliferone-8-methyleneglycine·½ H_2O

1. At pH 4
2. At pH 9

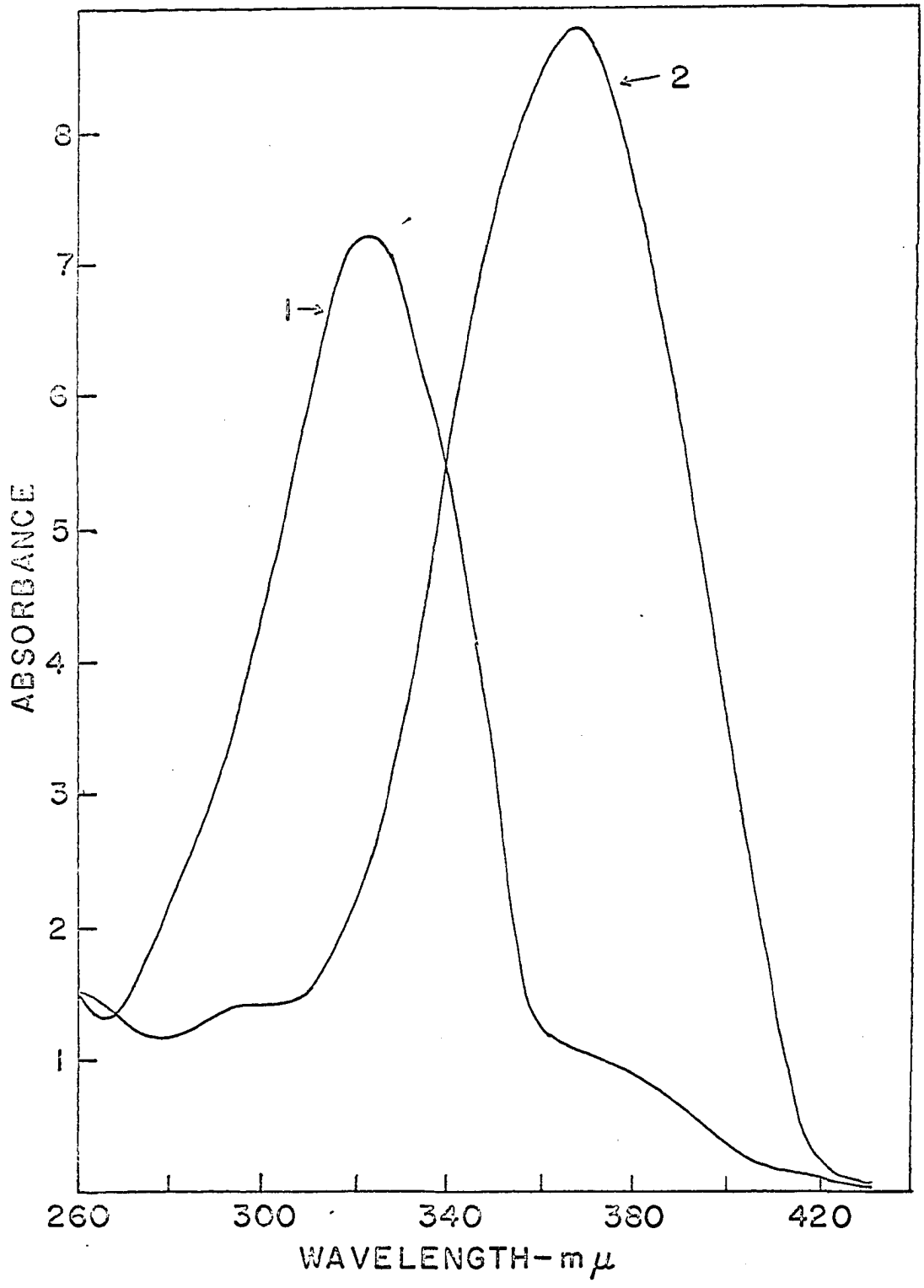

2
1
8
7
6
5
4
3
2
1
ABSORBANCE
260
300
340
380
420
WAVELENGTH- mμ

Figure 40. Absorption spectra of 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O

1. At pH 4
2. At pH 9

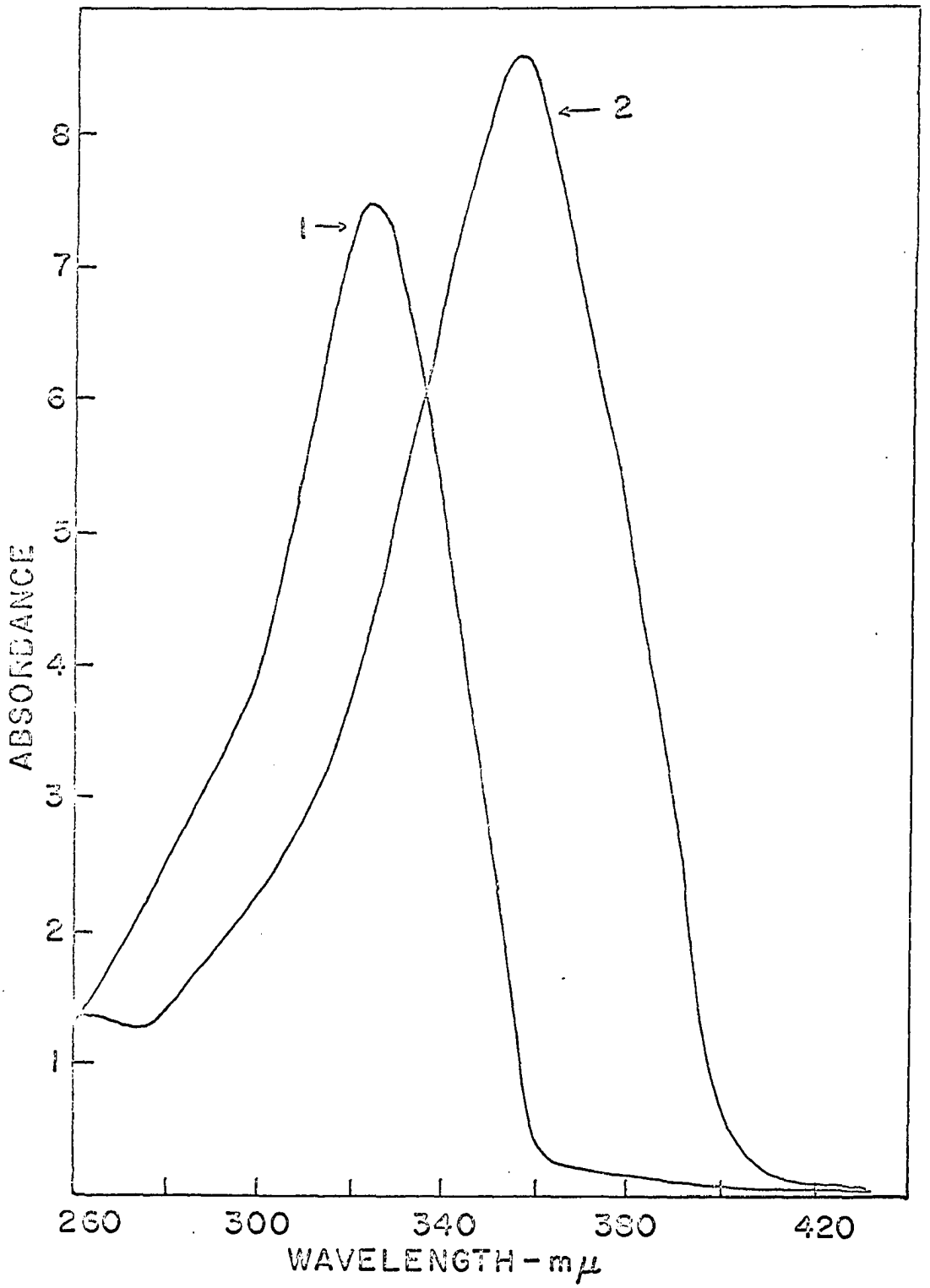

ABSORBANCE
8
7
6
5
4
3
2
1
1
2
260
300
340
380
420
WAVELENGTH - mμ

Figure 41. Absorbance of 4-methylumbelliferone as a function of pH

1. Absorbance at 320 mu
2. Absorbance at 360 mu

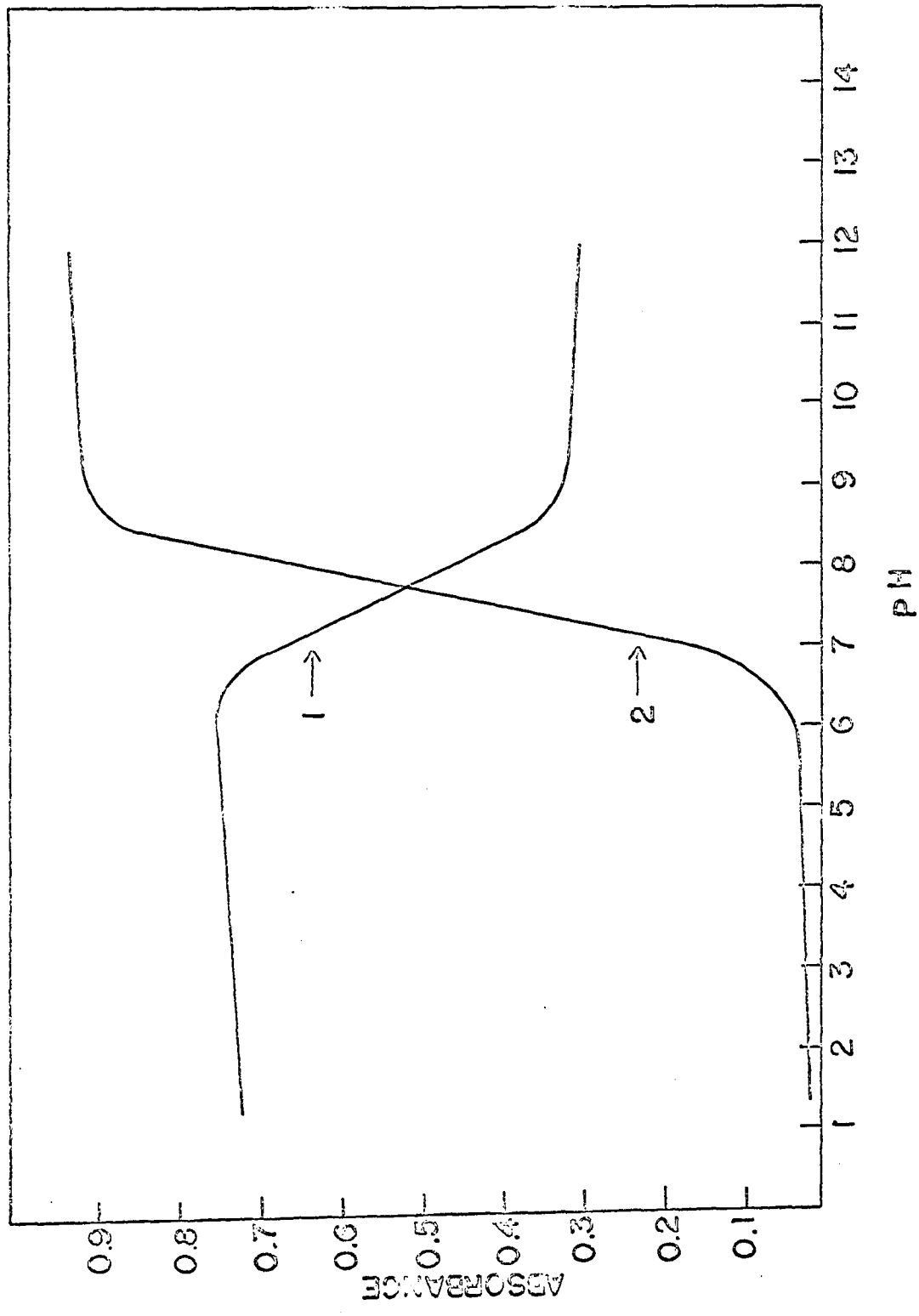

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
PH
1
2

Figure 42. Absorbance of umbelliferone as a function of pH

1. Absorbance at 325 mu
2. Absorbance at 365 mu

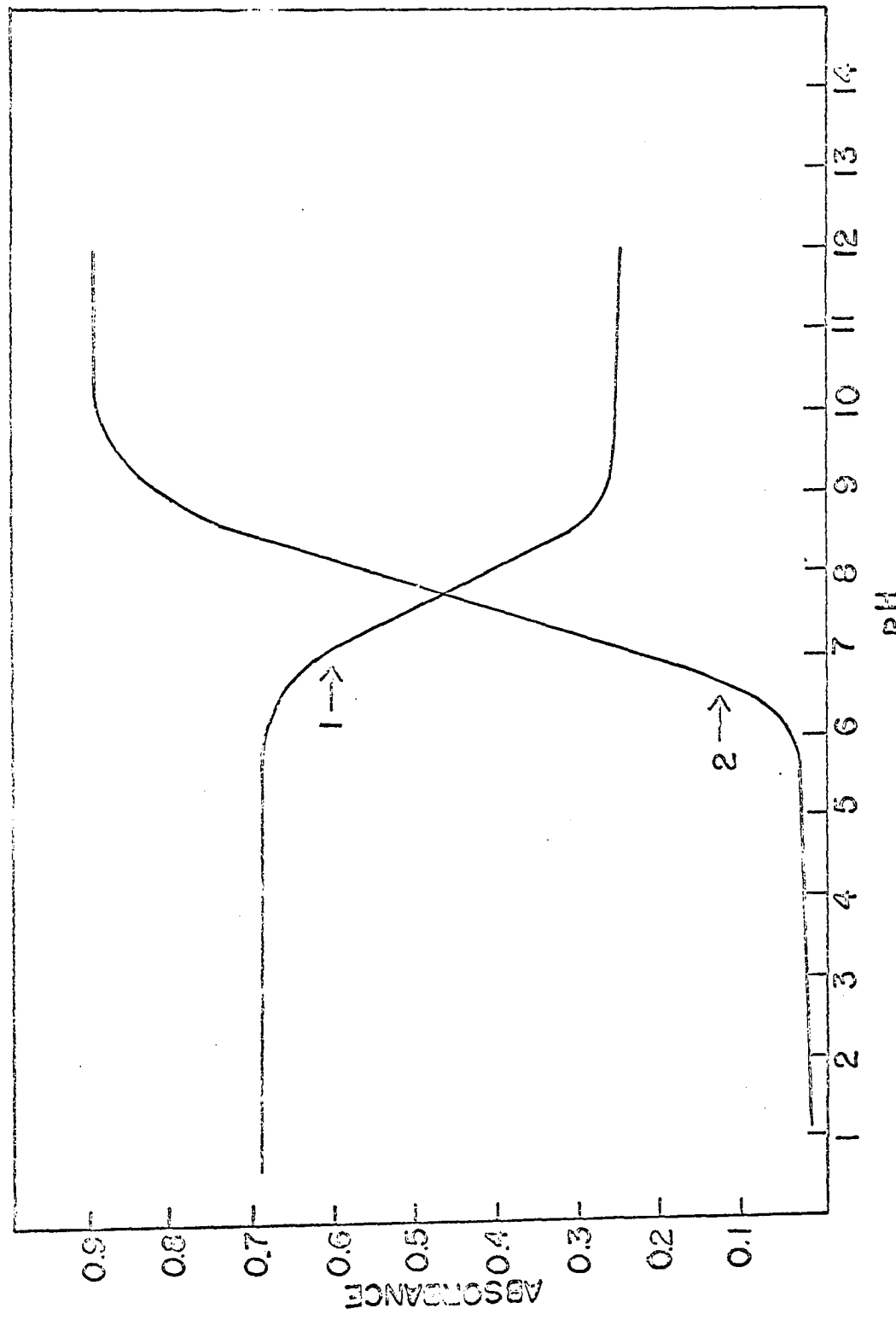

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
pH
1
2

Figure 43. Absorbance of umbelliferone-8-methyleneiminodiacetic acid as a function of pH

1. Absorbance at 325 mu
2. Absorbance at 367 mu

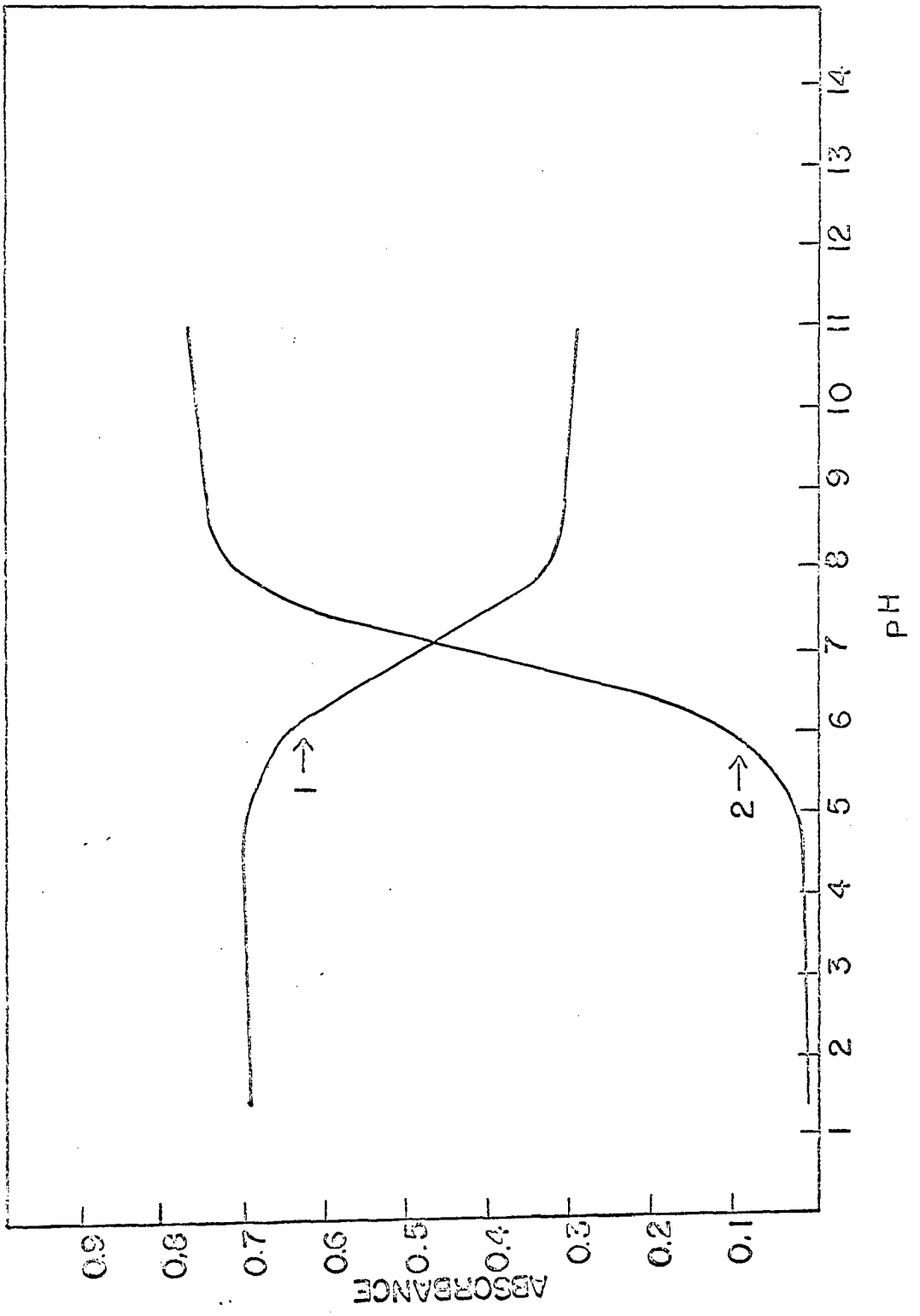

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
pH
1
2

Figure 44. Absorbance of 4-methylumbelliferone-8-methyleneglycine as a function of pH

1. Absorbance at 322 mu
2. Absorbance at 365 mu

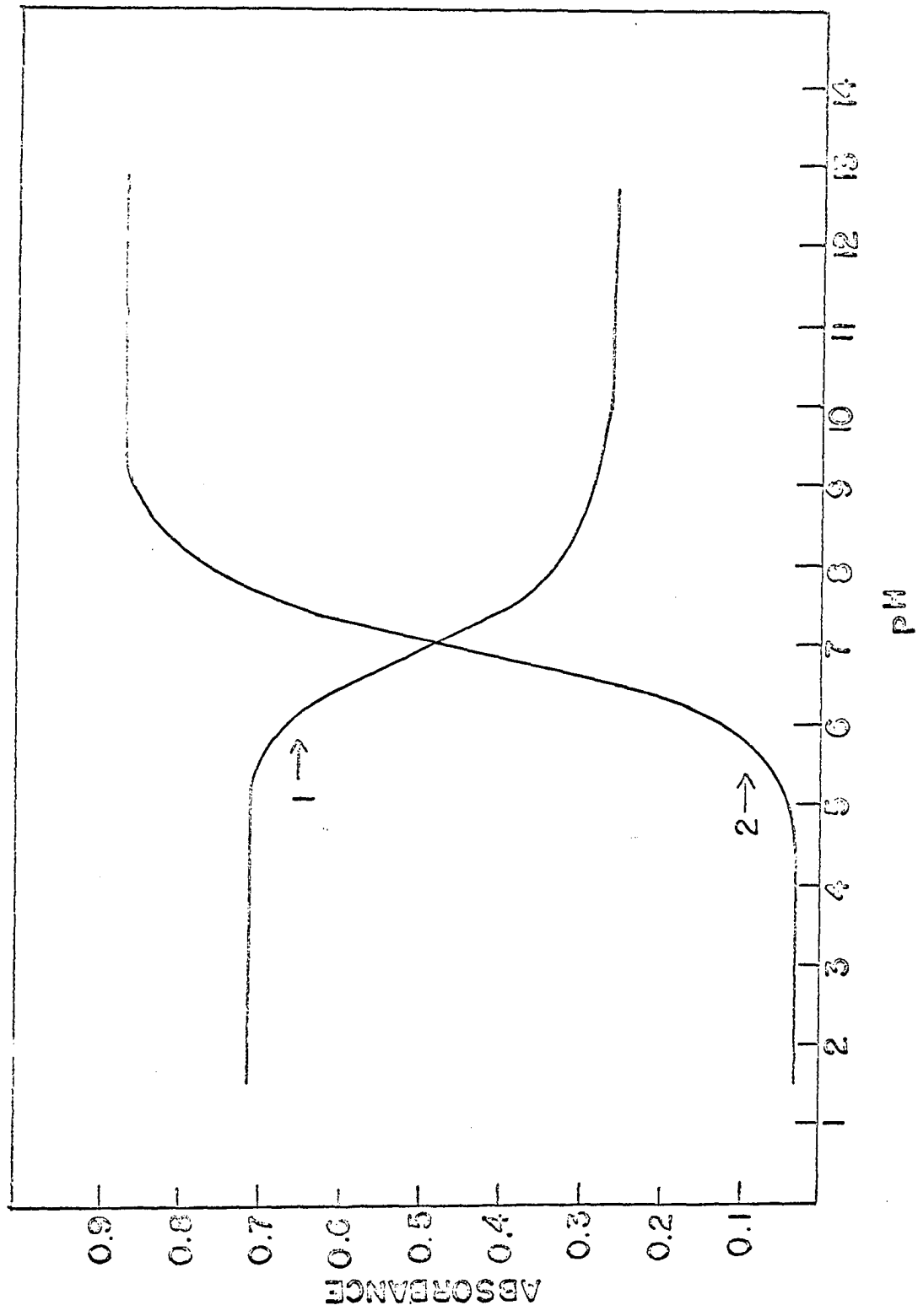

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
pH
1
2

Figure 45. Absorbance of umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O as a function of pH

1. Absorbance at 322 mu
2. Absorbance at 369 mu

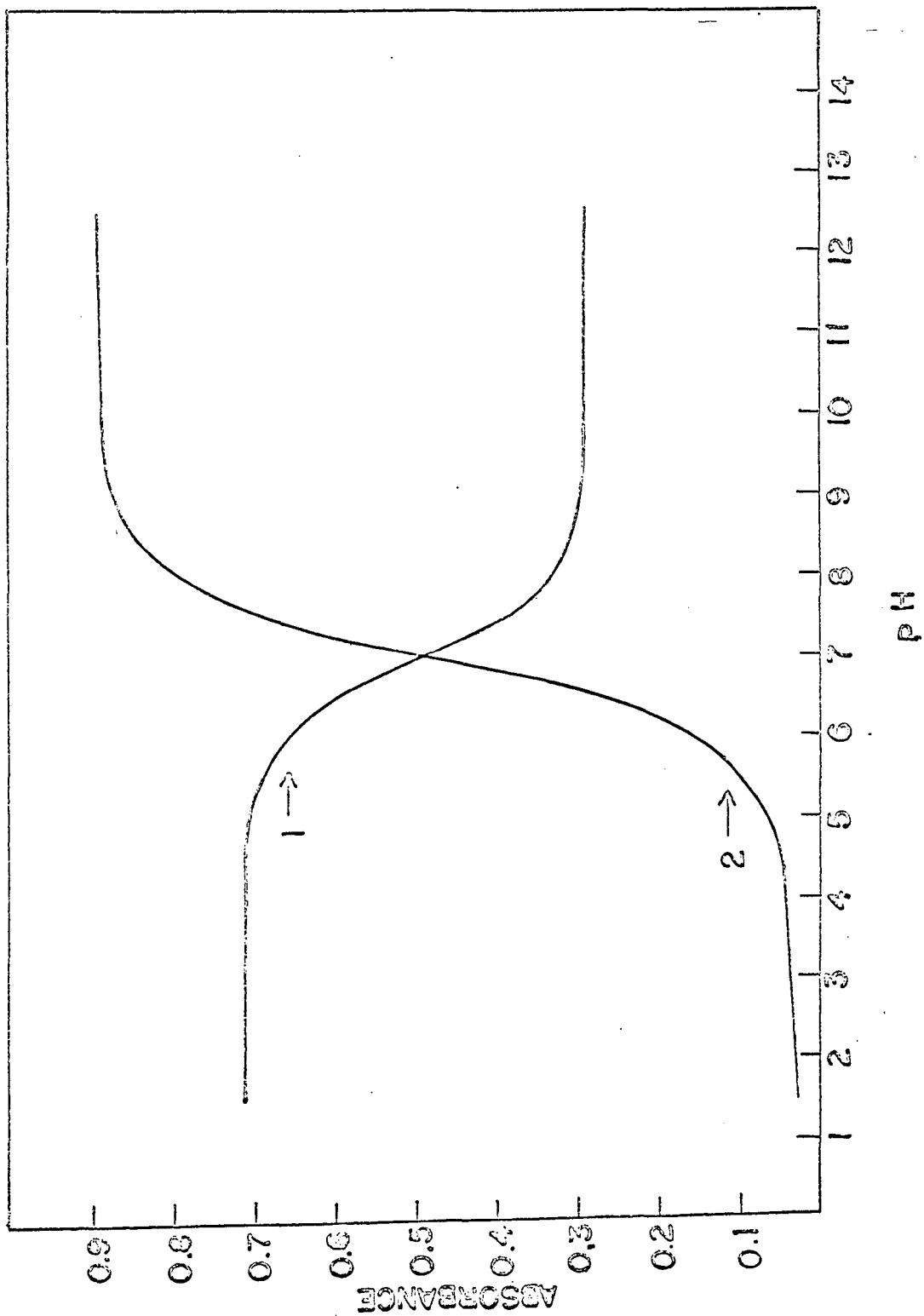

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
pH
1
2

Figure 46. Absorbance of 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O as a function of pH

1. Absorbance at 321 mu
2. Absorbance at 360 mu

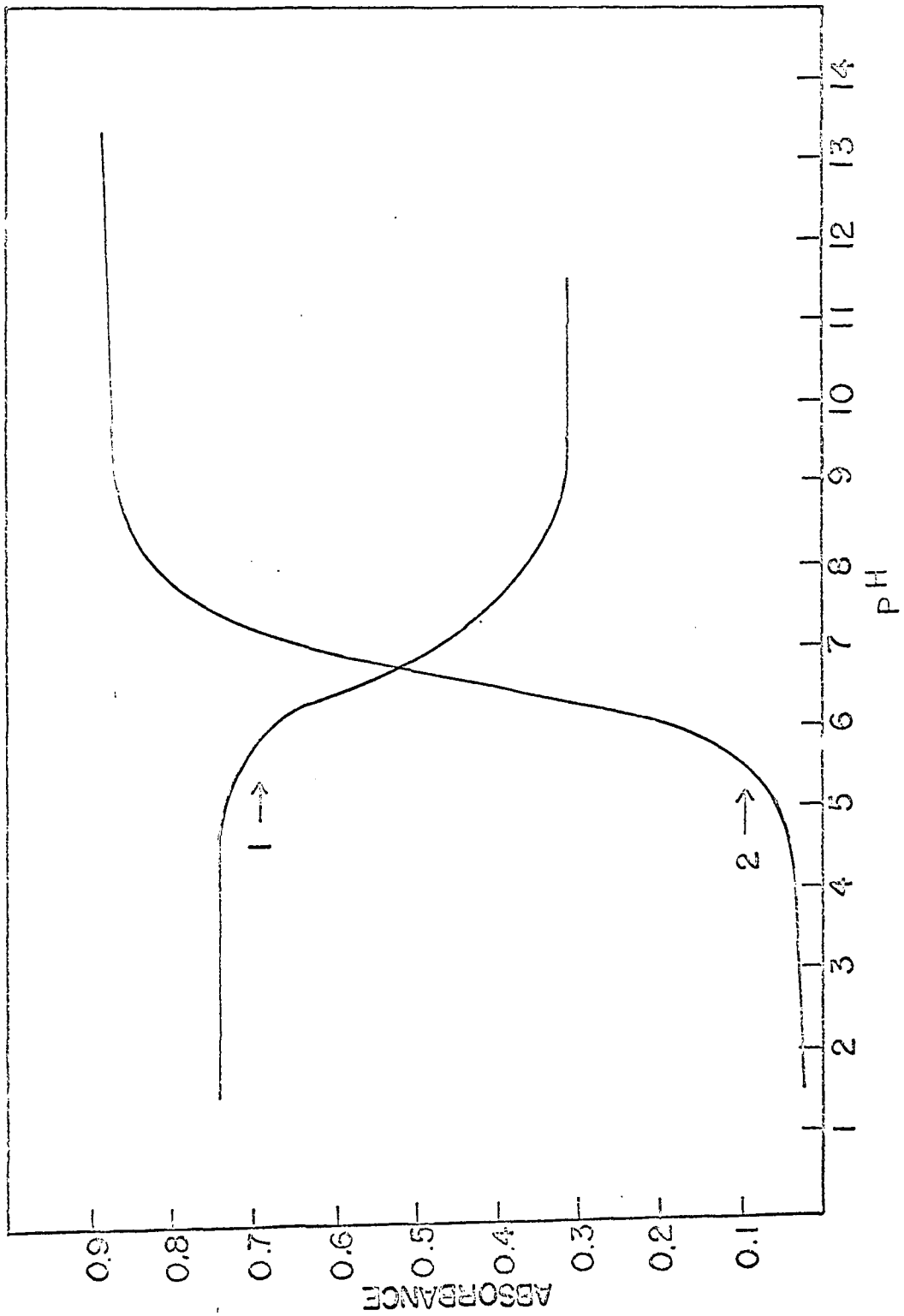

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
PH
1
2
3
4
5
6
7
8
9
10
11
12
13
14
1
2

Figure 47. Absorption spectra of 4-methylesculetin

1. At pH 4
2. At pH 9

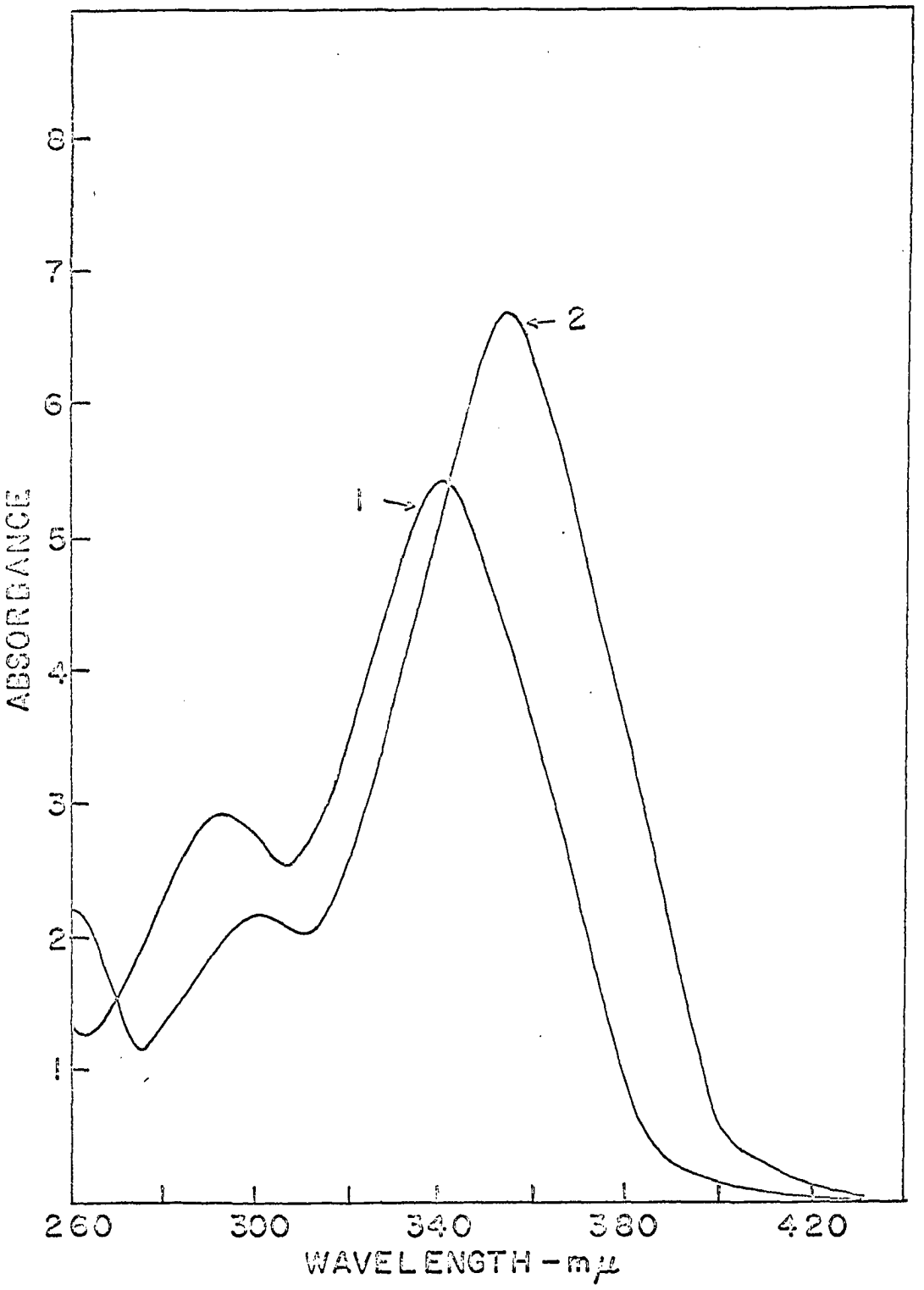
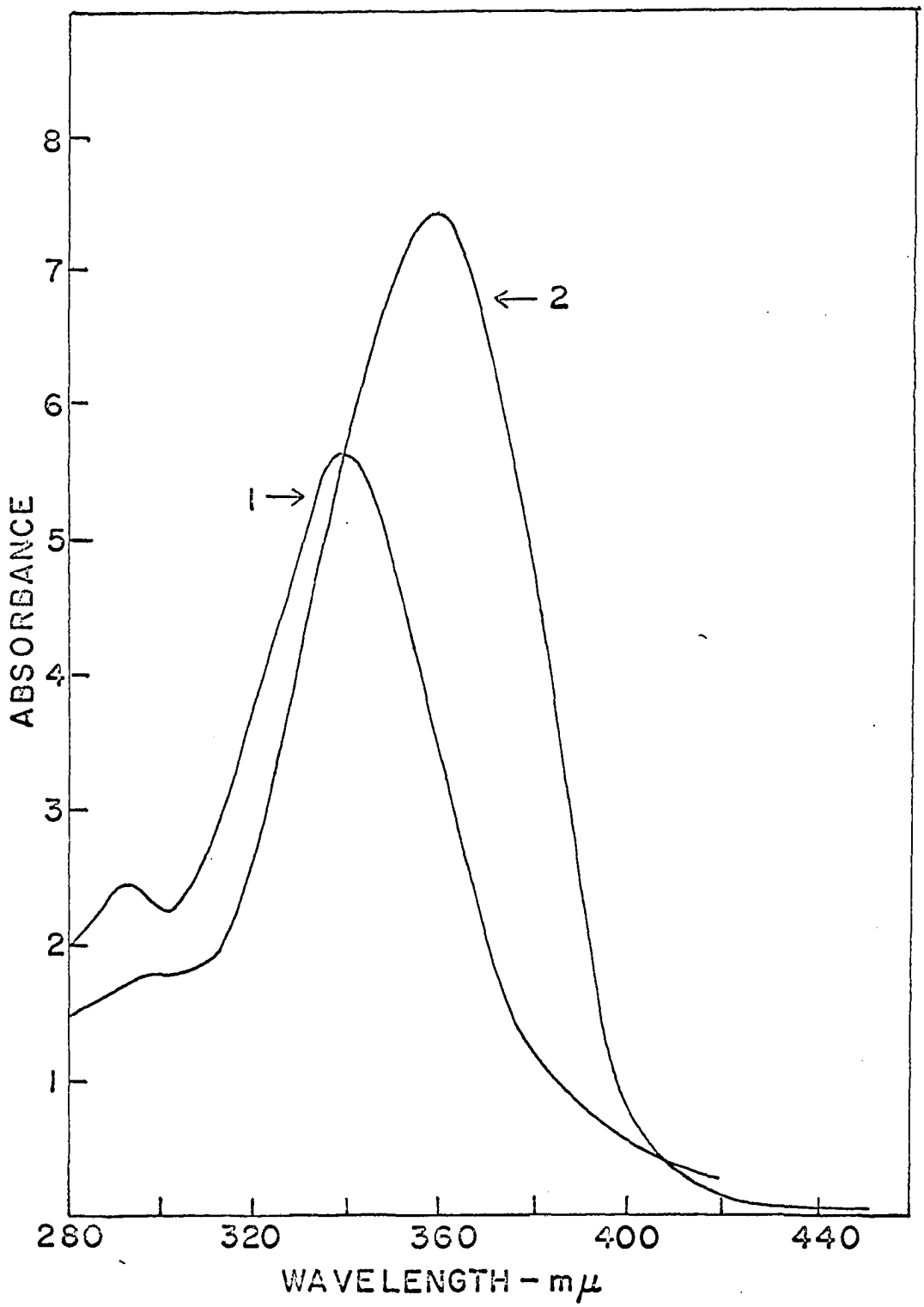

ABSORBANCE
8
7
6
5
4
3
2
1
2
1
260
300
340
380
420
WAVELENGTH - mμ

Figure 48. Absorption spectra of 4-methylesculetinmethylene-iminodiacetic acid·½ H_2O

1. At pH 4
2. At pH 9


8
7
6
5
4
3
2
1
ABSORBANCE
← 2
1 →
280
320
360
400
440
WAVELENGTH – mμ

equipped with a flow-through cell. Graphs showing the variation in absorbance with changes in pH are shown in Figures 49 and 50. The values for pK_a were determined from these graphs.

4. Determination of acid dissociation constants using fluorometric data

Graphs showing the change in fluorescence with pH for 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O are shown in Figures 22 and 23. The acid dissociation constant of the second phenolic proton was determined directly from these curves as being equal to the pH at the second inflection point.

Graphs showing the change in fluorescence with pH of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O are shown in Figures 15, 16, 17, 18 and 19. The acid dissociation constant of the ammonium group of each of the compounds was determined directly from the graphs and is equal to the pH at the second inflection point.

F. Formation Constants with Calcium and Copper

1. Apparatus and reagents

Measurements of pH were made with a Corning Model 10 pH meter equipped with a Beckman No. 40495 high alkalinity glass

Figure 49. Absorbance of 4-methylesculetin as a function of pH

1. Absorbance at 340 mu
2. Absorbance at 360 mu

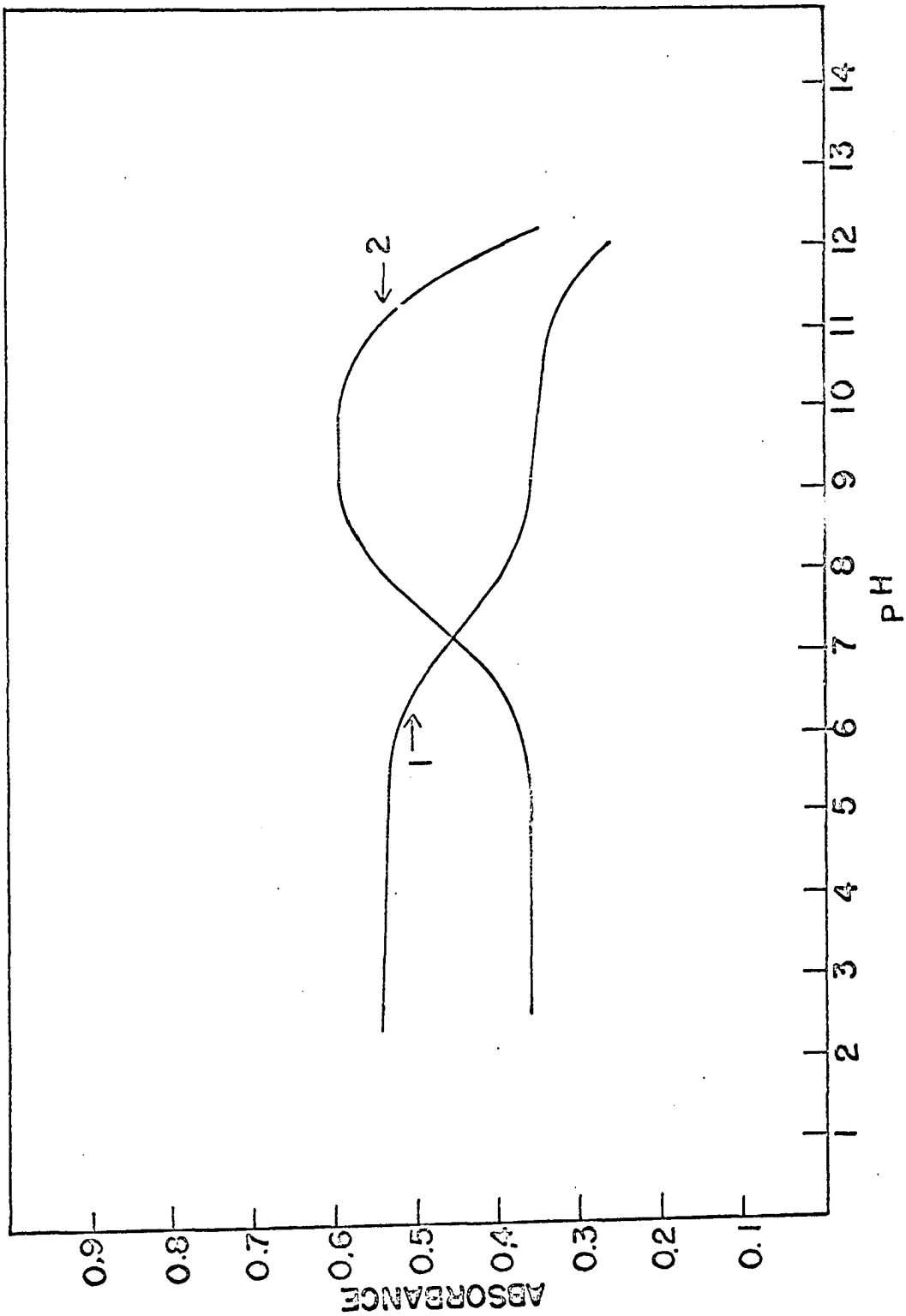
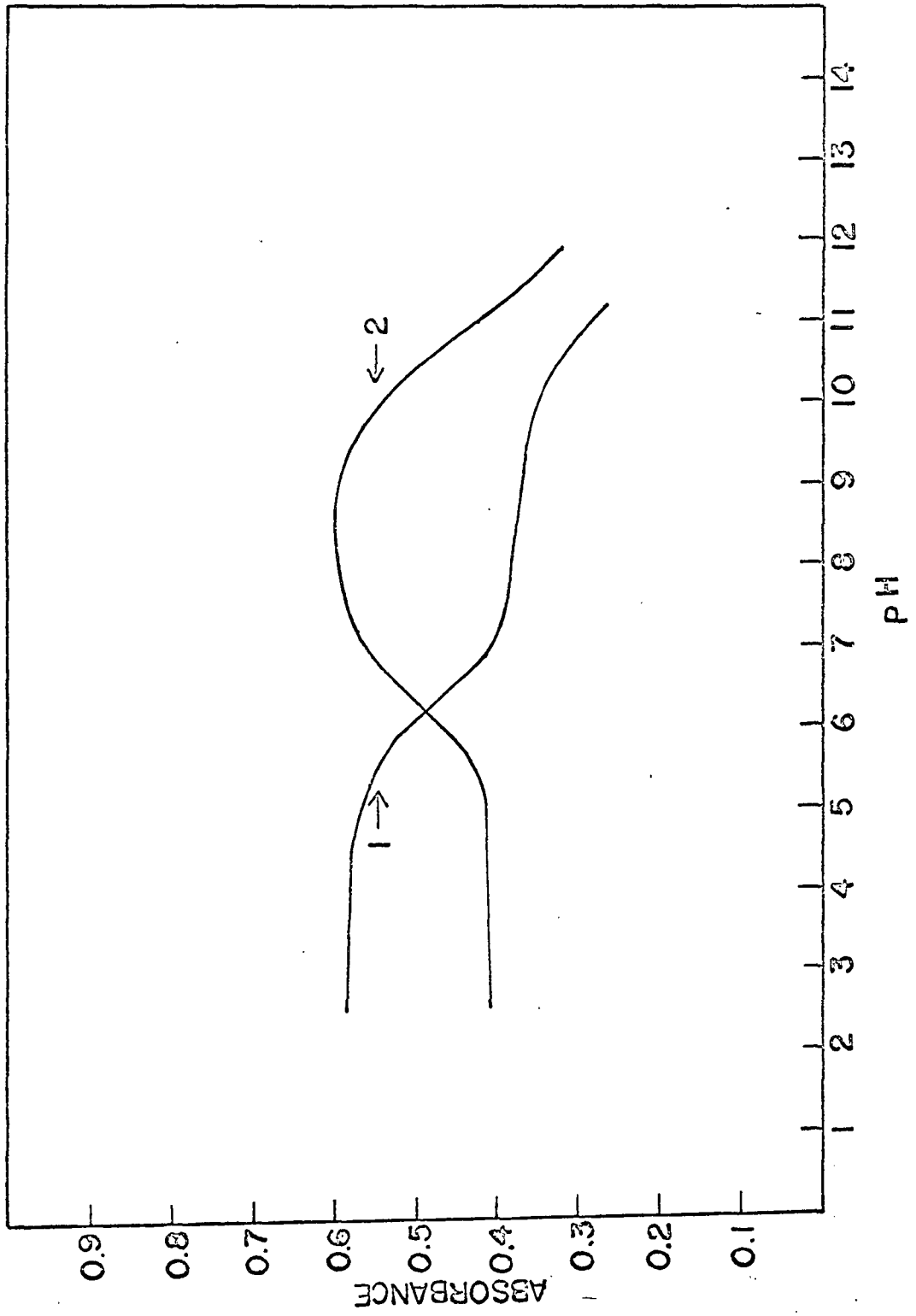

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
pH
1
2

Figure 50. Absorbance of 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O as a function of pH

1. Absorbance at 342 mu
2. Absorbance at 360 mu


ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
pH
1
2

electrode and a Beckman asbestos fiber type saturated calomel electrode.

All reagent chemicals were of reagent grade quality.

2. Determination of formation constants of calcium compounds

To determine the formation constants of the calcium compounds of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid and 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O, potentiometric titrations of mixtures containing 4.343×10^{-4} moles of compound and 0.6384 g. (4.343×10^{-3} moles) of calcium chloride dihydrate with 0.1185 M sodium hydroxide were made. These titration curves are shown in Figures 51, 52 and 53. The weight of compound used in each titration is listed in Table 4.

Table 4. Weight in grams of 4.343×10^{-4} moles of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid and 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O used in potentiometric titrations to determine the formation constants of the calcium derivatives

Compound	Weight (g.)
4-Methylumbelliferone-8-methyleneiminodiacetic acid	0.1400
Umbelliferone-8-methyleneiminodiacetic acid	0.1334
4-Methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O	0.1503

Figure 51. Titration of 4-methylumbelliferone-8-methyleneiminodiacetic acid in the presence of a ten-fold excess of metal ion with sodium hydroxide. Solvent: 0.1 M potassium chloride; sodium hydroxide: 0.1185 N in 0.1 M potassium chloride; sample: 0.1400 g

1. Ten-fold excess of cupric chloride dihydrate: 0.7403 g.

2. Ten-fold excess of calcium chloride dihydrate: 0.6384 g.

n Moles of base added per mole of acid

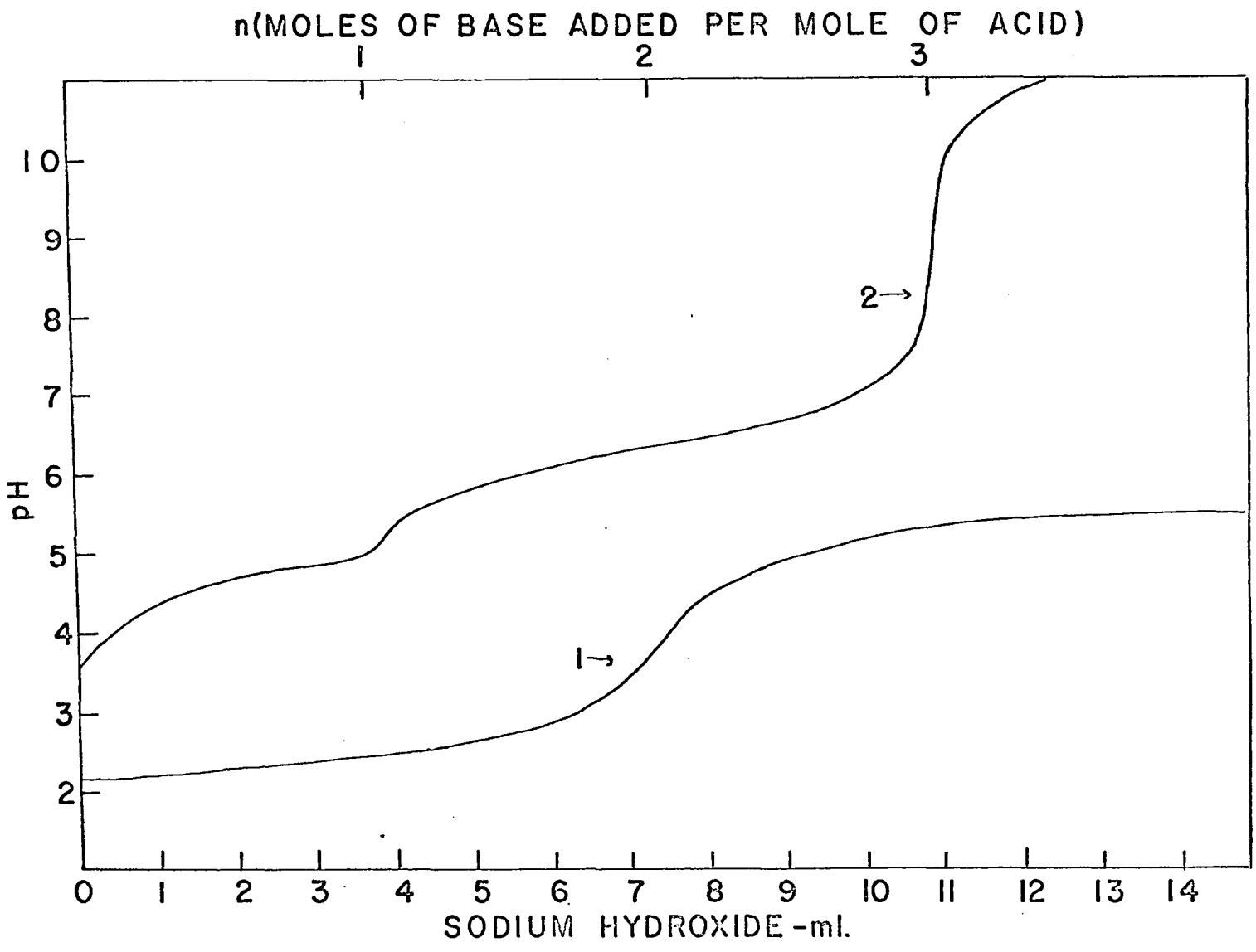

n(MOLES OF BASE ADDED PER MOLE OF ACID)
1
2
3
pH
10
9
8
7
6
5
4
3
2
2→
1→
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
SODIUM HYDROXIDE - ml.

Figure 52. Titration of umbelliferone-8-methyleneiminodiacetic acid in the presence of a ten-fold excess of metal ion with sodium hydroxide. Solvent: 0.1 M potassium chloride; sodium hydroxide: 0.1185 N in 0.1 M potassium chloride; sample: 0.1334 g.

1. Ten-fold excess of cupric chloride dihydrate: 0.7403 g.

2. Ten-fold excess of calcium chloride dihydrate: 0.6384 g.

n Moles of base added per mole of acid

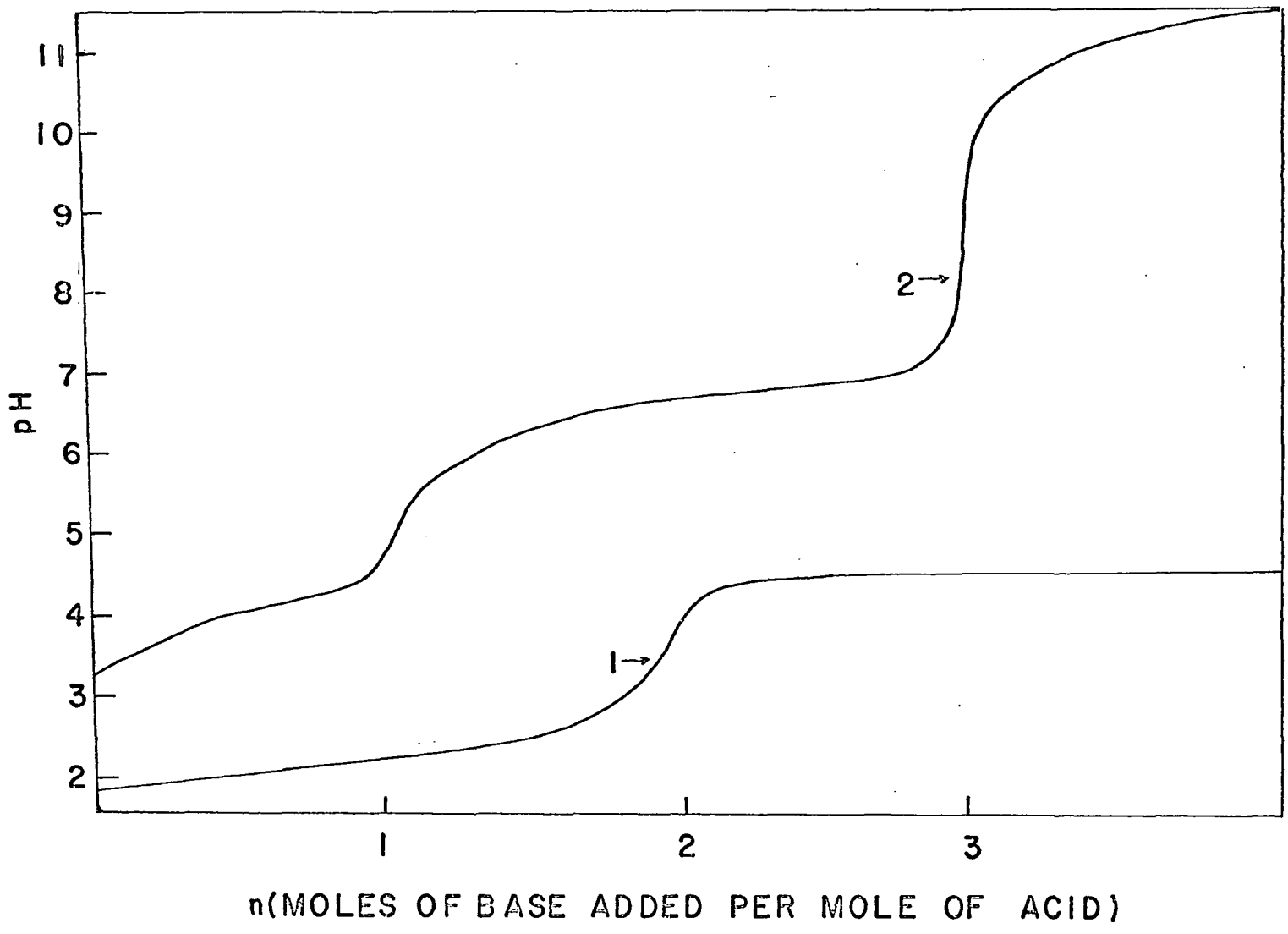
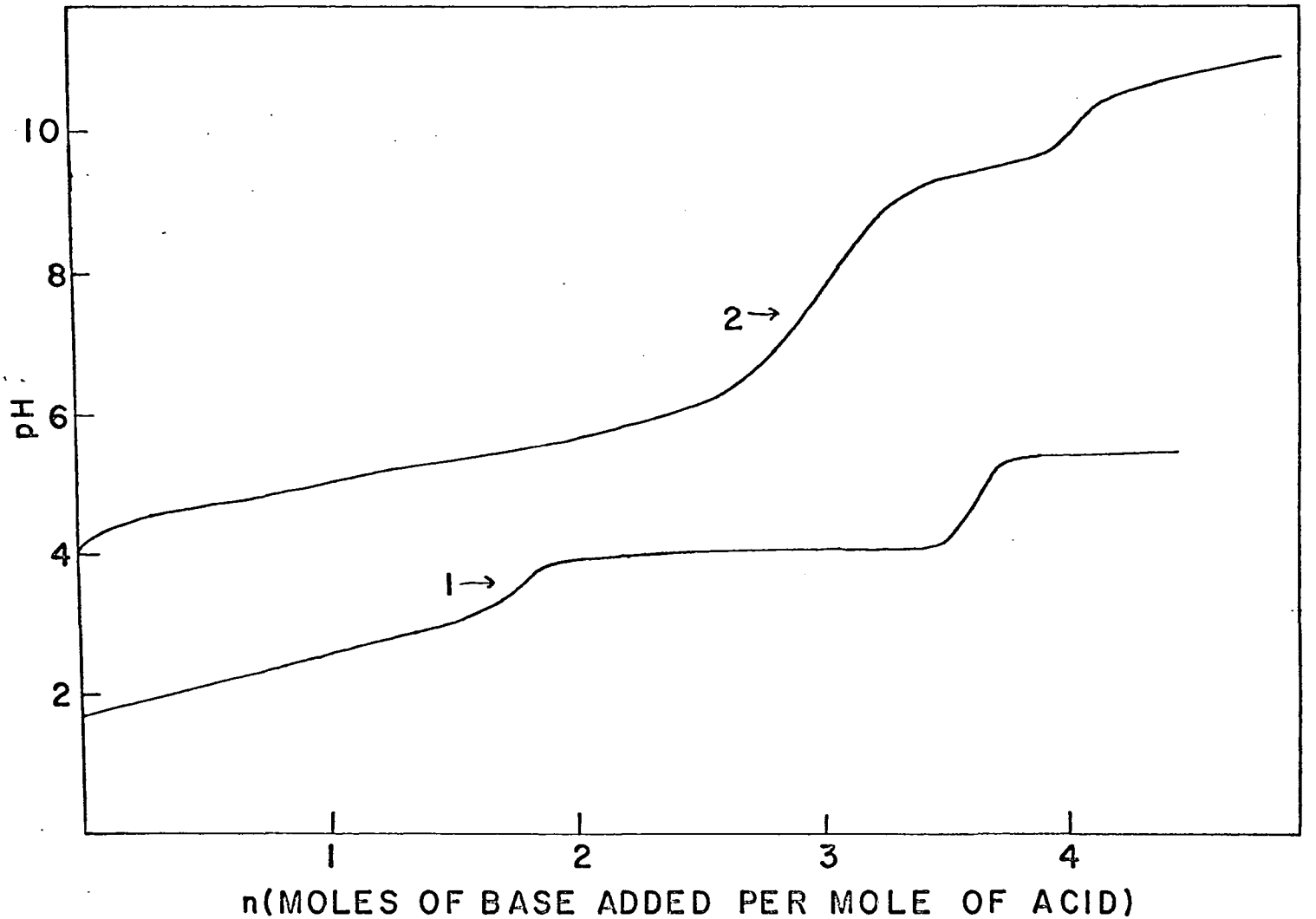

11
10
9
8
7
6
5
4
3
2
pH
2
1
1
2
3
n(MOLES OF BASE ADDED PER MOLE OF ACID)

Figure 53. Titration of 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O in the presence of a ten-fold excess of metal ion with sodium hydroxide. Solvent: 0.1 M potassium chloride; sodium hydroxide: 0.1185 N in 0.1 M potassium chloride; sample: 0.1503 g.

1. Ten-fold excess of cupric chloride dihydrate: 0.7403 g.

2. Ten-fold excess of calcium chloride dihydrate: 0.6384 g.

n Moles of base added per mole of acid


10
8
6
4
2
pH
2→
1→
1
2
3
4
n(MOLES OF BASE ADDED PER MOLE OF ACID)

3. Determination of formation constants of copper compounds

To determine the formation constants of the copper compounds of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylesculetin-methyleneiminodiacetic acid·½ H_2O, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O potentiometric titrations of mixtures containing 4.343×10^{-4} moles of compound and 0.7405 g. (4.343×10^{-3} moles) of cupric chloride dihydrate with 0.1185 M sodium hydroxide were performed. These titration curves are shown in Figures 51, 52, 53, 54, 55 and 56. The weight of compound used in each titration is listed in Table 5.

Table 5. Weight in grams of 4.343×10^{-4} moles of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylesculetinmethyleneiminodiacetic·½ H_2O, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O, 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O used in potentiometric titrations to determine the formation constants of the copper derivatives

Compound	Weight (g.)
4-Methylumbelliferone-8-methyleneiminodiacetic acid	0.1400
Umbelliferone-8-methyleneiminodiacetic acid	0.1334
4-Methylesculetinmethyleneiminodiacetic acid·½ H_2O	0.1503
4-Methylumbelliferone-8-methyleneglycine	0.1142
Umbelliferone-8-methyleneglycine·½ H_2O	0.1121
4-Methylumbelliferone-8-methylenesarcosine·1½ H_2O	0.1321

Figure 54. Titration of 4-methylumbelliferone-8-methyleneglycine in the presence of a ten-fold excess of metal ion with sodium hydroxide. Solvent: 0.1 M potassium chloride; sodium hydroxide: 0.1185 N in 0.1 M potassium chloride; sample: 0.1142 g.; cupric chloride dihydrate: 0.7403 g.

n Moles of base added per mole of acid

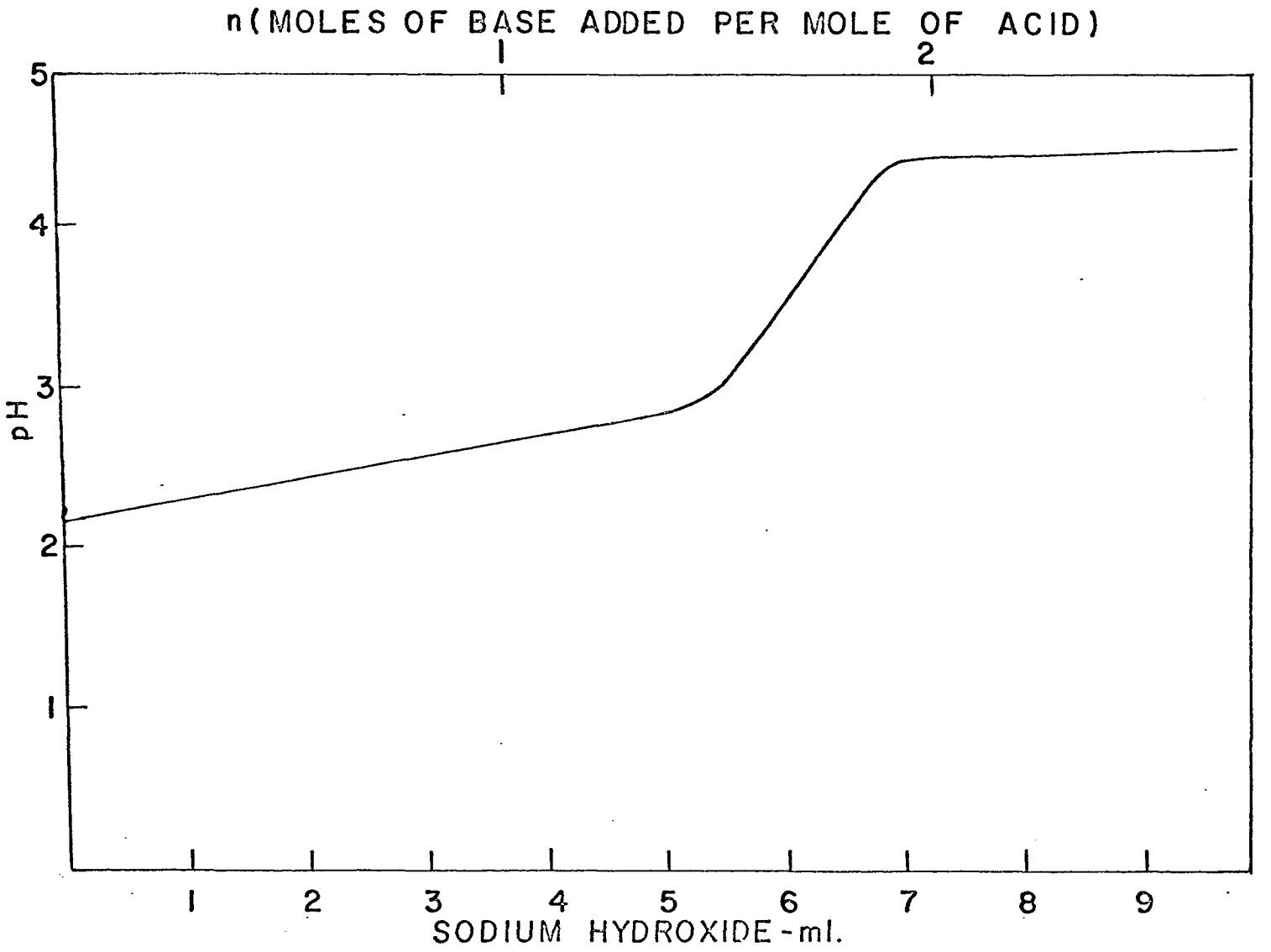

n(MOLES OF BASE ADDED PER MOLE OF ACID)
1
2
5
4
3
2
1
pH
1
2
3
4
5
6
7
8
9
SODIUM HYDROXIDE - ml.

Figure 55. Titration of umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O in the presence of a ten-fold excess of metal ion with sodium hydroxide. Solvent: 0.1 M potassium chloride; sodium hydroxide: 0.1185 N in 0.1 M potassium chloride; sample: 0.1121 g.; cupric chloride dihydrate: 0.7403 g.

n Moles of base added per mole of acid

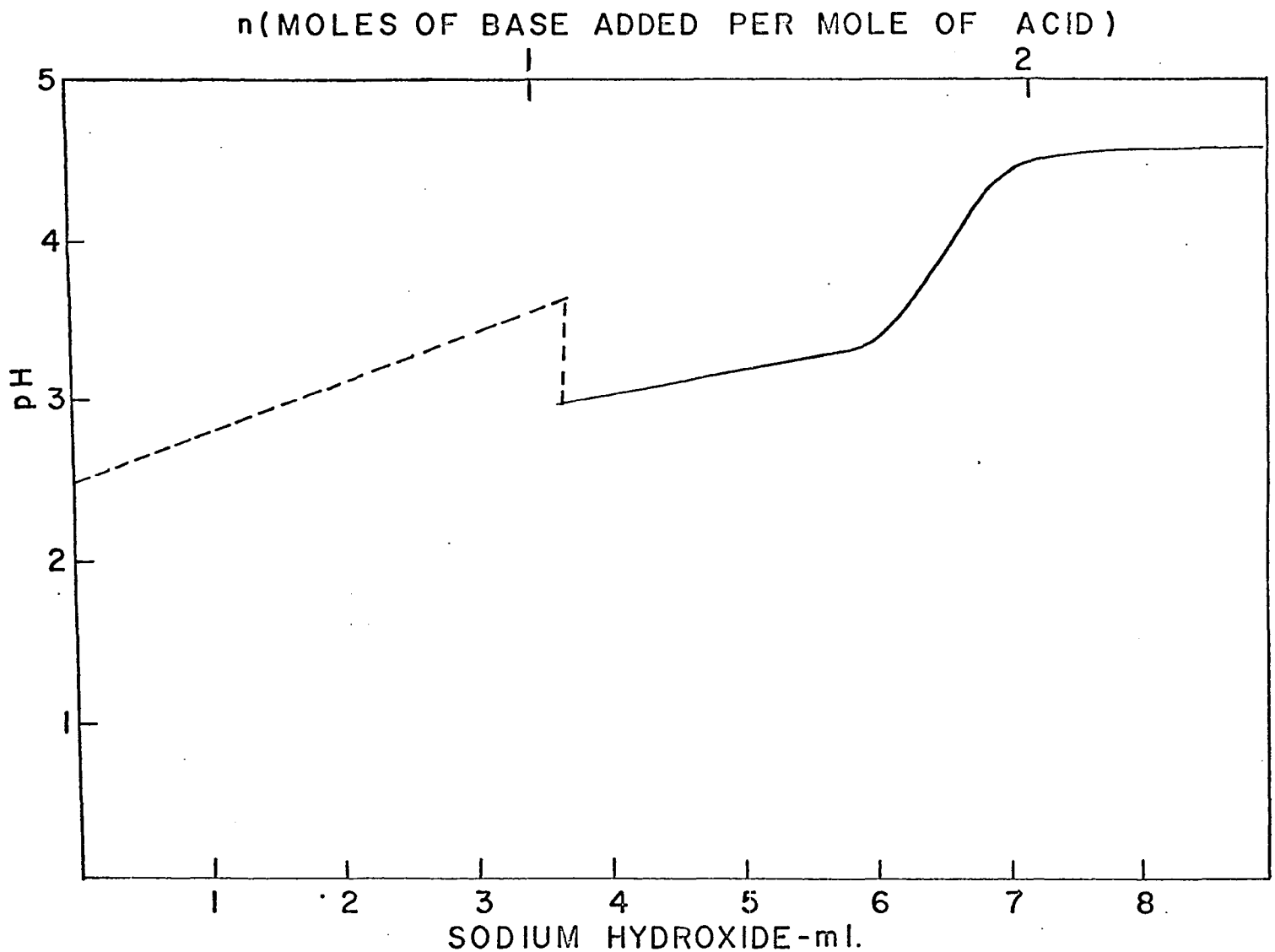
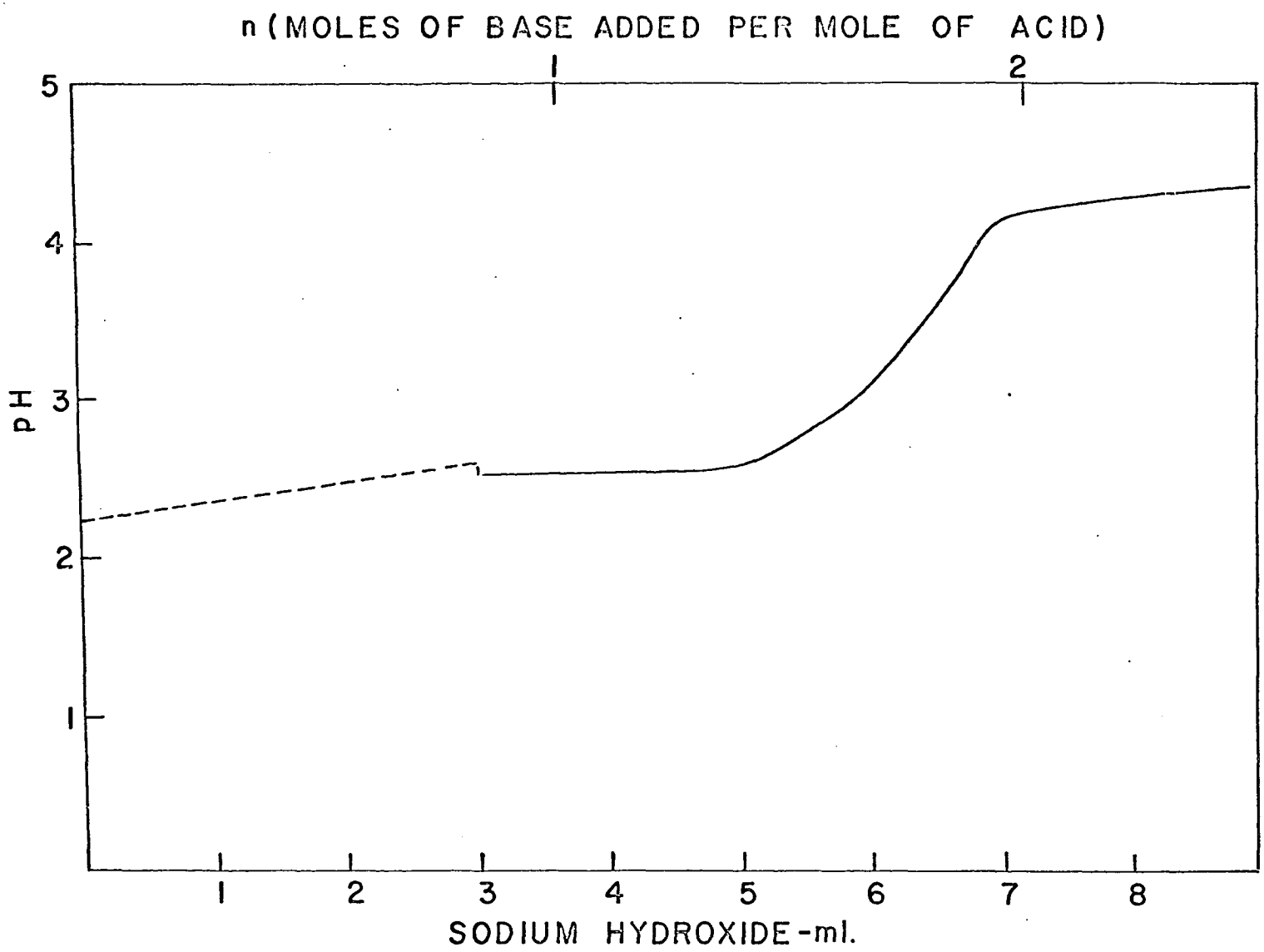

n(MOLES OF BASE ADDED PER MOLE OF ACID)
1
2
5
4
3
2
1
pH
1
2
3
4
5
6
7
8
SODIUM HYDROXIDE-ml.

Figure 56. Titration of 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O in the presence of a ten-fold excess of metal ion with sodium hydroxide. Solvent: 0.1 M potassium chloride; sodium hydroxide; 0.1185 N in 0.1 M potassium chloride; sample: 0.1321 g.; cupric chloride dihydrate; 0.7403 g.

n Moles of base added per mole of acid


n (MOLES OF BASE ADDED PER MOLE OF ACID)
1
2
5
4
3
2
1
pH
1
2
3
4
5
6
7
8
SODIUM HYDROXIDE - ml.

III. RESULTS AND DISCUSSION

A. Synthesis

Conditions under which the Mannich reaction was performed depended upon the amino acid being condensed with the hydroxycoumarin and formaldehyde. The conditions found best suited for the individual syntheses are given in Table 6.

Table.6. Conditions used in the Mannich condensation of 4-methylumbelliferone, umbelliferone, and 4-methylesculetin with formaldehyde and iminodiacetic acid, glycine and sarcosine

Reactants	Solvent	Temperature	pH of recrystallization medium
Iminodiacetic acid, formaldehyde and			
Umbelliferone	glacial acetic acid	70-75°	3.1
4-Methylesculetin	glacial acetic acid	70-75°	3.1
Glycine, formaldehyde and			
4-Methylumbelliferone	water	70-75°	4.7
Umbelliferone	water	70-75°	4.7
Sarcosine, formaldehyde and			
4-Methylumbelliferone	water	90-95°	4.5

B. Neutralization Equivalent

In the potentiometric titration of the Mannich condensation product of umbelliferone, formaldehyde and iminodiacetic acid two end-points were observed (Figure 1). The equivalent weight calculated from the volume of alkali used to reach the first end-point was 308.4, and the equivalent weight calculated from the volume of alkali used to reach the second end-point was 308.2. These values agree well with the molecular weight of an umbelliferone molecule bearing one methyleneiminodiacetic acid group, 307.25. Assuming the compound to be monomolecular, one hydrogen atom is replaced in each step of the neutralization.

The potentiometric titration of the Mannich condensation product of 4-methylesculetin, formaldehyde and iminodiacetic acid shows two end-points (Figure 2). The first break, however, occurred before all of the compound had dissolved and varied with the rate of addition of titrant. The second break corresponds to the neutralization of the second replaceable proton and the equivalent weight calculated from the volume of alkali used to reach this end-point was 349.1. This value agrees well with the molecular weight of a 4-methylesculetin molecule bearing one methyleneiminodiacetic acid group and one half of a molecule of water, 346.24.

One end-point was observed in the potentiometric titrations of the products obtained from the Mannich condensations

of glycine and formaldehyde with 4-methylumbelliferone and with umbelliferone and in the potentiometric titration of the product obtained from the Mannich condensation of sarcosine, formaldehyde and 4-methylumbelliferone. The equivalent weights of the compounds, calculated from the volume of alkali used to reach the end-point, are listed in Table 7.

Table 7. Equivalent weight of the products obtained from the Mannich condensation of 4-methylumbelliferone and umbelliferone with glycine and formaldehyde and of the product obtained from the Mannich condensation of 4-methylumbelliferone, sarcosine and formaldehyde calculated using the volume of alkali needed to reach the end-point

Compound	Equivalent weight	
	Found	Calculated*
Condensation product of 4-Methylumbelliferone, formaldehyde and glycine	264.9	263.1
Umbelliferone, formaldehyde and glycine	256.5	258.3
4-Methylumbelliferone, formaldehyde and sarcosine	303.6	304.3

*One replaceable hydrogen atom.

The experimental values agree well with the following theoretical values: the molecular weight of a 4-methylumbelliferone molecule bearing one methyleneglycine group, 263.1; the molecular weight of an umbelliferone molecule bearing one methyleneglycine group and one-half of a molecule of

water, 258.3; the molecular weight of a 4-methylumbelliferone molecule bearing one methylenesarcosine group and one and one-half molecules of water, 304.3. Elemental analysis confirms the values obtained from the potentiometric titration curves.

C. Structure

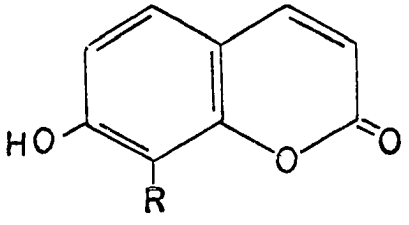
The position at which methyleneiminodiacetic acid enters umbelliferone, the position at which methyleneglycine enters 4-methylumbelliferone and umbelliferone and the position at which methylenesarcosine enters 4-methylumbelliferone have been determined through interpretation of NMR spectra. The peak positions and proton integrations of the NMR spectra are listed in Table 8.

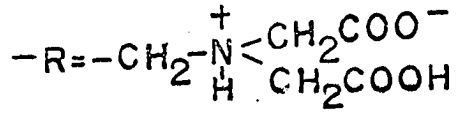
The only two positions at which the methyleneamino acid group could enter the parent molecules of 4-methylumbelliferone and umbelliferone are position 6 or position 8. If condensation occurred at position 6, the two aromatic protons would be para to each other and would exhibit an AB splitting constant of approximately 1 cycle per second or less. If, on the other hand, condensation occurred at position 8 the aromatic protons would be ortho to each other and would exhibit an AB splitting constant of approximately 10 cycles per second. The splitting constant exhibited by the aromatic protons was found to be nine cycles per second for each of the above compounds studied. Therefore, the methyleneamino acid group

Table 8. The peak positions, the proton integrations and the group bearing the proton obtained from the NMR spectra of umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O

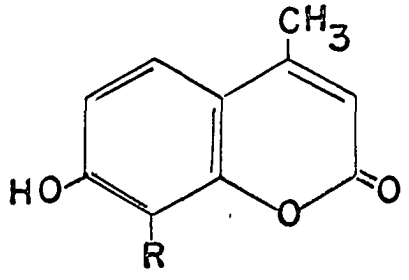
Compound	Peak position (ppm)	Proton integration	Group bearing protons
Umbelliferone-8-methyleneimino-diacetic acid	3.79	4	$-N-CH_2-COO^-$
	4.42	2	$-N-CH_2-Ar$
	5.95	1	olefinic proton
	6.63	1	aromatic proton
	7.30	1	aromatic proton
	7.70	1	olefinic proton
4-Methylumbelliferone-8-methyleneglycine	2.15	3	$Ar-CH_3$
	3.51	2	$-N-CH_2-COO^-$
	4.07	2	$-N-CH_2-Ar$
	5.72	1	olefinic proton
	6.60	1	aromatic proton
	7.20	1	aromatic proton
Umbelliferone-8-methyleneglycine·½ H_2O	3.52	2	$-N-CH_2-COO^-$
	4.05	2	$-N-CH_2-Ar$
	5.82	1	olefinic proton
	6.57	1	aromatic proton
	7.11	1	aromatic proton
	7.53	1	olefinic proton
4-Methylumbelliferone-8-methylenesarcosine·1½ H_2O	2.08	3	$Ar-CH_3$
	2.70	3	$-N-CH_3$
	3.67	2	$-N-CH_2-COO^-$
	4.15	2	$-N-CH_2-Ar$
	5.68	1	olefinic proton
	6.62	1	aromatic proton
	7.16	1	aromatic proton

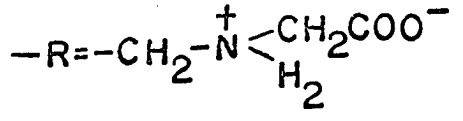
enters 4-methylumbelliferone and umbelliferone at position 8. The structure of the condensation product of umbelliferone, formaldehyde and iminodiacetic acid is



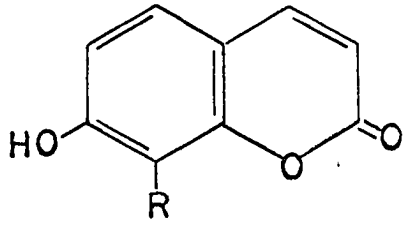


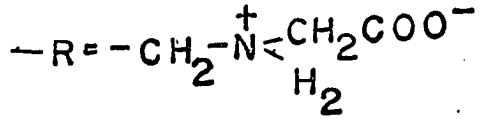
The structure of the condensation product of 4-methylumbelliferone, formaldehyde and glycine is



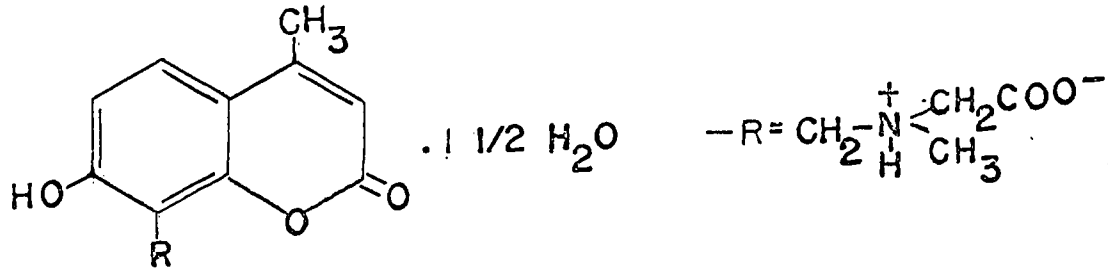


The structure of the condensation product of umbelliferone, formaldehyde and glycine is



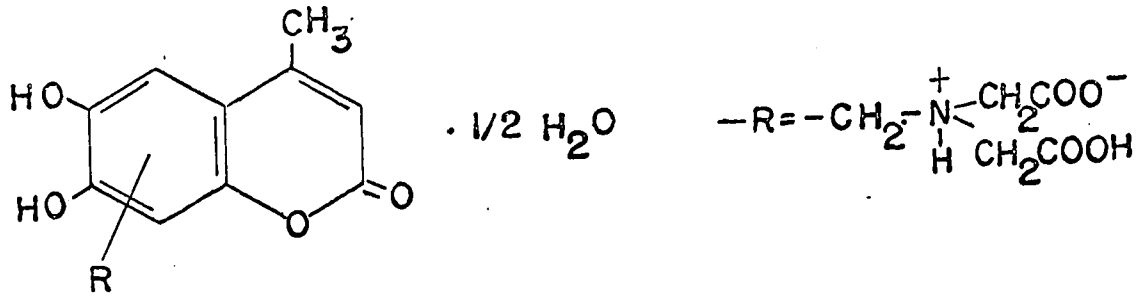


The structure of the condensation product of 4-methylumbelliferone, formaldehyde and sarcosine is



The peak positions and the proton integrations obtained from the NMR spectra of the condensation product of 4-methylesculetin, formaldehyde and iminodiacetic acid are 2.32 ppm (3), Ar-CH_3; 2.72 ppm (4), -N-CH_2COO^-; 3.01 ppm (2), -N-CH_2-Ar; 6.10 ppm (1), olefinic proton and 7.00 ppm (1), aromatic proton.

The position of attachment of methyleneiminodiacetic acid can not be determined from the data and the structure of the condensation product of 4-methylesculetin, formaldehyde and iminodiacetic acid must be written



D. Fluorescence Study

1. Effect of pH on fluorescence

The fluorescence excitation spectra of 4-methylumbelliferone (Figure 6), umbelliferone (Figure 7), 4-methylumbelliferone-8-methyleneiminodiacetic acid (Figure 8), umbelliferone-8-methyleneiminodiacetic acid (Figure 9), 4-methylumbelliferone-8-methyleneglycine (Figure 10), umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O (Figure 11) and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O (Figure 12) show one band, the wavelength of maximum excitation shifting from approximately 330 mu in acid solution to approximately 360 mu in alkaline solution. The fluorescence emission spectra also show one band with maximum intensity at a wavelength of approximately 455 mu, which does not shift with change in pH. The wavelength of maximum fluorescence excitation in acid and alkaline solution and the wavelength of maximum fluorescence emission of the above compounds are listed in Table 9.

The intensity of the emitted light varies with pH, the intensity of fluorescence at the emission maxima and at the excitation maxima in acid and alkaline solutions were measured (Figures 13, 14, 15, 16, 17, 18 and 19). At approximately 330 mu a fluorescence plateau is observed in all of the compounds studied. This plateau extends over a range of approximately 3 pH units. Umbelliferone and 4-methylumbelliferone, the parent molecules of the compounds under study,

Table 9. Wavelength of maximum fluorescence excitation in acid and alkaline solution and wavelength of maximum fluorescence emission of 4-methylumbelliferone, umbelliferone, 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O

Compound	Fluorescence excitation maximum		Fluorescence emission maximum
	Acid solution mu	Alkaline solution mu	mu
4-methylumbelliferone	328 ± 5	365 ± 5	455 ± 5
umbelliferone	330 ± 5	370 ± 5	460 ± 5
4-methylumbelliferone-8-methyleneiminodiacetic acid	330 ± 5	360 ± 5	455 ± 5
umbelliferone-8-methyleneiminodiacetic acid	328 ± 5	370 ± 5	455 ± 5
4-methylumbelliferone-8-methyleneglycine	328 ± 5	366 ± 5	450 ± 5
umbelliferone-8-methyleneglycine·½ H_2O	330 ± 5	370 ± 5	455 ± 5
4-methylumbelliferone-8-methylenesarcosine·1½ H_2O	328 ± 5	365 ± 5	448 ± 5

exhibit another plateau at approximately 370 mu, however, their amino acid derivatives all exhibit a maximum at approximately 370 mu. In the case of 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic the maximum is observed at pH 9.1 while in the case of 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O the maximum is observed at pH 8.5. The maxima are attributed to the neutralization of the ammonium ion which is accompanied by a decrease in fluorescence. The shift of the fluorescence excitation wavelength is attributed to the ionization of the phenolic proton.

The fluorescence data of 4-methylumbelliferone, umbelliferone and their amino acid derivatives was obtained in buffered solutions of constant ionic strength. The presence of ortho phenolic groups in 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O gave rise to serious buffer effects when boric acid buffers were employed. To circumvent the undesirable effects of buffer systems the intensity of emitted light versus pH was studied by titrating 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O with 0.2 M potassium hydroxide and monitoring the change in fluorescence with pH on a Turner Model 10 Fluorometer equipped with a flow through cell.

4-Methylesculetin and 4-methylesculetinmethyleneimino-

diacetic acid are non-fluorescent in acid solution. The fluorescence excitation spectra of 4-methylesculetin (Figure 20) and 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O (Figure 21) show one band in alakline solution and the fluorescence emission spectra also show one band. The wavelength of maximum fluorescence excitation and fluorescence emission of 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O are listed in Table 10.

Table 10. Wavelength of maximum fluorescence excitation and emission of 4-methylesculetin and 4-methylesculetinmethylamineiminodiacetic acid·½ H_2O

Compound	Fluorescence maximum Excitation	Emission
4-Methylesculetin	370 ± 5	460 ± 5
4-Methylesculetin-methyleneiminodiacetic acid·½ H_2O	369 ± 5	459 ± 5

The variation of intensity of emitted light with pH of 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O is shown in Figures 22 and 23. The first inflection point is caused by the ionization of one phenolic proton while the second inflection point is attributed to the neutralization of the second phenolic proton.

2. Effect of copper on fluorescence

The fluorescence of the amino acid derivatives of 4-methylumbelliferone and umbelliferone is quenched by copper.

This quenching, known as the "normal" indicator effect, extends over the pH range 4 to 10.5 for 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid and over the pH range 7 to 10.5 for 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O.

Turning to the case of the iminodiacetic acid derivatives, at the point where the pH reaches 4, the carboxylic proton has been removed and copper can combine with the carboxylate group thus quenching fluorescence. At higher pH values, the phenol group is neutralized and copper binds to the phenolate anion. At still higher pH values the ammonium group is neutralized and copper binds to the ammonium anion. Further proof that carboxylate, phenolate, and ammonium ions are involved in bonding with copper is obtained from a comparison of the titration curves of umbelliferone-8-methyleneiminodiacetic acid in the presence of excess copper (Figure 52) and of the compound alone (Figure 1). The shapes of the curves are markedly different. Copper combines with 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid in a ratio of 1 ligand to 1 copper ion, Figures 24 and 25.

The glycine and sarcosine derivatives of 4-methylumbelliferone and umbelliferone differ from the iminodiacetic acid

derivatives by one acetic acid group. This group is replaced by a hydrogen atom in the case of glycine and a methyl group in the case of sarcosine. Because no groups are present at low pH values with which copper can combine, quenching of fluorescence is not observed at pH values below pH 7, the point at which the phenolic proton has been neutralized. The combining ratio of copper with the methyleneglycine and methylenesarcosine derivatives of 4-methylumbelliferone and umbelliferone is 1 ligand to 1 copper ion (Figures 26, 27 and 28). The presence of a zwitter ion in these molecules renders the phenolic protons stronger acids than the analogous protons of the parent molecule. This increase in acid strength enables combination with copper to occur, whereas the parent molecules of 4-methylumbelliferone and umbelliferone do not combine with copper.

4-Methylesculetin forms compounds with copper in the ratio of 1 ligand to 1 copper ion (Figure 29), the union undoubtedly occurring through the ortho phenolic groups. 4-Methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O forms both 1:1 and 2:1 compounds with copper ion (Figure 30). The formation of a 2:1 compound (2 ligand to 1 copper ion) indicates that 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O has a greater affinity for copper than 4-methylesculetin. This greater affinity probably results from the presence of the carboxylate group and from the presence of the positive

charge on the nitrogen atom (zwitter ion) which makes both phenolic groups stronger acids. Apparently there are no steric effects hindering the formation of the 2:1 compound.

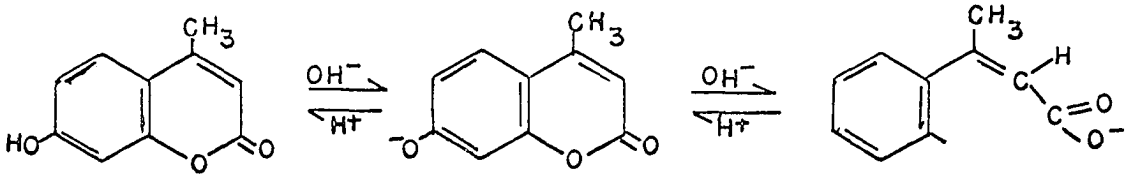
3. Effect of calcium on fluorescence

The calcium-4-methylumbelliferone-8-methyleneiminodiacetic acid and calcium-umbelliferone-8-methyleneiminodiacetic acid compounds show intense fluorescence at pH 12 and above while the free compounds are non-fluorescent at high pH (Figures 15 and 16). The bonding of calcium and 4-methylumbelliferone-8-methyleneiminodiacetic acid and the bonding of calcium and umbelliferone-8-methyleneiminodiacetic acid is through the phenolate and imino anions. Proof that the imino group is involved in bonding is obtained from the fact that the calcium compound exhibits intense fluorescence at high pH values where the decrease in the fluorescence of the free indicator is due to the neutralization of the imino proton. Further proof that the phenolate and imino groups are involved in bonding is obtained from the titration curve of 4-methylumbelliferone-8-methyleneiminodiacetic acid in the presence of a ten-fold excess of calcium with sodium hydroxide (Figure 51) and from the titration curve of umbelliferone-8-methyleneiminodiacetic acid in the presence of a ten-fold excess of calcium with sodium hydroxide (Figure 52). The titration curve up to the first end-point is not changed by the presence of calcium (Figure 1). Beyond the first end-

point, the curves are greatly altered, the second and third replaceable protons being much stronger acids and being neutralized in one step indicating displacement from the molecule by the copper.

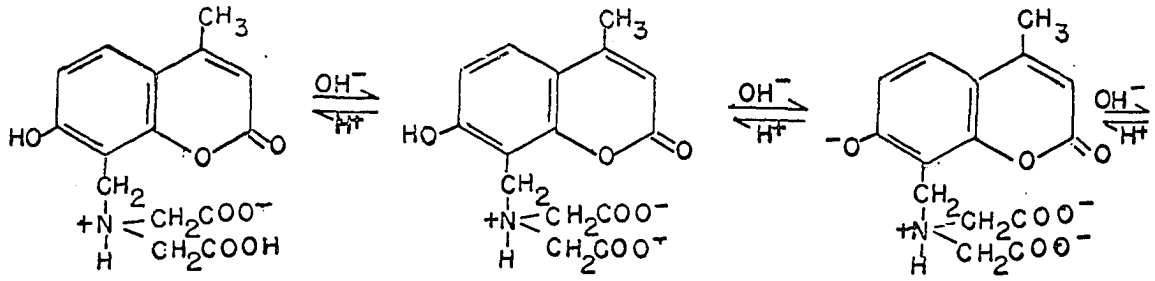
Although calcium does combine with 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid as indicated by a slight change in the fluorescence when calcium is present and as indicated by the shape of the titration curve of 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O, it is not useful as a fluorometric reagent for calcium because the fluorescence of the free compound is not different enough from the fluorescence of the calcium-4-methylesculetinmethyleneiminodiacetic acid compound.

The stability of the calcium compounds of 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid is directly related to the stability of 4-methylumbelliferone and umbelliferone. In basic solution 4-methylumbelliferone and umbelliferone are highly fluorescent but at high pH values the ∝pyrone ring opens, the corresponding hydroxycoumarinic acid is formed and fluorescence is lost.

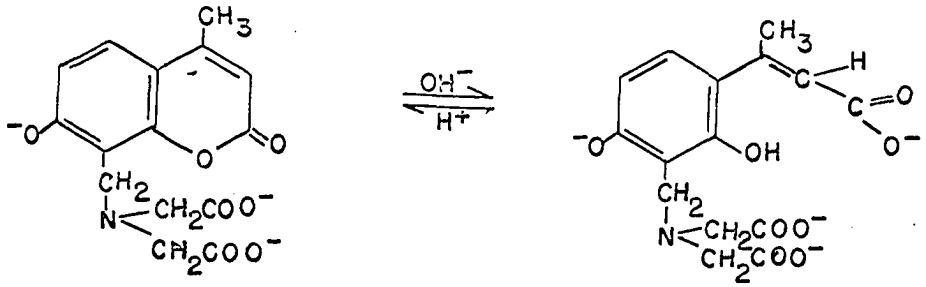


Fluorescent Fluorescent Non-fluorescent

Similar reactions occur when 4-methylumbelliferone-8-methylene-iminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid are titrated with alkali.



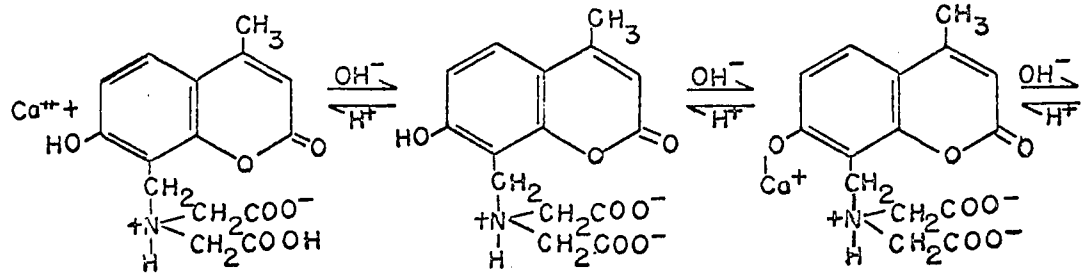
Fluorescent Fluorescent Fluorescent



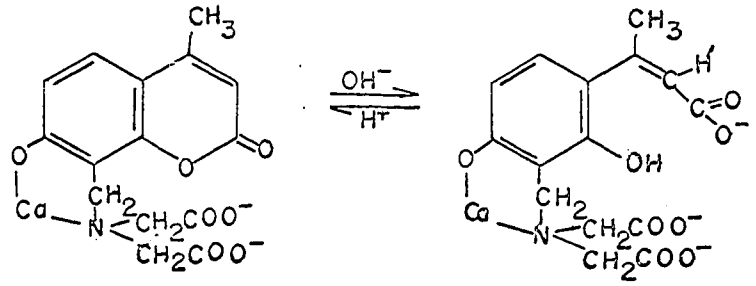
Non-fluorescent Non-fluorescent

The fluorescence of 4-methylumbelliferone-8-methylene-iminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid is lost when the imino proton is neutralized (before ring opening occurs); whereas, the loss of fluorescence in 4-methylumbelliferone and umbelliferone is caused by ring opening.

When calcium is present the following neutralization reactions occur



Fluorescent Fluorescent Fluorescent



Fluorescent Non-fluorescent

Union of the calcium with the carboxylate groups probably occurs after union with the phenolic group takes place but there is no evidence as to when this occurs.

It appears that the bonding of calcium to nitrogen has the same effect on fluorescence as the bonding of hydrogen to nitrogen, that is, when the imino nitrogen is bonded either to hydrogen or calcium the molecule is fluorescent, and when hydrogen or calcium is removed the fluorescence is lost.

The more basic the solution, the more readily the pyrone

ring will open (Figure 31). If an excess of compound is present, however, the calcium-4-methylumbelliferone-8-methyleneiminodiacetic acid compound is stable for one hour at pH 12.8 and hence can be used for the direct fluorometric determination of calcium.

Previous workers (10) have noted that the opening of the pyrone ring takes place more slowly when substituents are present in the pyrone ring. This correlation was reaffirmed by the study of the decrease in fluorescence of the calcium compound of 4-methylumbelliferone-8-methyleneiminodiacetic acid and the calcium compound of umbelliferone-8-methylene-iminodiacetic acid with time. The calcium-umbelliferone-8-methyleneiminodiacetic acid compound loses its fluorescence more quickly (Figure 31).

Calcium combines with 4-methylumbelliferone-8-methylene-iminodiacetic acid in a 1:1 ratio (Figure 32). It is assumed that calcium combines with umbelliferone-8-methyleneiminodi-acetic acid in the ratio 1:1 also but the opening of the pyrone ring was too rapid to permit proof of this.

4. Effect of aluminum on fluorescence

The titration of a mixture of 4-methylumbelliferone-8-methyleneiminodiacetic acid and aluminum with 0.2 M potassium hydroxide was followed fluorometrically on a Turner Model 10 fluorometer. The pH range covered was 1.5 to 4.0. Over this range the fluorescence was not affected by the presence of

aluminum. In this property 4-methylumbelliferone-8-methylene-iminodiacetic differs from Calcein which exhibits intense fluorescence in the pH range 2 to 2.5 when aluminum is present.

E. Acid Dissociation Constants

1. Determination of acid dissociation constants using solubility data

The determination of the acid dissociation constants of the compounds under study was made using various methods. The potentiometric titrations of 4-methylumbelliferone-8-methylene-iminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid (Figure 1) show two distinct end-points, one at a = 1 and one at a = 2, (a) being the number of moles of base added per mole of acid. However, the compounds did not dissolve completely until just before the first end-point at a = 1. Immediately before the first end-point, the pH changed very rapidly with very small increments of base added. This made it difficult to calculate accurately the acid dissociation constants from the potentiometric titration curve before the first end-point. The potentiometric titration of 4-methyl-esculetin-8-methyleneiminodiacetic acid (Figure 2) also shows two end-points. The position of the first end-point is dependent upon the rate of addition of base, the compound not dissolving until just after the point a = 1. Thus, in this case also it was not possible to calculate the first acid dissociation constant from the points on the titration curve.

Another procedure was therefore used, the method of Krebs and Speakman (9), to determine the acid dissociation constants of the carboxylic proton of 4-methylumbelliferone-8-methylene-iminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid and 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O. This method is based on a measurement of the solubility of the acid at various values of pH. The data obtained are given in Table 3.

The amount of material in solution at a specific pH, S, is the sum of the amount of the neutral molecule and of the anion in solution

$$S = [HA] + [A^-] . \qquad (1)$$

By definition

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

S is therefore

$$S = [HA] + \frac{K_a[HA]}{[H^+]} . \qquad (3)$$

At high hydrogen ion concentration S falls practically to S^o, the intrinsic solubility, which is equal to [HA] so (3) can be rewritten

$$S = S^o + \frac{K_a S^o}{[H^+]} \qquad (4)$$

or

$$S = S^o [1 + \text{antilog } (pH - pK_a)] \qquad (5)$$

rearranging

$$pK_a = pH - \log[(S/S^o) - 1]. \qquad (6)$$

Before Equation 6 can be applied, S^o must be determined. This is done by plotting S against $1/[H^+]$. According to Equation 4 extrapolation to $1/[H^+] = 0$ gives S^o (Figure 33). The acid dissociation constant can then be calculated using Equation 6 or can be determined by plotting log $[(S/S^o) - 1]$ against pH. In the second case the pK_a is the pH at which the line intersects the abscissa. Both methods were used in calculating the acid dissociation constants and yielded essentially the same results. The results are listed in Table 11.

2. Determination of acid dissociation constants using spectrophotometric data

The acid dissociation constants of the phenolic protons were determined spectrophotometrically. Absorption spectra of 4-methylumbelliferone, umbelliferone, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O shown in Figures 35, 36, 37, 38, 39 and 40 respectively, indicate one wavelength of maximum absorbance in acid solution, at approximately 320 mu, and one wavelength of maximum absorbance in alkaline solution, at approximately 365 mu. The wavelength of maximum absorbance in acid and alkaline solution for each compound is listed in Table 12.

Table 11. Acid dissociation constants of the carboxylic acid function of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid and 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O determined from solubility measurements

Compound	S^o (mg./100 ml.)	pH	S (mg./100 ml.)	pK_a (Equation 6)	pK_a (graph)
4-Methylumbelliferone-8-methyleneiminodiacetic acid	1.00	2.36	1.20	3.06	
		2.80	1.71	2.95	
		3.39	3.77	2.95	
		4.45	32.37	2.95	
		4.65	48.19	2.98	
		4.74	59.44	2.97	
				2.98 (Ave.)	2.97
Umbelliferone-8-methyleneiminodiacetic acid	7.20	3.26	22.66	2.92	
		3.84	65.66	2.93	
		3.96	84.80	2.92	
		4.06	94.33	2.97	
				2.94 (Ave.)	2.94
4-Methylesculetinmethyleneiminodiacetic acid·½ H_2O	0.52	3.34	1.58	3.03	
		3.76	3.55	2.99	
		4.06	5.63	3.07	
		4.41	12.94	3.03	
				3.03 (Ave.)	3.03

Table 12. Wavelength of maximum absorbance in acid and alkaline solution of 4-methylumbelliferone, umbelliferone, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O

Compound	Wavelength maximum	
	Acid solution mu	Alkaline solution mu
4-Methylumbelliferone	320	360
Umbelliferone	325	365
Umbelliferone-8-methylene-iminodiacetic acid	322	367
4-Methylumbelliferone-8-methyleneglycine	322	365
Umbelliferone-8-methylene glycine·½ H_2O	322	369
4-Methylumbelliferone-8-methylenesarcosine·1½ H_2O	321	360

The absorbance changes with pH, the absorbance at 320 mu decreasing with pH and the absorbance at 365 mu increasing with pH (Figures 41, 42, 43, 44, 45 and 46). This change in the absorption spectra corresponds to the change observed in the fluorescence spectra and is attributed to the neutralization of the phenolic proton. Only one inflection point is observed in each curve. The pH at the inflection point is a good approximation of pK_a and knowing the approximate value, the log-ratio method was employed to accurately determine the pK_a of the phenolic proton of 4-methylumbelliferone, umbel-

liferone and their derivatives.

The reasoning behind the log-ratio method is as follows. The reaction observed is

$$HA = H^+ + A^- \tag{7}$$

and the acid formation constant for the reaction is

$$K_a = \frac{[H^+]\,[A^-]}{[HA]} . \tag{8}$$

The ratio of $[A^-]/[HA]$ can be described by the ratio of the absorbances

$$\frac{A_{mix} - A_{HA}}{A_{A^-} - A_{mix}} \tag{9}$$

Substituting (9) into (8) and taking the negative logarithm

$$pK_a = pH - \log \frac{A_{mix} - A_{HA}}{A_{A^-} - A_{mix}} \tag{10}$$

By substituting the proper values of pH and absorbance into Equation 10 the pK_a values are obtained. The values calculated for the pK_a of the phenolic proton of 4-methylumbelliferone, umbelliferone and their derivatives are given in Table 13.

The log-ratio method was not used to determine the acid dissociation constants of 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O because of serious buffer effects caused by chemical reaction of buffer and the ortho phenolic groups of 4-methylesculetin. The acid dissociation constants of these compounds were determined

Table 13. Acid dissociation constants of the phenolic proton of 4-methylumbelliferone, umbelliferone, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O

Compound	pK_a
4-Methylumbelliferone	7.82
Umbelliferone	7.83
Umbelliferone-8-methyleneiminodiacetic acid	6.95
4-Methylumbelliferone-8-methyleneglycine	6.88
Umbelliferone-8-methyleneglycine·½ H_2O	6.79
4-Methylumbelliferone-8-methylenesarcosine·1½ H_2O	6.71

directly from the graph of absorbance versus pH obtained from the titration of the compounds with 0.1 M potassium hydroxide (Figures 49 and 50). Many readings of absorbance were taken in the vicinity of the inflection point to assure the certainty of its measurement. The acid dissociation constant of the first phenolic proton of 4-methylesculetin obtained from the graph of absorbance versus pH is 7.40 and the acid dissociation constant of the first phenolic proton of 4-methylesculetinmethyleneiminodiacetic acid is 6.35.

3. Determination of acid dissociation constants using fluorometric data

Two inflection points are observed in the graphs of

absorbance versus pH (Figures 49 and 50) and in the graphs of fluorescence versus pH (Figures 22 and 23) of 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O. The pH at the first inflection point corresponds to the pK_a of one phenolic proton and the pH at the second inflection point corresponds to the pK_a of the second phenolic proton. Because the second inflection point is not well defined by absorbance data, the fluorescence data was used to calculate the acid dissociation constant of the second phenolic proton. The values obtained are 11.65 for 4-methylesculetin and 11.35 for 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O.

The acid dissociation constants of the ammonium ions were determined using the data shown in Figures 15, 16, 17, 18 and 19. Two changes in fluorescence are observed with changes in pH for the amino acid derivatives of 4-methylumbelliferone and umbelliferone, namely the simultaneous decrease in fluorescence at 330 mu and increase in fluorescence at 370 mu at pH 6 and the decrease in fluorescence at 370 mu at pH 9.5. Only one change in fluorescence with pH is found with 4-methylumbelliferone and umbelliferone, Figures 13 and 14. This change or inflection is attributed to the neutralization of the phenolic proton and is common to parent molecule and amino acid derivative while the second inflection is present only in the curves for the amino acid derivatives and is attributed to the neutralization of the ammonium

proton. The pH at the second inflection point, then, is equal to the pK_a of the ammonium group. The values of pK_a determined for the ammonium groups are listed in Table 14.

Table 14. Acid dissociation constants of the ammonium proton or 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O

Compound	pK_a
4-Methylumbelliferone-8-methyleneiminodiacetic acid	11.30
Umbelliferone-8-methyleneiminodiacetic acid	11.11
4-Methylumbelliferone-8-methyleneglycine	10.25
Umbelliferone-8-methyleneglycine$1\frac{1}{2}$ H_2O	10.25
4-Methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O	10.80

The acid dissociation constant of the imino proton of 4-methylesculetinmethyleneiminodiacetic acid,$\frac{1}{2}$ H_2O could not be determined from data obtained from the potentiometric titration curve, from absorbance data or from fluorescence data and no value for it is given.

A summary of the acid dissociation constants of 4-methylumbelliferone, umbelliferone, 4-methylesculetin, 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylesculetinmethyleneiminodiacetic acid, 4-methylumbelliferone-8-methyleneglycine,

umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O is given in Table 15.

The only acid functions in 4-methylumbelliferone, umbelliferone and 4-methylesculetin are the phenolic groups. The values of pK_{a1} are 7.83, 7.83 and 7.40 respectively. These are reasonable values, agreeing with values for substituted phenols which vary from 7.1 to 10.3 depending on the nature and position of the substituent. The second acid dissociation constant of 4-methylesculetin is 11.65 which is a considerably weaker than that of the first phenolic group. This is expected because of the presence of a negative charge on the neighboring oxygen.

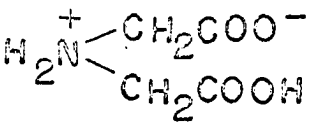
Turning to 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid and 4-methylesculetinmethyleneiminodiacetic acid, the replaceable hydrogen atoms come from the carboxyl group, one phenolic group in the case of 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid and two phenolic groups in the case of 4-methylesculetin-8-methyleneiminodiacetic acid·½ H_2O, and from the imino group. Of the two carboxyl groups present, one is already ionized owing to the formation of a zwitter ion (the carboxyl proton is transferred to the imino group). The second carboxyl group is a stronger acid than expected because of the positive charge on the neighboring ammonium group. This phenomenon

Table 15. Summary of the acid dissociation constants of 4-methylumbelliferone, umbelliferone, 4-methylesculetin, 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylesculetin-8-methyleneiminodiacetic acid·½ H_2O, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O

Compound	pK_1	pK_2	pK_3	pK_4
4-Methylumbelliferone	7.82 P_1			
Umbelliferone	7.83 P_1			
4-Methylesculetin	7.40 P_1	11.65 P_2		
4-Methylumbelliferone-8-methyleneiminodiacetic acid	2.97 C	6.92 P_1	11.28 A	
Umbelliferone-8-methyleneiminodiacetic acid	2.94 C	6.95 P_1	11.11 A	
4-Methylesculetinmethyleneiminodiacetic acid·½ H_2O	3.03 C	6.35 P_1	11.35 P_2	___A
4-Methylumbelliferone-8-methyleneglycine	6.88 P_1	10.25 A		
Umbelliferone-8-methyleneglycine·½ H_2O	6.79 P_1	10.25 A		
4-Methlyumbelliferone-8-methylenesarcosine·1½ H_2O	6.71 P_1	10.80 A		

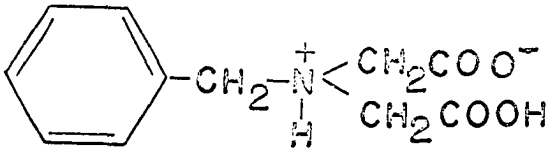
C = Carboxyl group
P_1 = Replaceable hydrogen of first phenolic group
P_2 = Replaceable hydrogen of second phenolic group
A = Ammonium ion

also occurs in iminodiacetic acid.



$pK_1 = 2.54$ (Carboxyl) $pK_2 = 9.12$ (Ammonium)

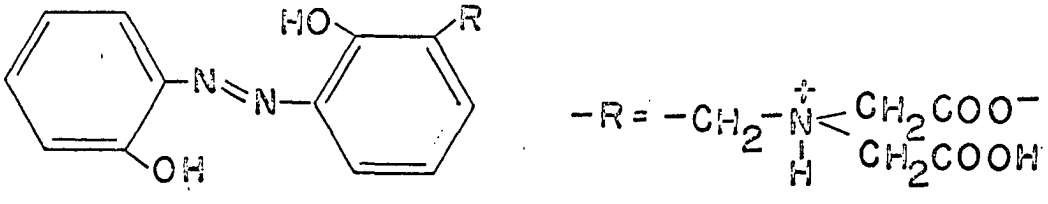
The second replaceable hydrogen atom of 4-methylumbelliferone-8-methyleneiminodiacetic acid ($pK_{a2} = 6.92$) and umbelliferone-8-methyleneiminodiacetic acid ($pK_{a2} = 6.95$) comes from the phenolic groups while the second and third replaceable hydrogen atoms of 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O ($pK_{a2} = 6.35$, $pK_{a3} = 11.35$) come from phenolic groups. The phenolic groups of 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid and the first phenolic group of 4-methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O are stronger acids than the corresponding phenols of the parent molecules by approximately one order of magnitude. This again results from the positive charge on the ammonium group. The same argument is adopted by Schwarzenbach, Anderegg and Sallman (11) for the analogous compound 2[N,N-bis(carboxymethyl)aminomethyl]phenol



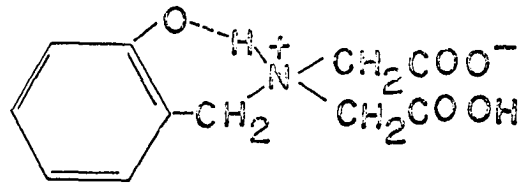
$pK_1 = 2.2$	$pK_2 = 8.17$	$pK_a = 11.79$
(Carboxyl)	(Phenol)	(Ammonium)

The acid dissociation constant of the second phenolic group of 4-methylesculetinmethyleneiminodiacetic acid is 11.35 which as in the case of 4-methylesculetin is a much weaker acid than the first phenolic proton. It is a slightly stronger acid, however, than the corresponding phenolic group of 4-methylesculetin (11.60) owing to the presence of a positive charge on the neighboring ammonium group.

The acid dissociation constants of the ammonium group of 4-methylumbelliferone-8-methyleneiminodiacetic acid (pK_{a3} = 11.11) are approximately the same as that of 1-[3'(N,N-bis (carboxymethyl)aminomethyl)-2'-hydroxy-1'-benzeneazo]-2-hydroxybenzene



(pK_{a4} = 11.2) but somewhat greater than the corresponding one for 2[N,N-bis(carboxymethyl)aminomethyl]phenol (pK_3 = 11.79). They are considerably weaker than the ammonium group of iminodiacetic acid (pK_{a2} = 9.12). Schwarzenbach (11) explains this by assuming hydrogen bond formation of the ammonium hydrogen atom with the neighboring phenolate group.



The more highly bonded hydrogen would have less tendency to dissociate.

The reasoning is much the same for 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O the only difference being the absence of one acetic acid group. A zwitter ion is formed by the transfer of the carboxyl proton to the ammonium group and thus no free carboxyl groups are present. The phenolic groups of 4-methylumbelliferone-8-methyleneglycine (pK_{a1} = 6.88), umbelliferone-8-methyleneglycine·½ H_2O (pK_{a1} = 6.79) and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O (pK_{a1} = 6.71) are slightly stronger than the corresponding ones for the methyleneiminodiacetic acid derivatives. This presumably is due to stronger repulsion by the more positive charge on the ammonium groups of

glycine and sarcosine. The positive charge on the ammonium group of the iminodiacetic acid derivatives is partially neutralized by a negative charge on each of its carboxylate groups whereas in glycine and sarcosine it is partially neutralized by only one carboxylate group.

The acid dissociation constants of the ammonium group of 4-methylumbelliferone-8-methyleneglycine (pK_{a2} = 10.25), umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O (pK_{a2} = 10.25) and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O (pK_{a2} = 10.80) are somewhat lower than the corresponding ones for 4-methylumbelliferone-8-methyleneiminodiacetic acid (pK_{a3} = 11.28) and umbelliferone-8-methyleneiminodiacetic acid (pK_{a3} = 11.11). This is also explained by the fact that the positive charge on the ammonium group is partially neutralized by only one carboxylate group in the case of the glycine and sarcosine derivatives (the ammonium group is more positive and the positive hydrogen is more readily removed) but it is partially neutralized by two carboxylate groups in the case of the iminodiacetic acid derivatives (the positive hydrogen is repulsed less by the less positive ammonium group and hence is a weaker acid).

F. Formation Constants with Calcium and Copper

1. Determination of formation constants of calcium compounds

The formation constants for calcium uniting with 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid and 4-methylesculetin-methyleneiminodiacetic acid were determined by the method devised by G. Schwarzenbach, G. Anderegg, and R. Ballman (11). Use of this method requires data obtained from the potentiometric titration of the compounds in the presence of excess metal ion with sodium hydroxide. Such titration curves for 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid are shown in Figures 51 and 52.

The argument is as follows. The graph of the titration curve with a ten-fold excess of calcium ion shows two distinct end-points. The portion of the curve up to the first end-point corresponds to the neutralization of the carboxyl group, pK_1, and is not altered by the presence of calcium. The second end-point results from compound formation and the overall apparent acid formation constant, K'_{H_2Ind}, was calculated using Equations 11 and 12.

$$2H^+ + Ind^{-3} = H_2Ind^- \qquad (11)$$

$$K'_{H_2Ind} = \frac{[H_2Ind^-]}{[H^+]^2[Ind^{-3}]} \qquad (12)$$

The results of the determination of $K'_{H_2Ind^-}$ for 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid are 3.44×10^{15} and 2.74×10^{16} respectively.

The reactions which take place on neutralization in the presence of excess metal are:

$$H_3Ind = H_2Ind^- = \begin{bmatrix} HInd^{-2} \\ + \\ MHInd \end{bmatrix} = \begin{bmatrix} Ind^{-3} \\ + \\ MInd^- \end{bmatrix} \qquad (13)$$

Therefore, $K'_{H_2Ind^-}$ can be expressed in terms of the species present in solution in the pH range indicated.

$$K'_{H_2Ind^-} = \frac{[H_2Ind^-]}{[H^+]^2([Ind^{-3}] + [MInd^-])} \qquad (14)$$

Substituting

$$K^M_{MHInd} = \frac{[MHInd]}{[M^{+2}][HInd^{-2}]} \qquad (15)$$

$$K^M_{MInd^-} = \frac{[MInd^-]}{[M^{+2}][Ind^{-3}]} \qquad (16)$$

and

$$K_{a_1}K_{a_2} = \frac{[H_2Ind^-]}{[H^+]^2[Ind^{-3}]} \qquad (17)$$

into Equation 14 gives

$$K'_{H_2Ind^-} = K_{a_1}K_{a_2} \frac{1}{1 + [M^{+2}]K^M_{MInd^-}} \qquad (18)$$

The value of $K^{M}_{MInd^-}$ can be calculated using Equation 18, the value obtained for $K'_{H_2Ind^-}$ and the acid formation constants. The values for the formation constants of the calcium compound of 4-methylumbelliferone-8-methyleneiminodiacetic acid and the calcium compound of umbelliferone-8-methyleneiminodiacetic acid are 1.06×10^5 and 8.26×10^3 respectively. The two values differ by approximately one order of magnitude. This difference stems from the two titration curves, the curve for the titration of umbelliferone-8-methyleneiminodiacetic acid being higher than the titration curve of 4-methylumbelliferone-8-methyleneiminodiacetic acid in the presence of excess calcium in the region between 1 and 3 moles of base added per mole of acid.

In the case of 4-methylesculetinmethyleneiminodiacetic acid$\cdot\frac{1}{2}$ H_2O, the problem of determining $K^{M}_{MInd^-}$ is more complex owing to the presence of the additional phenolic group but basically the reasoning is the same. The graph of the titration curve with excess metal has two distinct end-points. The first portion of the titration curve is assigned to the dissociation of the carboxyl group and the two phenolic groups and the second portion is assigned to the neutralization of the ammonium group. The first region extends over the pH range of three replaceable hydrogen atoms and the overall apparent acid formation constant K'_{H_4Ind} can be calculated using Equations 19 and 20.

$$3H^{+} + HInd^{-3} = H_4Ind \qquad (19)$$

$$K'_{H_4Ind} = \frac{[H_4Ind]}{[H^{+}]^3[HInd^{-3}]} \qquad (20)$$

It is assumed that the carboxyl group is not involved in compound formation as in the case of 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid, compound formation being the result of combination of calcium with the ortho phenolic groups. Knowing K for the reaction

$$H^{+} + H_3Ind^{-} = H_4Ind \qquad (21)$$

$$K = \frac{[H_4Ind]}{[H^{+}][H_3Ind^{-}]} \qquad (22)$$

the overall apparent acid formation constant $K'_{H_3Ind^-}$ can be calculated

$$2H^{+} + HInd^{-3} = H_3Ind^{-} \qquad (23)$$

$$K'_{H_3Ind} = \frac{[H_3Ind^{-}]}{[H^{+}]^2[HInd^{-3}]} \qquad (24)$$

because

$$K(K'_{H_3Ind^-}) = K'_{H_4Ind} \qquad (25)$$

The second region extends over the range of neutralization of one replaceable hydrogen, thus $K'_{HInd^{-3}}$ can be determined from Equations 26 and 27

$$H^{+} + Ind^{-4} = HInd^{-3} \qquad (26)$$

$$K'_{HInd-3} = \frac{[HInd^{-3}]}{[H^+][Ind^{-4}]} \qquad (27)$$

The reactions which take place on neutralization in the presence of excess metal ion are

$$H_4Ind = H_3Ind^- = \begin{bmatrix} H_2Ind^{-2} \\ + \\ MH_2Ind \end{bmatrix} = \begin{bmatrix} HInd^{-3} \\ + \\ MHInd^- \end{bmatrix} = \begin{bmatrix} Ind^{-4} \\ + \\ MInd^{-2} \end{bmatrix} \qquad (28)$$

Therefore, $K'_{H_3Ind^-}$ can be expressed in terms of the species present in solution in the pH range indicated.

$$K'_{H_3Ind^-} = \frac{[H_3Ind^-]}{[H^+]^2([HInd^{-3}] + [MHInd^-])} \qquad (29)$$

Substituting

$$K_2K_3 = \frac{[H_3Ind^-]}{[H^+]^2[HInd^{-3}]} \qquad (30)$$

$$K^M_{MHInd^-} = \frac{[MHInd^-]}{[M^{+2}][HInd^{-3}]} \qquad (31)$$

and

$$K^M_{MH_2Ind} = \frac{[MH_2Ind]}{[M^{+2}][H_2Ind^{-2}]} \qquad (32)$$

into Equation 29 gives

$$K'_{H_3Ind^-} = K_2K_3 \frac{1}{1 + [M^{+2}]K^M_{MHInd^-}} \qquad (33)$$

Similarly K'_{HInd-3} can be expressed as

$$K'_{HInd-3} = \frac{[HInd^{-3}] + [MHInd^{-}]}{[H^{+}]([Ind^{-4}] + [MInd^{-2}])} \qquad (34)$$

Substituting

$$K_4 = \frac{[HInd^{-3}]}{[H^{+}][Ind^{-4}]} \qquad (35)$$

$$K^{M}_{MInd-2} = \frac{[MInd^{-2}]}{[M^{+2}][Ind^{-4}]} \qquad (36)$$

and

$$K^{M}_{MHInd^{-}} = \frac{[MHInd^{-}]}{[M^{+2}][HInd^{-3}]} \qquad (37)$$

into Equation 34 gives

$$K'_{HInd-3} = K_4 \frac{1 + [M^{+2}]K^{M}_{MHInd^{-}}}{1 + [M^{+2}]K^{M}_{MInd-2}} \qquad (38)$$

This equation however, could not be solved because K_4 could not be determined (see page 167). Also, because the calcium-4-methylesculetinmethyleneiminodiacetic acid compound was not highly fluorescent and, therefore, was not a fluorescent indicator for calcium the problem was abandoned.

None of the other compounds acted as fluorescent indicators for calcium. The titration curves of 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O and 4-methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O in the presence of a ten-fold excess of calcium were identical to the titration curves of the compound alone, indicating that

no compound formation occurred.

2. Determination of formation constants of copper compounds

The formation constants of copper reacting with 4-methylumbelliferone-8-methyleneiminodiacetic acid, umbelliferone-8-methyleneiminodiacetic acid, 4-methylesculetinmethyleneiminodiacetic acid·½ H_2O, 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylenesarcosine·1½ H_2O were determined by the method devised by G. Schwarzenbach, G. Anderegg and R. Sallman (11) in the cases where it proved possible to determine them. Use of this method required data obtained from the potentiometric titration of the compound in the presence of a ten-fold excess of copper ion with sodium hydroxide. Such titration curves are shown in Figures 51, 52, 53, 54, 55 and 56. The argument is as follows. The graphs of the titration curve of 4-methylumbelliferone-8-methyleneiminodiacetic acid and of umbelliferone-8-methyleneiminodiacetic acid in the presence of excess copper with sodium hydroxide have one end-point. The buffer region extends over the range of neutralization of two replaceable hydrogen atoms and the overall apparent acid formation constant K'_{H_3Ind} was calculated using Equations 39 and 40.

$$2H^+ + HInd^{-2} = H_3Ind \qquad (39)$$

$$K'_{H_3Ind} = \frac{[H_3Ind]}{[H^+]^2[HInd^-]} \qquad (40)$$

The values of K'_{H_3Ind} for 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid are 1.04×10^4 and 1.85×10^3 respectively.

The reactions which take place on neutralization in the presence of excess metal ion are

$$H_3Ind = \begin{bmatrix} H_2Ind^- \\ + \\ MH_2Ind^+ \end{bmatrix} = \begin{bmatrix} HInd^{-2} \\ + \\ MHInd \end{bmatrix} = \begin{bmatrix} Ind^{-3} \\ + \\ MInd^- \end{bmatrix} \qquad (41)$$

Therefore, K'_{H_3Ind} can be expressed in terms of the species present in solution in the pH range indicated

$$K'_{H_3Ind} = \frac{[H_3Ind]}{[H^+]^2([HInd^{-2}] + [MHInd])} \qquad (42)$$

Defining

$$K_{a_1}K_{a_2} = \frac{[H_3Ind]}{[H^+]^2[HInd^{-2}]} \qquad (43)$$

$$K^M_{MHInd} = \frac{[MHInd]}{[M^{+2}][HInd^{-2}]} \qquad (44)$$

$$K^M_{MH_2Ind} = \frac{[MH_2Ind^+]}{[M^{+2}][H_2Ind^-]} \qquad (45)$$

Equation 42 can be expressed as

$$K'_{H_3Ind} = K_{a_1}K_{a_2}\frac{1}{1 + [M^{+2}]K^M_{MHInd}} \qquad (46)$$

The ammonium group also appears to be involved in compound formation with copper because in the titration of 4-methylumbelliferone-8-methyleneiminodiacetic acid and

umbelliferone-8-methyleneiminodiacetic acid in the presence of excess copper the curve at n = 3, where n is the number of moles of copper added per mole of acid, is displaced to lower pH values.

The apparent acid formation constant of the second portion of the titration curve can be determined using equations 47 and 48

$$H^+ + Ind^{-3} = HInd^{-2} \qquad (47)$$

$$K'_{HInd-2} = \frac{[HInd^{-2}]}{[H^+][Ind^{-3}]}$$

The values of K'_{HInd-2} calculated for 4-methylumbelliferone-8-methyleneiminodiacetic acid and for umbelliferone-8-methyleneiminodiacetic acid are 3.0×10^6 and 3.60×10^5 respectively.

K'_{HInd-2} can be expressed in terms of the species present in solution in the pH range indicated

$$K'_{HInd-2} = \frac{[HInd^{-2}] + [MHInd]}{[H^+]([Ind^{-3}] + [MInd^-])} \qquad (48)$$

Defining

$$K_{a_3} = \frac{[HInd^{-2}]}{[H^+][Ind^{-3}]} \qquad (49)$$

$$K^M_{MInd^-} = \frac{[MInd^-]}{[M^{+2}][Ind^{-3}]} \qquad (50)$$

and

$$K^M_{MHInd} = \frac{[MHInd]}{[M^{+2}][HInd^{-2}]} \qquad (51)$$

K'_{HInd-2} can be expressed as

$$K'_{HInd-2} = K_{a_3} \frac{1 + [M^{+2}]K^M_{MHInd}}{1 + [M^{+2}]K^M_{MInd^-}} \qquad (52)$$

The values of $K^M_{MInd^-}$ calculated for 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid are 1.15×10^{13} and 3.67×10^{14} respectively.

It is apparent that corresponding values of K^M_{MHInd} and $K^M_{MInd^-}$ for 4-methylumbelliferone-8-methyleneiminodiacetic acid and umbelliferone-8-methyleneiminodiacetic acid differ by approximately one order of magnitude. The reason for this is not obvious, however, it may be traced back to the titration curves. The titration of 4-methylumbelliferone-8-methyleneiminodiacetic acid in the presence of a ten-fold excess of copper with sodium hydroxide was carried out in a clear blue solution - everything being in solution. However, the solution containing umbelliferone-8-methyleneiminodiacetic acid in the presence of a ten-fold excess of copper was cloudy and did not become clear during the course of the titration.

The graph of the potentiometric titration curve of 4-methylesculetinmethyleneiminodiacetic acid in the presence of a ten-fold excess of copper with sodium hydroxide contains two distince end-points but instead of occurring at n = a whole number, the end-points occur at n = 1.70 and at n =

3.52. Likewise the graphs of the titration curves of 4-methylumbelliferone-8-methyleneglycine, umbelliferone-8-methyleneglycine·½ H_2O and 4-methylumbelliferone-8-methylene-sarcosine·1½ H_2O in the presence of a ten-fold excess of copper show one end-point, at n = 1.77.

IV. SUMMARY

Five substituted coumarins have been prepared, purified and studied as potential metallofluorochromic indicators:

Umbelliferone-8-methyleneiminodiacetic acid

4-Methylesculetinmethyleneiminodiacetic acid·½ H_2O

4-Methylumbelliferone-8-methyleneglycine

Umbelliferone-8-methyleneglycine·½ H_2O

4-Methylumbelliferone-8-methylenesarcosine·1½ H_2O.

In addition, 4-methylumbelliferone-8-methyleneiminodiacetic acid (Calcein Blue), prepared in earlier work, has been studied more extensively.

The methyleneamino acid group of these molecules was introduced into the molecule of the parent hydroxycoumarin by condensation with formaldehyde and the appropriate amino acid (Mannich reaction). The composition and structure of these compounds was established by elemental analysis, determination of equivalent weight by neutralization, and nuclear magnetic resonance spectroscopy. The methyleneamino acid was introduced into 4-methylumbelliferone and umbelliferone at position 8.

The acid dissociation constants of each of the parent hydroxycoumarins, of each of the Mannich condensation products, and of 4-methylumbelliferone-8-methyleneiminodiacetic acid have been determined and assignments made of the various acid functions to specific groups in the molecules. The methylene-

amino acid groups are present as zwitter ions. The negative logarithms of the dissociation constants and the corresponding groups are:

4-Methylumbelliferone

$pK_1 = 7.82$ (P)

Umbelliferone

$pK_1 = 7.83$ (P)

4-Methylesculetin

$pK_1 = 7.40$ (P_1) $pK_2 = 11.65$ (P_2)

4-Methylumbelliferone-8-methyleneiminodiacetic acid

$pK_1 = 2.97$ (C) $pK_2 = 6.92$ (P) $pK_3 = 11.28$ (A)

Umbelliferone-8-methyleneiminodiacetic acid

$pK_1 = 2.94$ (C) $pK_2 = 6.95$ (P) $pK_3 = 11.11$ (A)

4-Methylesculetinmethyleneiminodiacetic acid·$\frac{1}{2}$ H_2O

$pK_1 = 3.03$ (C) $pK_2 = 6.35$ (P_1) $pK_3 = 11.35$ (P_2)

pK_4 = unknown

4-Methylumbelliferone-8-methyleneglycine

$pK_1 = 6.88$ (P) $pK_2 = 10.25$ (A)

Umbelliferone-8-methyleneglycine·$\frac{1}{2}$ H_2O

$pK_1 = 6.79$ (P) $pK_2 = 10.25$ (A)

4-Methylumbelliferone-8-methylenesarcosine·$1\frac{1}{2}$ H_2O

$pK_1 = 6.71$ (P) $pK_2 = 10.80$ (A)

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

The absorption spectra of 4-methylumbelliferone, umbel-

liferone and their methyleneamino acid derivatives exhibit one maximum which shifts from 320 mu in acid solution to 365 mu in alkaline solution. Above pH 6, the absorbance of 4-methylumbelliferone and umbelliferone at 320 mu decreases and the absorbance at 365 mu increases while above pH 5 the absorbance of the methyleneamino acid derivatives of 4-methylumbelliferone and umbelliferone at 320 mu decreases and the absorbance at 365 mu increases. This shift in absorbance is associated with the neutralization of the phenolic group which is a stronger acid in the methyleneamino acid derivatives owing to the presence of the positive charge on the neighboring nitrogen atom (zwitter ion).

The fluorescence excitation spectra indicate that excitation occurs over the same wavelength range as absorbance, undergoing a shift from 330 mu in acid solution to 370 mu in alkaline solution. Beyond pH 9.5 the fluorescence decreases owing to neutralization of the ammonium group. This decrease is not observed for 4-methylumbelliferone and umbelliferone. The fluorescence emission spectra show one maximum in both acid and alkaline solution at 450 mu which does not change with changes in pH.

The absorption spectra of 4-methylesculetin and 4-methylesculetinmethyleneiminodiacetic acid exhibit one maximum shifting from 340 mu in acidic solution to 360 mu in basic solution. This shift is associated with the neutralization

of one phenolic group. The absorbance of 4-methylesculetin in basic solution decreases above pH 10 while the absorbance of its methyleneiminodiacetic acid derivative decreases above pH 9. This decrease in absorbance is associated with the neutralization of the second phenolic group. The compounds exhibit negligible fluorescence in acid solution, however, the fluorescence excitation spectrum in alkaline solution corresponds to the absorbance spectrum in alkaline solution. The fluorescence emission spectrum shows one maximum in basic solution.

Copper forms 1:1 and 2:1 compounds with 4-methylesculetin-methyleneiminodiacetic acid while it forms 1:1 compounds with all of the other Mannich condensation products. The fluorescence of the methyleneamino acid derivatives is quenched by copper over the pH range 4 through 10.5 owing to the combination of copper with carboxylate, phenolate and ammonium ions. The behavior of the methyleneglycine and methylenesarcosine derivatives in the presence of copper is similar, the fluorescence being quenched over the pH range 7 through 10.5 owing to combination of copper with phenolate and ammonium ions.

Calcium forms a 1:1 compound with 4-methylumbelliferone-8-methyleneiminodiacetic acid. The fluorescence of the methyleneiminodiacetic derivatives of 4-methylumbelliferone and umbelliferone is enhanced above pH 9.5 in the presence of calcium ions owing to combination with the phenolate and

ammonium anions.

It is assumed that a 1:1 compound is also formed with umbelliferone-8-methyleneiminodiacetic acid, however, owing to the instability of the calcium umbelliferone-8-methyleneiminodiacetic acid compound at high pH this was not proven.

The fluorescence of 4-methylesculetinmethyleneiminodiacetic acid is not affected by calcium ions.

The capacity to combine with calcium is terminated with the loss of one acetic acid group. Methyleneglycine and methylenesarcosine derivatives do not combine with calcium.

The formation constants of the calcium and copper derivatives of the methyleneiminodiacetic acid compounds of 4-methylumbelliferone and umbelliferone have been determined and are:

4-Methylumbelliferone-8-methyleneiminodiacetic acid

$$K^{Ca}_{CaInd^-} = 1.06 \times 10^5 \quad K^{Cu}_{CuHInd} = 1.72 \times 10^8$$

$$K^{Cu}_{CuInd^-} = 1.15 \times 10^{13}$$

Umbelliferone-8-methyleneiminodiacetic acid

$$K^{Ca}_{CaInd^-} = 8.3 \times 10^3 \quad K^{Cu}_{CuHInd} = 1.05 \times 10^9$$

$$K^{Cu}_{CuInd^-} = 3.67 \times 10^{14}$$

Of the six methyleneamino acid derivatives studied, 4-methylumbelliferone-8-methyleneiminodiacetic acid (Calcein Blue), has the greatest potential as an analytical reagent. It is relatively easy to prepare. It combines with copper over the pH range 4 to 10.5 with complete quenching of fluorescence and it combines with calcium above pH 12 with enhancement of fluorescence. At high pH its calcium derivative is stable long enough (1 hour) to allow its use not only in the EDTA titration of calcium but also in the direct fluorometric determination of calcium.

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