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THE 7-HYDROXY-8-(N,N BISCARBOXYMETHLAMINOMETHYL)-BENZO-γ-PYRONES AS METALLOFLUORESCENT INDICATORS

by

Gerald Joseph Scheppers

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INTRODUCTION

Ethylenediaminetetraacetic acid, commonly known as EDTA, possesses the ability to react with numerous metals under a wide variety of conditions. This makes its use as a titrant in the volumetric determination of any given metal primarily dependent upon the availability of an indicator to mark the equivalence point of the titration. Such an indicator must necessarily react with a metal to give a metal-indicator complex ion with a stability substantially less than the corresponding metal-EDTA complex. In addition, the metal-indicator complex ion must yield an absorption spectrum quite different from that of the indicator itself in order to observe a suitable color transition in the equivalence point region of a titration. This situation has stimulated both the synthesis and characterization of compounds that can function as "metallochromic" indicators.

Most indicators that have been developed to date can be classed as either azo- derivatives or methyleneiminodiacetic acid derivatives of common acid-base indicators. It is the latter derivatives that have received much attention, owing to their ability to function in strongly alkaline media. They are commonly prepared by the Mannich reaction, starting with iminodiacetic acid, formaldehyde, and an acid-base indicator having one or more phenolic groups. Schwarzenbach, Anderegg, and coworkers (31, 2) prepared the methyleneiminodiacetic

acid derivatives of nitrophenol and o-cresophthalein. They found shifts of the visible absorption spectra when many metal ions, including the alkaline earths, were added. Although the o-cresolphthalein derivative has since found use as an indicator for titration of alkaline earths, the slight difference in color of the metal-indicator complex ions (red) and the free indicator (pink) makes location of titration end points very difficult.

Korbl and Pribil (22, 23, 21) also prepared metallochromic indicators via the Mannich reaction. The most widely used of these indicators is the condensation product of iminodiacetic acid, formaldehyde, and o-cresolsulfonephthalein which they gave the trivial name Xylenol Orange. Xylenol Orange has been used as an indicator for the EDTA titrations of bismuth (III) at pH 1-3, thorium (IV) at pH 2.5-3.5, lanthanum (III) at pH greater than 5, scandium (III) at pH 5, lead and zinc at pH 5, cadmium at pH 5.9 and mercury (II) at pH 6.

In their search for a metallochromic indicator for calcium, Diehl and Ellingboe (9) reacted iminodiacetic acid, formaldehyde and fluorescein to form a condensation product containing two methyleneiminodiacetic acid groups. They gave this compound the trivial name of Calcein. Although their first procedure for the EDTA titration of calcium using Calcein as indicator was based on a color change at the end

point, Calcein has since been recognized as a fluorescent indicator. Calcein in aqueous solution exhibits strong yellow-green fluorescence in the acidity range from pH 4 to pH 11. With calcium ions present the solution is fluorescent from pH 4 to pH 14. Although the magnesium-Calcein complex ion is also fluorescent, interference from magnesium can be masked by high hydroxide ion concentration.

Kepner and Hercules (20) developed a method for the fluorometric determination of calcium in blood serum using Calcein. The method involved the measurement of the fluorescence of the non-dissociated ion formed by calcium and Calcein in strongly alkaline solution. Although results obtained by Kepner and Hercules compared favorably with previously existing clinical methods, Calcein had several short comings as a fluorometric reagent. One, there was a fluorescence in the reagent blank; two, the reagent was not stable for more than a few days in aqueous alkaline solution.

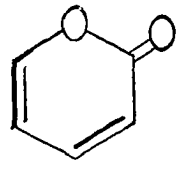
Other difficulties encountered in the use of Calcein for calcium analysis have been reported by Wallach and Steck (35). They concluded that it required two moles of calcium to one mole of Calcein to yield a fluorescent species. They also stated that calcium must be in excess of that required for a one to one mole ratio of calcium to Calcein before the relation between fluorescence and calcium concentration becomes linear. Kepner and Hercules (20) did not add extra calcium to reach the linear portion of the curve; however, the most likely

possibility is that they unkowingly added sufficient calcium with their other reagents. These problems as well as uses of Calcein are summarized in a recent monograph by Diehl (8).

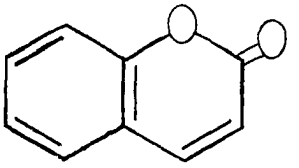
Wilkins (36) introduced a condensation product of 4-methylumbelliferone, formaldehyde, and iminodiacetic acid which he called Calcein Blue. Calcein Blue functions as an indicator in a manner very similar to that of Calcein. However, Wilkins states that Calcein Blue is superior to Calcein because fluorescent excitation occurs in the 370 mμ region which is near the maximum output of the radiation of most commercial ultraviolet light sources. Moreover, Calcein Blue has only one methyleneiminodiacetic acid group, and presumably this implies that only one calcium per Calcein Blue is necessary to form a fluorescent species.

In a study of Calcein Blue by Eggers (10) it was found that Calcein Blue was unstable in strongly alkaline solutions. Further work by Huitink (16) in this laboratory revealed that solid Calcein Blue decomposed in day light. These observations discourage the use of Calcein Blue as a fluorometric reagent.

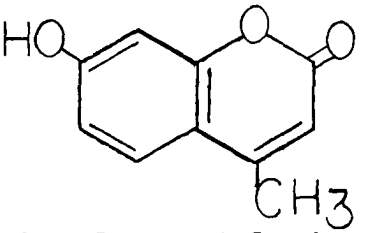
The reason for the decomposition of Calcein Blue in aqueous alkali is apparent from the discussion of pyrones by Badger (3). Although they do not seem to make any marked contribution, resonance structures can be drawn for α-pyrone,



as it behaves very much like a lactone. For benzo-α-pyrone,

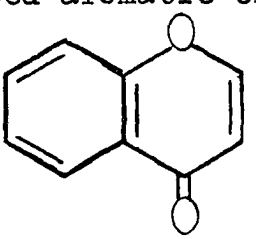


the lactonic character is somewhat depressed; however, the α-pyrone ring can be opened with slight difficulty by treatment with alkali. Because 4-methylumbelliferone,



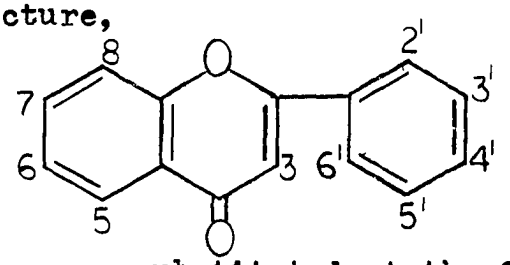
is the parent molecule of Calcein Blue, the same type ring opening would be expected for this compound.

An improvement on Calcein Blue is suggested in the discussion of γ-pyrone by Badger (3). In contrast to α-pyrone, γ-pyrone has pronounced aromatic character, and benzo-γ-pyrones,



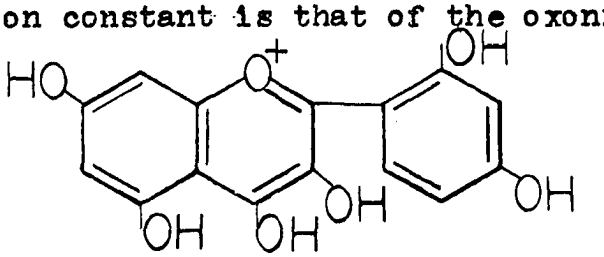
behave like αβ-unsaturated ketones. Therefore, a methylene-iminodiacetic acid derivative of a benzo-γ-pyrone should be more stable in strongly alkaline solution than the corresponding derivative of a benzo-α-pyrone. Certain derivatives of benzo-γ-pyrone have already been used as analytical reagents, notably the hydroxyl derivatives. Morin and quercetin have found application as fluorometric reagents. Both are flavones

with the basic structure,



Morin has hydroxyl groups substituted at the 2', 4', 3, 5, and 7 position, and quercetin at the 3', 4', 3, 5, and 7 positions. Both are natural products.

Several workers (24, 29, 32, 33) have reported methods for the determination of beryllium with morin. These methods were based on the fluorescence of beryllium-morin compounds in aqueous sodium hydroxide solutions. Fletcher (11) who has studied the beryllium-morin system in detail, reported the formation of a one to one beryllium : morin monomeric compound, a one to one dimeric compound and a one to two compound and the corresponding formation constants. Five approximate ionization constants ranging from 10^{1} to 10^{-13} were reported for morin, each supposedly corresponding to the ionization of a phenol to a phenolate ion. It would appear very likely, however, that the first ionization constant is that of the oxonium ion,



and not that of hydroxy group as indicated by Fletcher (11).

Tomic and Hecht (34) reported a microanalytical determination of uranyl ion with morin based on the quenching of fluorescence proportional to the concentration of uranyl ion.

Geiger and Sandell (13) reported a method for the determination of zirconium with morin in two molar hydrochloric acid solution. The method was based on the decrease in the fluorescence when EDTA was added to the solution. Fletcher and Milkey (12) have studied morin as a spectrophotometric reagent for thorium.

Quercetin, which differs from morin only by the position of one hydroxyl group, has been studied as a spectrophotometric reagent for zirconium (15) and thorium (25).

Flavonol, 3-hydroxyflavone, has been used for the fluorometric determination of zirconium in minerals by Alford, Shapiro, and White (1). The method is based on the blue-fluorescence of zirconium plus flavonol in moderately strong sulfuric acid solution when exposed to ultraviolet light. Coyle and White (7) developed a similar procedure for the fluorometric determination of tin (IV) with flavonol. Bottei and Trusk (6) have used flavonol for the fluorometric determination of tungsten. This method is based on the blue fluorescence of flavonol plus tungstate ion at pH 4.

Murata (26, 27) and co-workers have studied 3-hydroxychromone and 5-hydroxychromone as analytical reagents. They found that 5-hydroxychromone forms complexes with Cu^{2+}, Be^{2+}, Al^{3+}, Sn^{4+}, Ti^{4+}, Bi^{3+}, U^{6+}, Fe^{3+}, and Pd^{2+} which were extractable into organic solvents. Complexes with Th^{4+}, Co^{2+}, and Ni^{2+} were not extractable. 5-Hydroxychromone may be used as a reagent for the spectrophotometric determination of Cu^{2+},

Be^{2+}, Al^{3+}, Ti^{4+}, Fe^{3+}, and Pd^{2+}. The beryllium complex fluoresces strongly under ultraviolet radiation, and beryllium can be determined fluorometrically. 3-Hydroxychromone reacts with Zn^{2+}, Cd^{2+}, Hg^{2+}, U^{6+}, Mn^{2+}, Cu^{2+}, Al^{3+}, Ga^{3+}, In^{3+}, Tl^{3+}, Sn^{4+}, Pb^{2+}, Zr^{4+}, Th^{4+}, Fe^{3+}, Co^{2+}, Ni^{2+}, and Pd^{2+}. All except Zn^{2+}, Cd^{2+}, Hg^{2+}, U^{6+}, and Mn^{2+} are extractable into organic solvents.

Each of the reagents, morin, quercetin, flavonol, 3-hydroxychromone, and 5-hydroxychromone, has a hydroxyl group in the 3 position, 5 position or both. From the data of Murata et al. (26, 27) it is evident that a hydroxyl group in the 3 or 5 position is sufficient for a benzo-γ-pyrone to react with a metal. In the consideration of a benzo-γ-pyrone as a parent molecule for a Calcein type indicator, it would be advantageous to avoid a pyrone with a hydroxyl group in the 3 and/or 5 position because a hydroxyl group in one of these positions could cause a reaction which would be independent of the methyleneiminodiacetic acid group. It would also be advisable to avoid the hydroxyl group in the 3 position because flavonols are readily oxidized by air in alkaline solutions (28, p. 257).

Another property of the benzo-γ-pyrones to be considered is their fluorescence and the effect of substituent groups on their fluorescence. Jatkar and Mattoo found that for chromones one hydroxyl group was necessary, but not sufficient, condition to impart fluorescence, (17). For example, fluorescence was not found with 2-methyl-5-hydroxychromone, but was found for

2-methyl-7-hydroxychromone. Acetylation of the 2-methyl-7-hydroxychromone destroyed the fluorescence. The total relative fluorescence of 2-methyl-7-hydroxychromone changed from 10,250 in 10^{-4}M potassium hydroxide in 1,350 in 10^{-4}M hydrochloric acid.

A hydroxyl group was not necessary for fluorescence of flavones (18). It was found that replacing the methoxy group of 4'-methoxyflavone with a benzoyl group caused a slight increase in fluorescence. The introduction of a benzoyl group in the 6 position of 4'-methoxyflavone caused a sharp decrease in the fluorescence. The introduction of a methoxy group in the 4' position of flavonol greatly increased the fluorescence (19).

It was apparent that the 7-hydroxybenzo-γ-pyrones fulfilled the conditions for substrates in the preparation of Calcein type indicators. It was also apparent that the fluorescent intensity could be varied by the substitution of various groups in the molecule.

This work is concerned with the 8-(N,N biscarboxymethyl-aminomethyl) derivatives of 2-methyl-7-hydroxychromone, 7-hydroxyflavone, and 4'-methoxy-7-hydroxyflavone. Preparation for the derivatives are given along with the methods for determination of product purities. The fluorescence and absorption spectra of the methyleneiminodiacetic acid derivatives and their parent molecules as a function of acidity are given. The effect of substitution in the 2 position on the fluorescence of chromones is evaluated. The decomposition of all three

methyleneiminodiacetic acid derivatives and their calcium complex ions in strongly alkaline solution was found to be first order in acid derivative, and the pseudo first order rate constants are reported. The change in the fluorescent spectra of the derivatives upon addition of either calcium, barium, strontium, magnesium, zinc, or copper is reported. The stoichiometry of the calcium-derivative complex ion is given for each iminodiacetic acid derivative. A method for the estimation of metals in potassium hydroxide capable of giving fluorescent metal-iminodiacetic acid derivative complex ions at pH greater than 13 is given. The fluorescence-concentration relations for calcium and magnesium in 0.5 to 2 M potassium hydroxide are reported. The iminodiacetic acid derivatives are suitable for use as indicators for EDTA titrations and for fluorometric reagents for calcium in the presence of magnesium.

EXPERIMENTAL WORK

Reagents

2', 4',-Dihydroxyacetophenone and p-anisoyl chloride were obtained from Aldrich Chemical Company, ethyl benzoylacetate from Eastman Organic Chemicals, and disodium iminodiacetate monohydrate from Geigy Chemical Company. All other chemicals were reagent grade and were used without further purification.

Approximately 0.1 M sodium hydroxide solutions were prepared by the dilution of 50 per cent sodium hydroxide solution and were standardized against potassium acid phthalate. Approximately 0.1 M hydrochloric acid solutions were standardized by titration with the standard sodium hydroxide solutions. One molar and four molar potassium hydroxide solutions were prepared from reagent grade potassium hydroxide pellets from Allied Chemical.

A 0.00999 M solution of calcium chloride was prepared by dissolving primary standard grade calcium carbonate from G. F. Smith Chemical Company in dilute hydrochloric acid. A 0.01012 M zinc chloride solution was made by dissolving electrolytic zinc of unknown origin in hydrochloric acid. Pure copper wire was dissolved in nitric acid to make a 0.01001 M copper nitrate solution. A 0.0868 M EDTA solution was prepared from ethylenediaminetetraacetic acid and potassium hydroxide. The EDTA solution was standardized by titration with standard calcium chloride solution. Approximately 0.01 M solutions of magnesium

chloride, barium chloride, and strontium chloride were prepared and were standardized against a standard EDTA solution.

A solution of 0.998 x 10^{-3} M 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone was prepared by the titration of 0.3579 grams of the solid chromone with 0.1176 N sodium hydroxide. The molarity of the chromone solution was calculated from the volume of base, 16.98 ml., required to reach the second end point. After addition of 0.1 M hydrochloric acid to lower the pH to about 8, the solution was diluted to one liter. A 0.969 x 10^{-3} M solution, 0.3954 g./l, of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone and a 1.067 x 10^{-3} M solution, 0.4594 g./l, of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone were prepared in a similar manner.

Solutions of 10^{-3} M 2-methyl-7-hydroxychromone, 10^{-3} M 7-hydroxyflavone, and 10^{-3} M 4'-methoxy-7-hydroxyflavone were prepared by dissolving weighed amounts of the solids in dilute potassium hydroxide. Before diluting to volume the acidity was adjusted to about pH 10 with hydrochloric acid.

Synthesis of 2-methyl-7-hydroxychromone

The method used to synthesize 2-methyl-7-hydroxychromone was similar to that used by Blaise (5) for the preparation of 7-hydroxyflavone. The reaction was carried out in a two liter round bottom flask which was fitted with a glass fractionating column and condenser. A mixture of 110 g. of resorcinol, 130 g. of ethyl acetoacetate, and 100 ml. of nitrobenzene was

heated for two hours so as to reflux nitrobenzene and distill the ethanol formed in the reaction. After cooling to room temperature, 300 ml. of ether were added to precipitate the 2-methyl-7-hydroxychromone. The precipitate was filtered, washed with benzene, dissolved in hot ethanol and decolorized with charcoal. After cooling, the yellow crystalline product was filtered from solution. A second recrystallization from glacial acetic acid followed by washing with ethanol gave a slightly yellow product which melted at 254-255° C. Geissman (14) reported a melting point of 253-254° C. for 2-methyl-7-hydroxychromone. The calculated percentages of carbon and hydrogen are 68.18 and 4.56 per cent respectively, and those found by elemental analysis are 68.08 and 4.44 per cent.

Synthesis of 7-hydroxyflavone

Part of the 7-hydroxyflavone was prepared by the method of Blaise (5), and part by the method of Baker (4). For that prepared by the method of Blaise, 50 g. of ethyl benzoylacetate, 35 g. of resorcinol, and 60 ml. of nitrobenzene were placed in a 500 ml. round bottom flask which was fitted with a glass fractionating column and condenser. The mixture was heated for approximately two hours so as to reflux nitrobenzene and distill the ethanol formed in the reaction. The mixture was allowed to cool and 100 ml. of ether were added to precipitate crude 7-hydroxyflavone. This material was washed with hot benzene and dried at 75° C. overnight. The solid was then dissolved in hot

ethanol, the solution was decolorized with charcoal, and 7-hydroxyflavone was reprecipitated by cooling. This recrystallization procedure was repeated until the silky needle crystals finally obtained were only slightly colored. These crystals started to discolor at 235° C. and melted at 241-242° C. Blaise (5) reported a melting point of 240° C.

For the preparation of 7-hydroxyflavone by the method of Baker (4) 30.4 g. of 2', 4'-dihydroxyacetophenone was dissolved in 60 ml. of pyridine, and to this 56.2 g. of benzoyl chloride were added. The mixture was heated on a steam bath with stirring for 15 minutes, then poured into water and stirred for approximately five minutes. The water layer was poured off the heavier oil layer and discarded. The oil was then slurried with 600 ml. of dilute hydrochloric acid solution. After allowing the oil to settle the aqueous layer was discarded. The oil was then slurried with 200 ml. of ethanol. At this point the oil began to solidify, and after the ethanol layer was discarded the rest of the oil solidified. This solid, crude resacetophenone dibenzoate, was washed with ethanol and then recrystallized from about 500 ml. of methanol. Twenty-seven grams of the recrystallized product were placed in a one liter flask along with 60 g. of anhydrous potassium carbonate and 250 ml. of toluene. The mixture was heated on a steam bath with stirring for four hours. The yellow solid, potassium salt of ω: 4-dibenzoylresacetophenone or 2-hydroxy-4-benzoyloxyphenyl benzoylmethyl ketone, and the excess potassium carbonate were

filtered from the solution while hot. This material was then washed with several portions of hot benzene, air dried, and then added to 400 ml. of glacial acetic acid in a one liter flask. As the mixture was heated, the solid dissolved, and the resulting solution was refluxed overnight. The acetic acid solution was then poured into 1400 ml. of water to precipitate the crude 7-hydroxyflavone. The flavone was filtered from the solution, recrystallized from ethanol and dried at 70° C. The melting point was 242-243° C. while that reported by Baker (4) was 240° C.

Synthesis of 4'-methoxy-7-hydroxyflavone

The method of Baker (4) was used for the preparation of 4'-methoxy-7-hydroxyflavone starting with 2',4'-dihydroxyacetophenone and p-anisoyl chloride. The procedure was identical to that described for 7-hydroxyflavone except that the intermediate, resacetophenone dianisate, was heated with potassium carbonate in toluene for 12 hours instead of four. The melting point of the product was 268-269° C. Baker (4) reported a melting point of 263-264° C. with rapid heating.

Synthesis and characterization of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone

2-Methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-chromone was prepared by the Mannich reaction starting with 8.8 g., 0.045 moles, of disodium iminodiacetate monohydrate, 5.3 g., 0.030 moles, of 2-methyl-7-hydroxychromone, and 5 ml. of 37 per cent formaldehyde. These reactants were heated in 140

ml. of glacial acetic acid to approximately 70° C. After a few minutes all solids dissolved and after four hours of constant stirring at 70° C. a solid began to form. The heating and stirring were continued for approximately ten hours after the first solid appeared. The mixture was allowed to cool, and the crude product was filtered from solution. The crude material was dissolved in dilute sodium hydroxide and reprecipitated with hydrochloric acid. The resulting white crystalline material was dried at 100° C. The melting point is 155-178° C. with decomposition.

The neutralization equivalent weight, 177.6 g. per equivalent, was determined by the potentiometric titration of 0.2635 g. of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-chromone with 0.1173 N sodium hydroxide. The titration curve is shown in figure 1. The theoretical neutralization equivalent weight for anhydrous 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone, assuming two replaceable hydrogens, is 160.6 per equivalent. Increasing the drying time and temperature caused a decrease in the neutralization equivalent weight; however, the compound began to discolor before the theoretical equivalent weight was reached. A thermogravimetric analysis showed a steadily increasing loss of weight as the temperature changed from 70° C. to 170° C. At 170° C. the rate of decrease in weight became much more rapid. Therefore, it appears that there are no optimum drying conditions to yield a compound of definite composition.

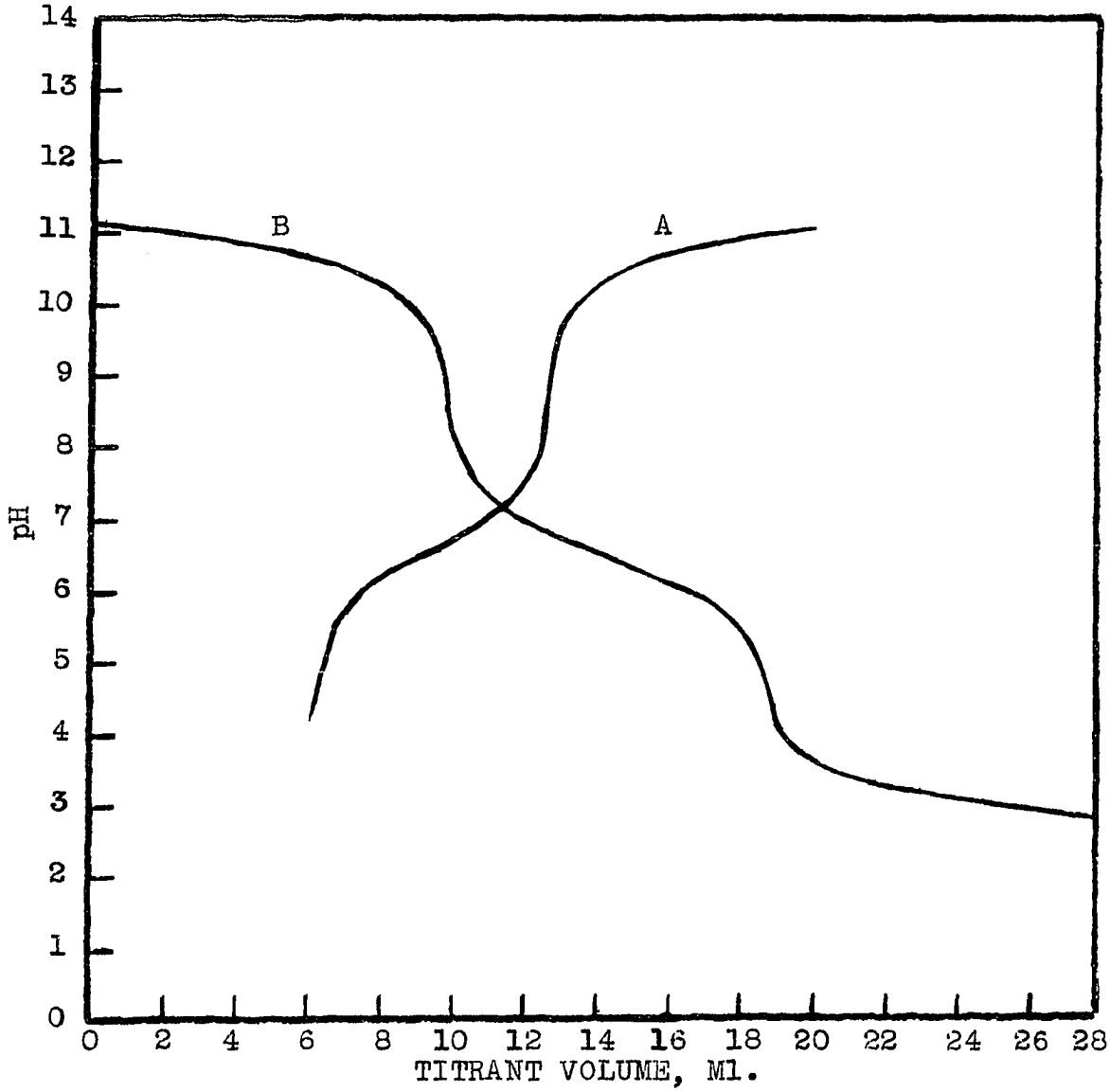


Figure 1. Titration of 0.2635 g. of 2-methyl-7-hydroxy--8-(N,N biscarboxymethylaminomethyl)chromone

A Titration with 0.1173 N sodium hydroxide

B Back titration with 0.0859 N hydrochloric acid

Table 1. Water analysis of 2-methyl-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)chromone

Grams of sample	Equivalent weight	Ml. of reagent	Per cent[a] water	Per cent[b] water
0.1353	179.1	4.10	10.3	10.3
0.1308	179.1	4.25	11.0	10.3
0.1509	179.1	4.50	10.1	10.3
0.0674	164.1	.50	2.5	2.5

[a]Calculated from the Karl Fischer titration.

[b]Calculated from the difference between the experimental and theoretical equivalent weights.

The amount of water present in different samples of the compound was determined by Karl Fischer titrations. Weighed samples of about 0.1 g. were suspended in methanol and titrated with Karl Fischer reagent which was standardized against weighed amounts of water. The end point was detected by the dead stop method. Table 1 gives the size of the sample taken, the neutralization equivalent weight determined by a potentiometric titration, the ml. of Karl Fischer reagent required, the per cent of water as determined by the Karl Fischer titration and the per cent water calculated from the difference in the equivalent weight and the theoretical equivalent weight.

Elemental analysis by Galbraith Laboratories, Inc. on two samples of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone gave carbon, 51.28 per cent; hydrogen 5.16 per cent; and nitrogen, 4.97 per cent for one sample, and carbon,

50.93 per cent; hydrogen, 5.40 per cent; and nitrogen, 4.17 per cent for the other sample. The analysis of the former corresponds to the formula $C_{15}H_{18.2}N_{1.25}O_{8.48}$ and the later corresponds to $C_{15}H_{19.1}N_{1.06}O_{8.74}$. The excess hydrogen and oxygen in these formulas compared to the theoretical formula of $C_{15}H_{15}NO_7$ is $H_{3.2}O_{1.5}$ in the one case and $H_{4.1}O_{1.7}$ in the other. These amounts of excess hydrogen and oxygen, assumed to be present as water, agree reasonably well with the findings in the equivalent weight titrations and the water analyses.

Because of the large discrepency in the amount of nitrogen found in one of the above elemental analyses, various samples of the compound were analyzed for nitrogen by the kjeldahl method. Weighed amounts were digested in concentrated sulfuric acid with copper selenite as a catalyst. Ammonia was the distilled by boiling the digested sample in a strong sodium hydroxide solution. The ammonia was collected in 0.0859 N hydrochloric acid, and the excess acid was back titrated with 0.1173 N sodium hydroxide. The per cent nitrogen found and the per cent calculated from the neutralization equivalent weight of each sample are given in table 2.

A 0.8692 g. sample of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone left no weighable residue upon ignition in a platinum crucible.

Nuclear magnetic resonance spectra of 2-methyl-7-hydroxychromone, figure 2, and its methyleneiminodiacetic acid derivative, figure 3, were obtained on a Varian Model A 60

Table 2. Kjeldahl analysis of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone

Grams of sample	Equivalent weight	Per cent nitrogen found	Per cent nitrogen calculated[a]
0.3594	176.6	4.01	3.97
0.3332	170.4	4.11	4.11
0.3090	165.2	4.27	4.24

[a]Calculated assuming one-half nitrogen per neutralization equivalent.

spectrometer in order to confirm the structure of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone. The solvent used was deuterium oxide and potassium carbonate, the radio frequency field was 80,000 gauss, and the reference standard was Tiers' salt, sodium 3-(trimethylsilyl)-1-propanesulfonate.

Two acid dissociation constants were determined from the potentiometric titration of 0.2635 g. of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone dissolved in 70 ml. of water and 2.346 millimoles of sodium hydroxide using 0.0859 N hydrochloric acid as titrant, figure 1. The constants obtained are $10^{-2.9}$ and $10^{-6.45}$.

Evidence of the reaction of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone with calcium ion was observed when 0.1904 grams, 0.599 millimoles, of the derivative, 0.1010 grams, 1.009 millimoles, of primary standard grade calcium carbonate, and 25.00 ml. of 0.0859 N hydrochloric acid were

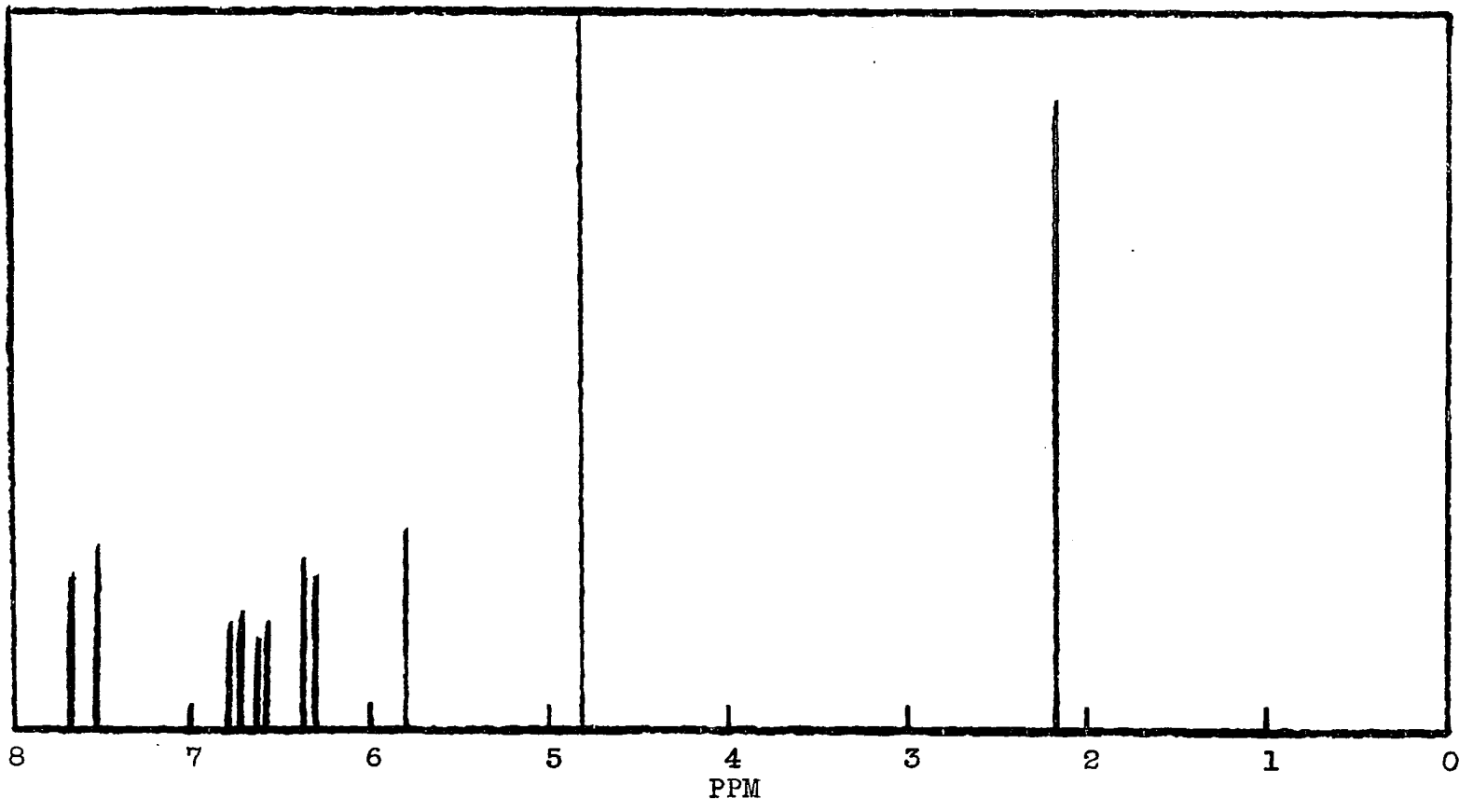


Figure 2. Representation of the N.M.R. spectrum of 2-methyl-7-hydroxychrome

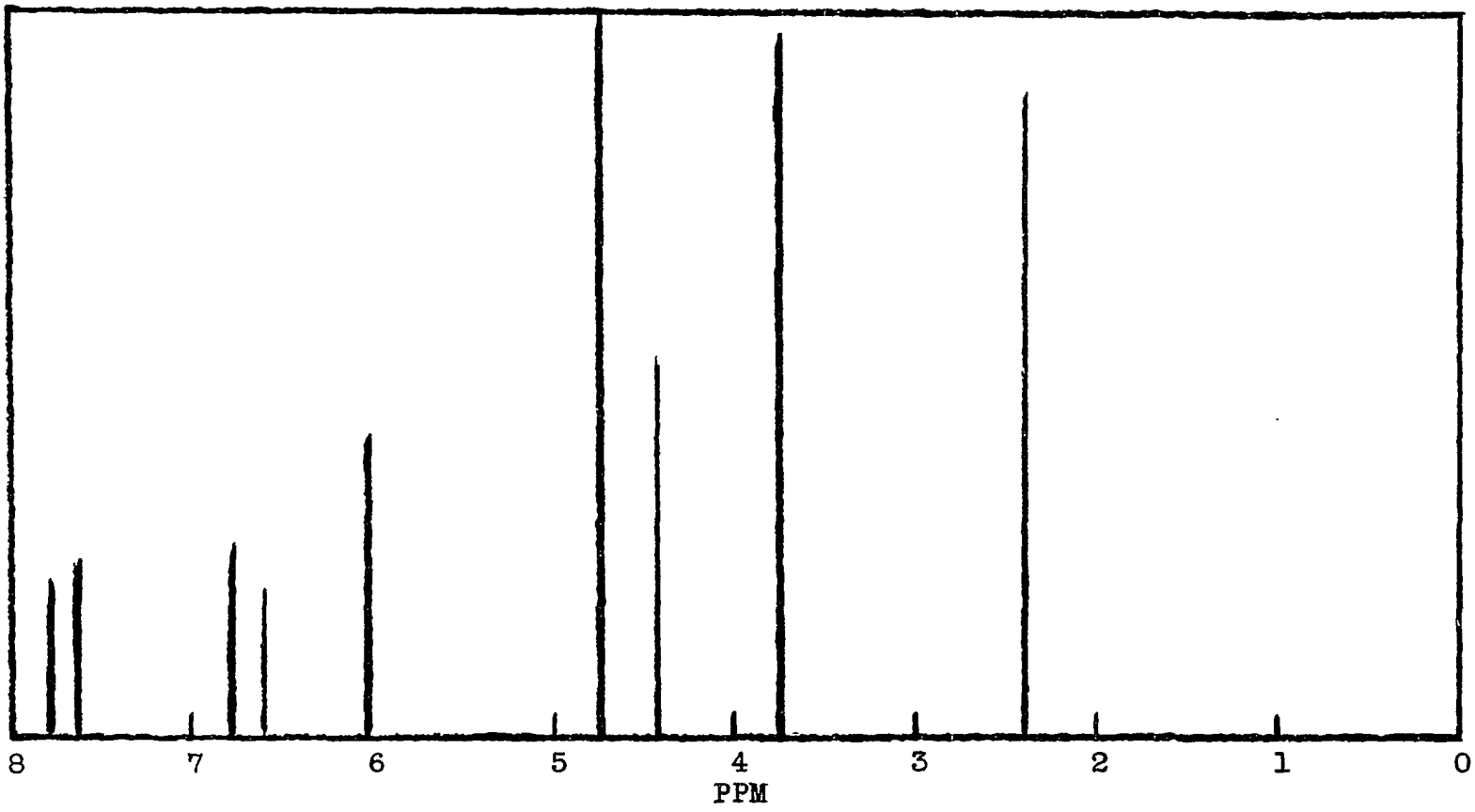


Figure 3. Representation of the N.M.R. spectrum of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone

titrated potentiometrically with 0.1173 N sodium hydroxide solution, figure 4. The 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone and calcium carbonate were slurried in 50 ml. of water until much of the solid had dissolved. Then the hydrochloric acid was added slowly causing all the solid to dissolve; however, a white precipitate appeared after a few minutes of stirring. The mixture was purged of carbon dioxide by a stream of nitrogen and was then titrated with sodium hydroxide.

Synthesis and characterization of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

7-Hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone was prepared by the Mannich reaction starting with 5.8 grams, 0.03 moles, of disodium iminodiacetate monohydrate, 4.8 grams, 0.02 moles, 7-hydroxyflavone, and 5 ml. of 37 per cent formaldehyde. These materials were heated in 140 ml. of glacial acetic acid to approximately 70° C. After a few minutes all solids dissolved and after one-half hour of constant stirring at 70° C. a solid began to form. The heating and stirring were continued for approximately 12 hours after the first solid appeared. The mixture was allowed to cool, and the crude product was filtered from solution. The crude material was dissolved in dilute sodium hydroxide and reprecipitated with hydrochloric acid. The precipitate was yellow-green in color and gelatinous. After drying at 70° C. the solid was yellow

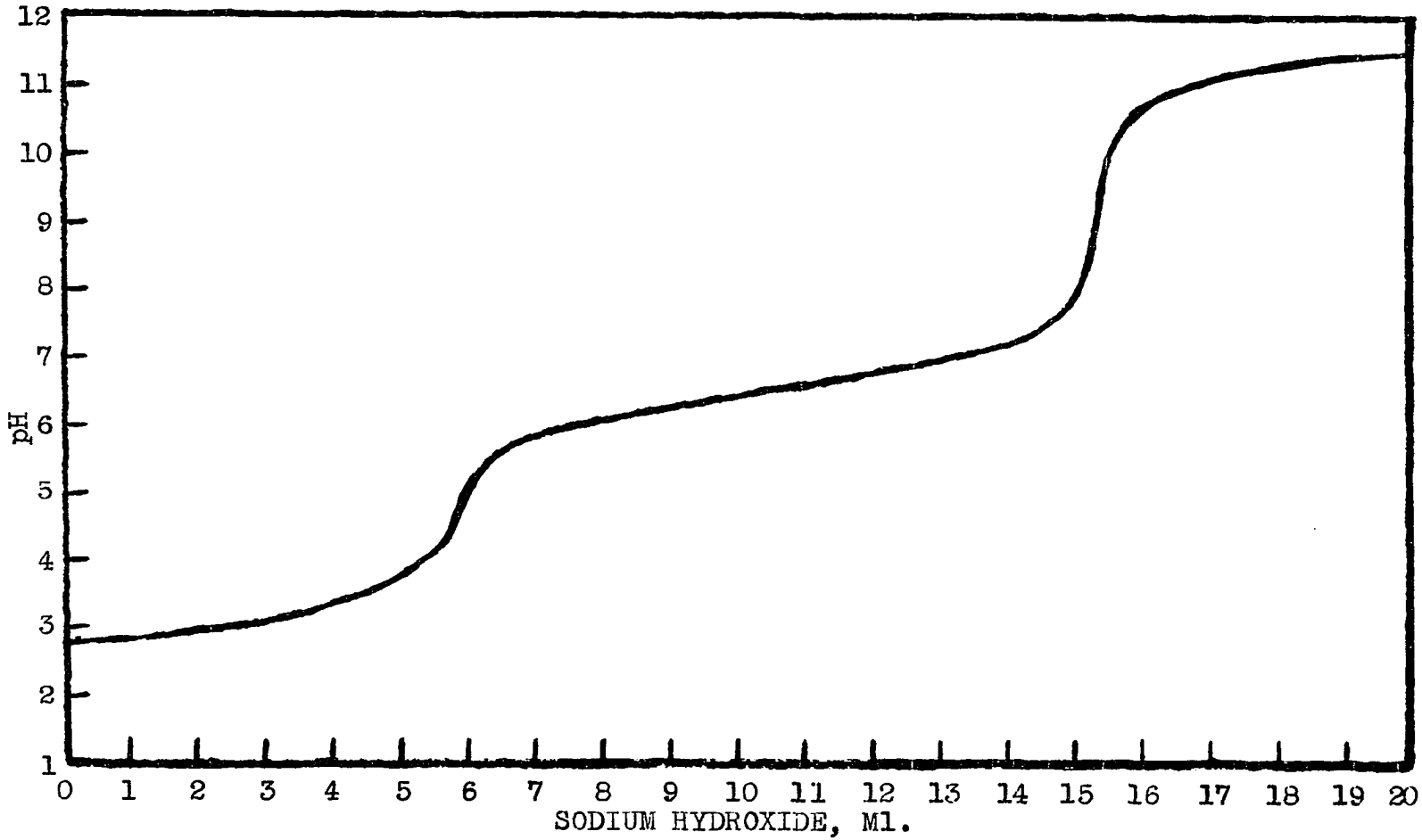


Figure 4. Titration of 0.559 millimoles of 2-methyl-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)chromone, 1.01 millimoles of calcium carbonate, and 2.15 millimoles of hydrochloric acid with 0.1173 N sodium hydroxide

and caked. The yellow cakes were powdered in an agate mortar and dried again. The material shows no melting point, but it decomposes at 150° C.

The neutralization equivalent weight, 197.7 g. per equivalent, was determined by the potentiometric titration of 0.1194 grams of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone with 0.1173 N sodium hydroxide, figure 5. The theoretical neutralization equivalent weight for the 7-hydroxyflavone derivative, assuming two replaceable hydrogens, is 191.6 grams per equivalent.

Elemental analysis by Galbraith Laboratories Inc. showed carbon to be 60.41 per cent; hydrogen, 4.61 per cent; and nitrogen, 3.60 per cent. This corresponds to a compound of $C_{20}H_{18.43}N_{1.02}O_{7.80}$. The excess hydrogen and oxygen in this formula compared to the theoretical formula of $C_{20}H_{17}NO_7$ is $H_{1.43}O_{0.80}$ or 7.1 grams per equivalent which is in reasonable agreement with the difference between the theoretical and experimental value of the neutralization equivalent weight, 6.1 grams per equivalent.

The 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone was analyzed for water by a Karl Fischer titration. The procedure was identical to that used for the analysis of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone. The water found in a 0.0747 gram sample was 5.95 per cent. This was considerably higher than the three per cent calculated from the elemental analysis and neutralization

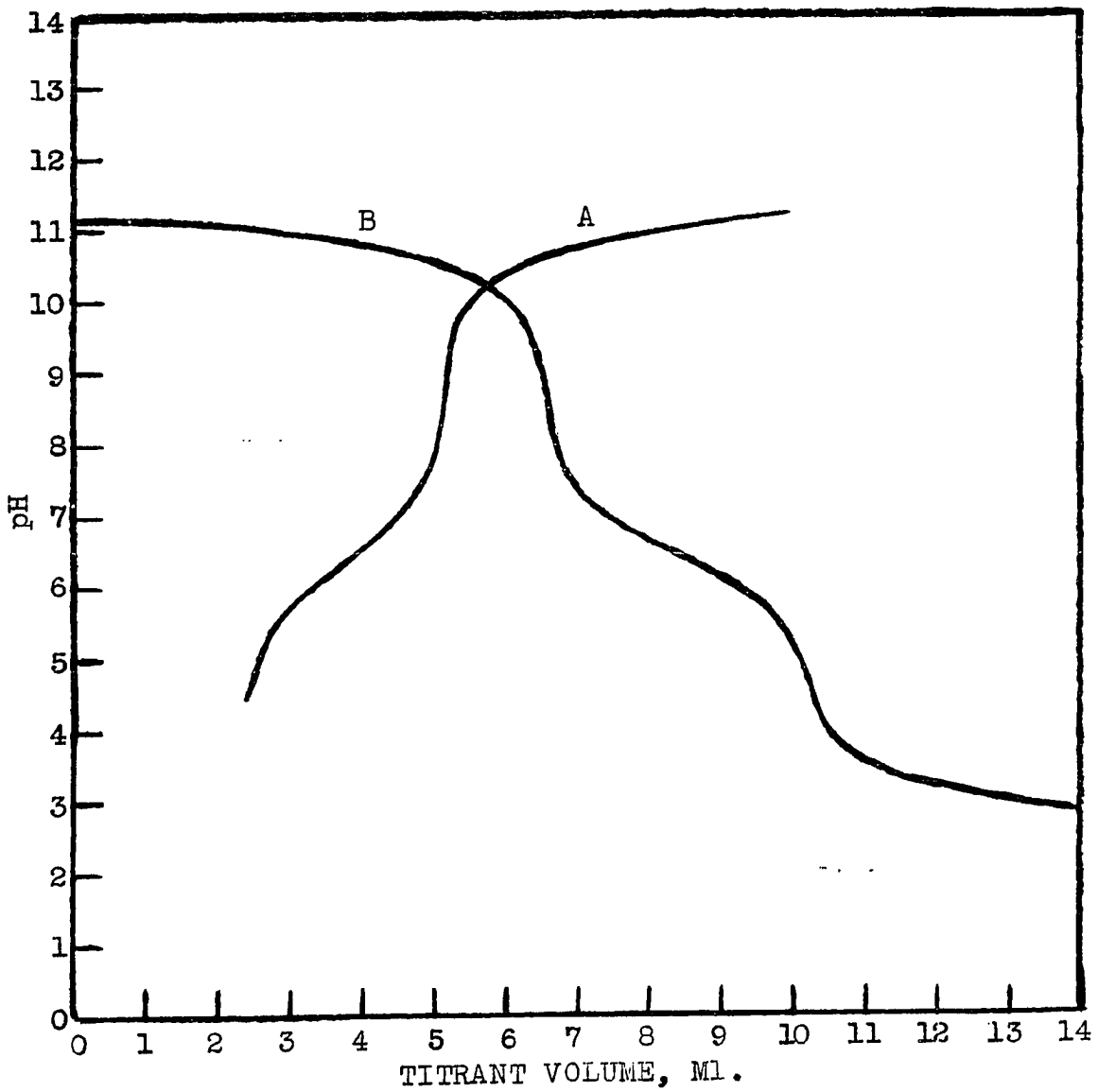


Figure 5. Titration of 0.1194 g. of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

A Titration with 0.1173 N sodium hydroxide

B Back titration with 0.0859 N hydrochloric acid

equivalent weight titration. Therefore, it was suspected that the material absorbed moisture. The per cent water calculated from the data obtained from a different potentiometric titration performed shortly before the water analysis was 6.12 per cent which is in good agreement with 5.95 per cent found by the Karl Fischer titration.

A nuclear magnetic resonance spectrum of 7-hydroxy-8-(N, N biscarboxymethylaminomethyl)flavone, figure 6, was obtained on a Varian Model H R 60 in order to confirm its structure. The solvent was deuterium oxide and potassium carbonate, the radio frequency field was 14,100 gauss and the reference standard was Tiers' salt.

Two acid dissociation constants were determined from the potentiometric titration of 0.1194 grams of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone dissolved in 60 ml. of water and 1.173 millimoles of sodium hydroxide using 0.0859 N hydrochloric acid as titrant, figure 5. The constants obtained were $10^{-2.8}$ and $10^{-6.37}$.

Synthesis and characterization of 4'-methyoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

4'-Methyoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone was also prepared by the Mannich reaction starting with 2.9 grams, 0.015 moles, of disodium iminodiacetate monohydrate, 2.7 grams, 0.01 moles, of 4'-methoxy-7-hydroxyflavone, 3 ml. of 37 per cent formaldehyde and 100 ml. of glacial

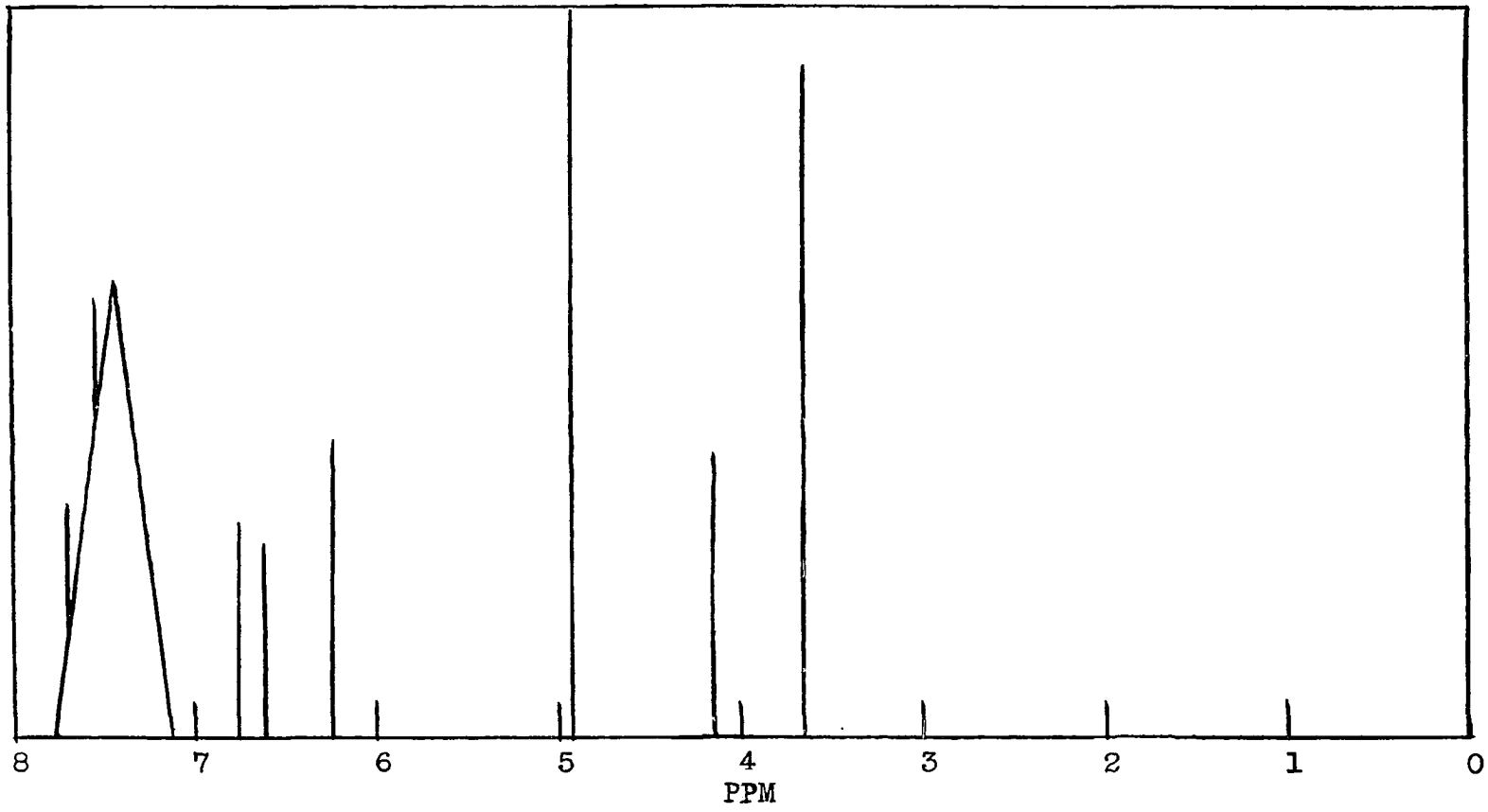


Figure 6. Representation of the N.M.R. spectrum of 7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)flavone

acetic acid. The procedure was identical to that used for 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone. The physical appearance of the solid 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone is the same as that of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone. However, its decomposition temperature, 190° C., is considerably higher.

The neutralization equivalent weight, 215.1 grams per equivalent, was determined by potentiometric titration of 0.2297 grams of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone with 0.1176 N sodium hydroxide, figure 7. The theoretical neutralization equivalent weight, assuming two replaceable hydrogens, is 206.7 grams per equivalent.

Elemental analysis by Galbraith Laboratories Inc. showed carbon to be 59.18 per cent; hydrogen, 4.90 per cent; and nitrogen, 3.30 per cent. This corresponds to a compound with a formula of $C_{21}H_{20.9}N_{1.01}O_{8.69}$. The excess hydrogen and oxygen in the formula compared to the theoretical, $C_{21}H_{19}NO_8$ is $H_{1.9}O_{.69}$ or 6.4 grams per equivalent which compares favorably with differences between the theoretical and experimental value of the neutralization equivalent weight, 8.4 grams per equivalent.

The 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone was analyzed for water by a Karl Fischer titration. The procedure was identical to that used for the analysis of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-chromone. The water found in a 0.0980 gram sample was 3.93

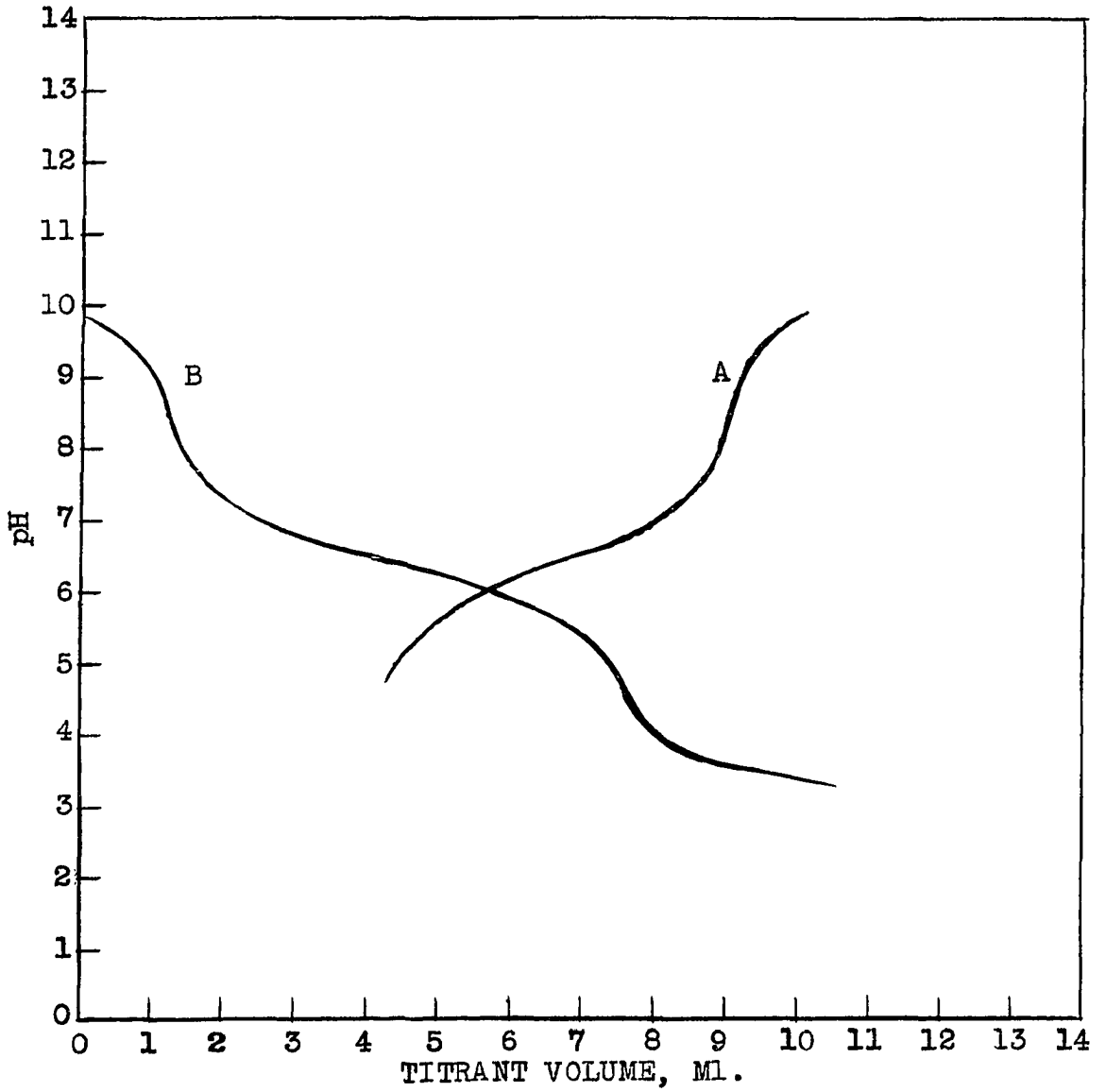


Figure 7. Titration of 4'-methoxy-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)flavone

A Titration with 0.1176 N sodium hydroxide

B Back titration with 0.0859 N hydrochloric acid

per cent compared to 3.88 per cent calculated from the difference in the experimental and theoretical equivalent weights.

A nuclear magnetic resonance spectrum of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone, figure 8, was obtained on a Varian Model H R 60 in order to confirm its structure. The solvent was dueterium oxide and potassium carbonate, the radio frequency field was 14,100 gauss and the reference standard was Tiers' salt.

Two acid dissociation constants, $10^{-3.0}$ and $10^{6.37}$, were determined from the potentiometric titration of 0.2297 grams of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone dissolved in 60 ml. of water and 1.173 millimoles of sodium hydroxide using 0.0859 N hydrochloric acid as titrant, figure 7.

Absorption and Fluorescence

Spectra of 2-methyl-7-hydroxychromone

The absorption spectra of 2×10^{-5} M solutions of chromone at different acidities were obtained with a Carey Model 15 recording spectrophotometer, figure 9. One buffered solution was 0.01 M in citric acid and 0.01 M in dihydrogen citrate ions to give a pH of 3.23. The other solution was 0.02 M in potassium carbonate and had a pH of 10.80. Both solutions were 0.002 M in EDTA in order to prevent interference of metal ions. The blank solutions contained the appropriate buffer and EDTA.

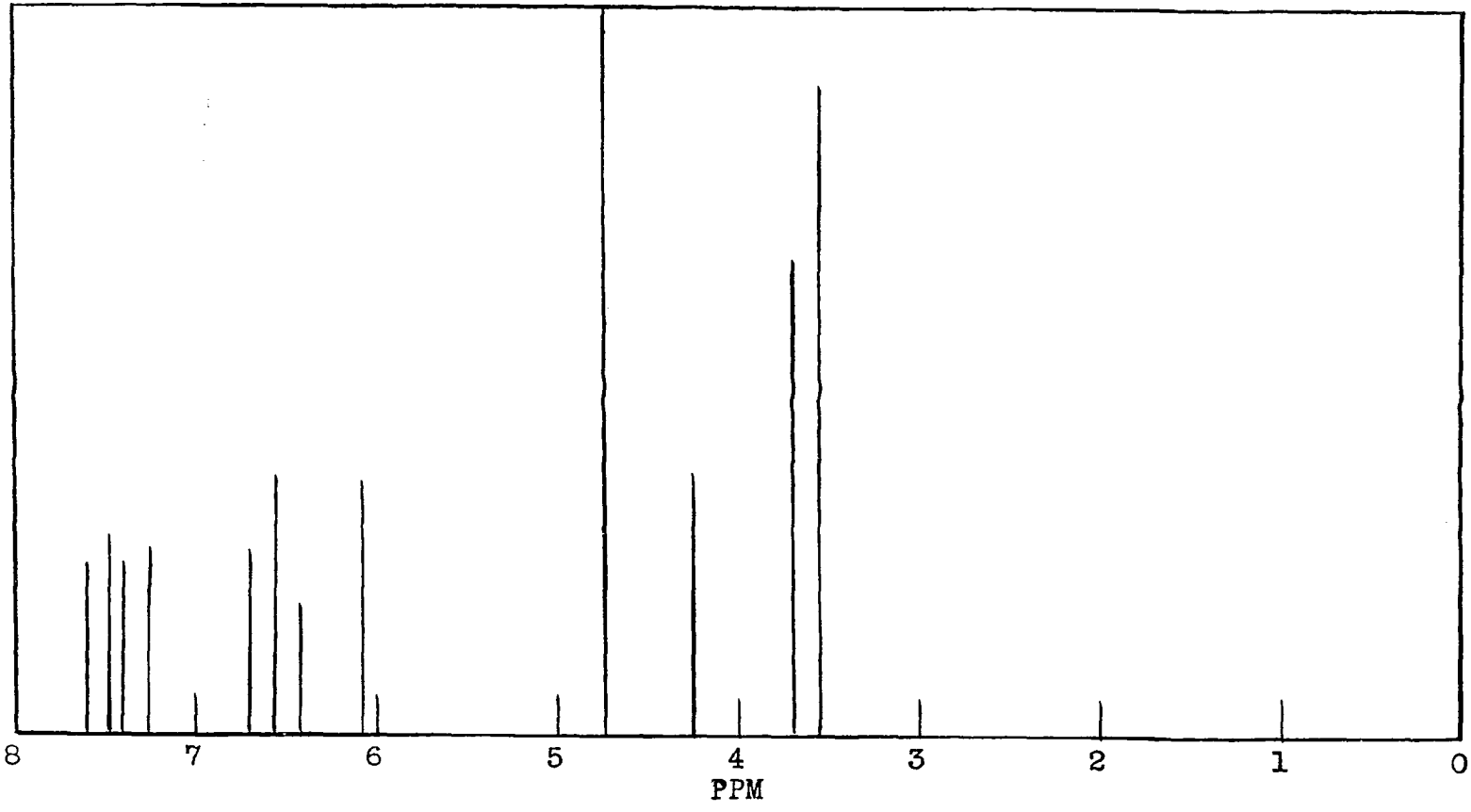


Figure 8. Representation of the N.M.R. spectrum of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

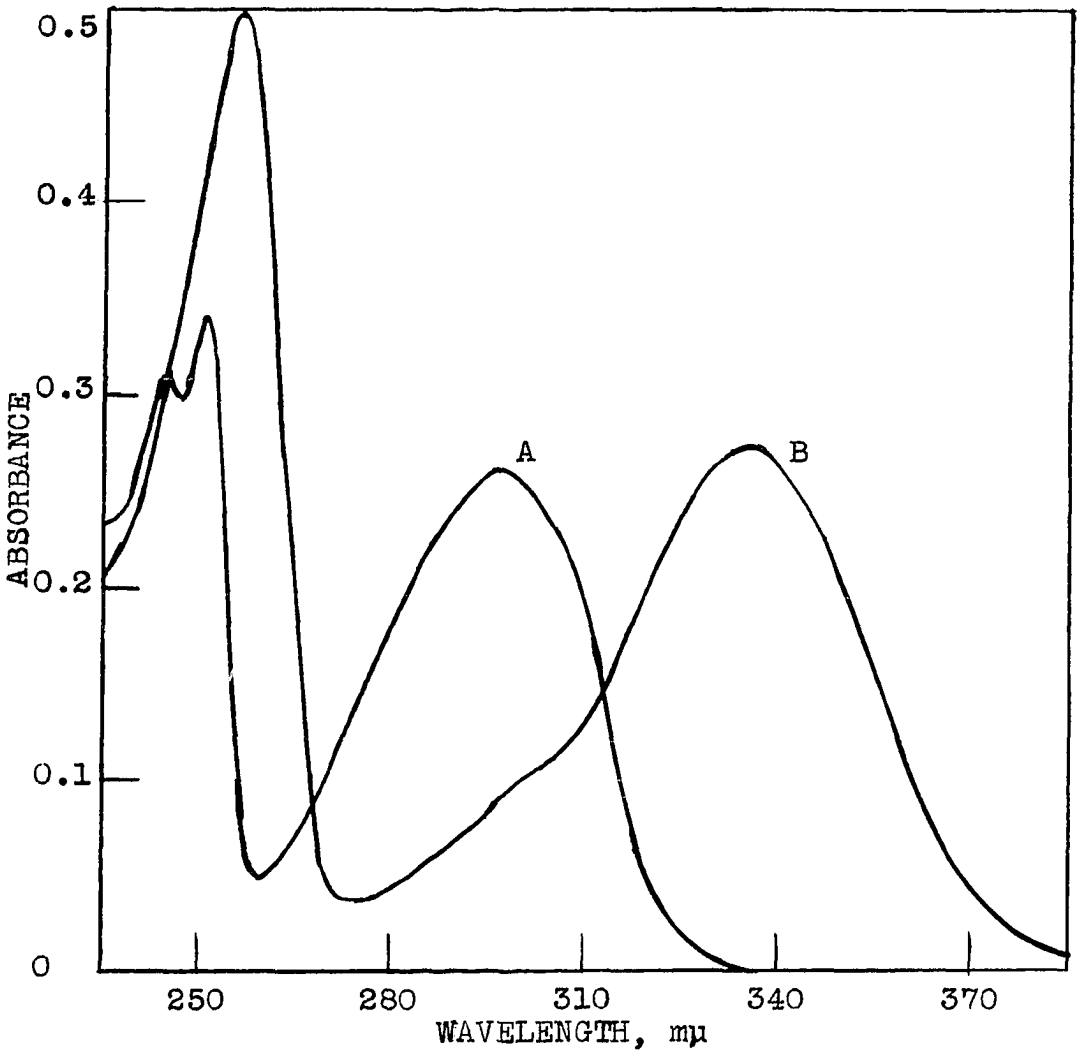


Figure 9. Absorption spectra of 2-methyl-7-hydroxychromone

A pH 3.23

B pH 10.80

The effect of acidity of the solution on the absorbance of a 3 x 10^{-5} M 2-methyl-7-hydroxychromone solution is shown in figure 10. The data for absorbance versus pH at 335 and 295 mμ wavelengths of maximum absorbance obtained from figure 9, were obtained from the potentiometric titration of a solution containing 10 ml. of 0.10 M citric acid, 10 ml. of 0.10 M trishydroxymethylaminomethane, 5 ml. 1 M potassium hydroxide, 3.00 ml. of 1 x 10^{-3} M 2-methyl-7-hydroxychromone and 72 ml. of water, the titrant being 1 M hydrochloric acid. The pH was followed with a Leeds and Northrup pH meter, and the absorbance was followed with a Beckman Model DU spectrophotometer. Absorbance readings were corrected for the effect of the volume change resulting from the addition of titrant. An acid dissociation constant for 2-methyl-7-hydroxychromone calculated from these data is $10^{-7.36}$.

Fluorescent spectra, both fluorescence versus excitation wavelength and fluorescence versus emission wavelength, figure 11, for a 2 x 10^{-6} M aqueous solution of 2-methyl-7-hydroxychromone at pH 10.15 were obtained for comparison with the spectra of the methyleneiminodiacetic acid derivative. There was no significant fluorescence in acidic solutions. Spectra were obtained with an Aminco-Bowman spectrofluorometer.

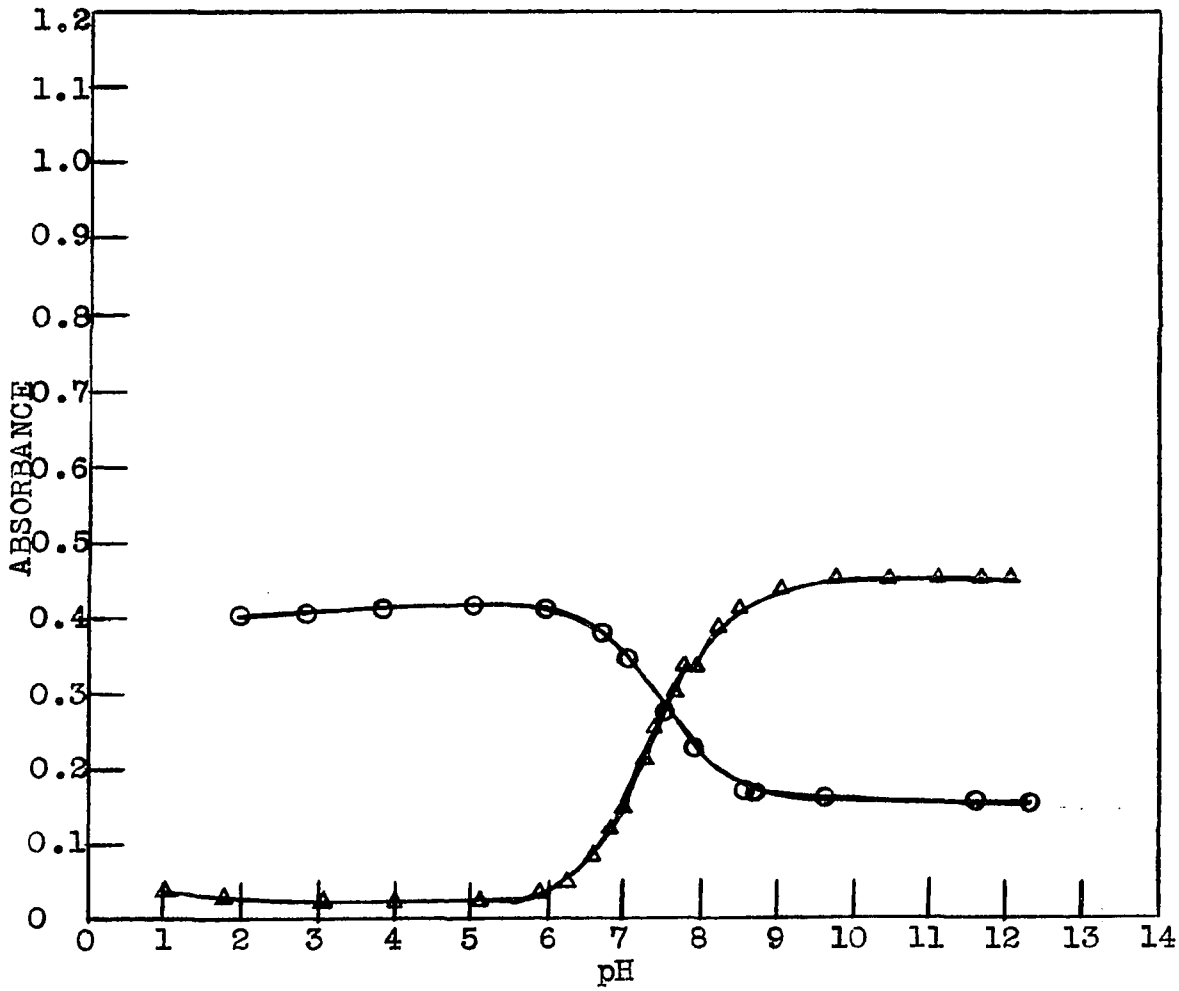


Figure 10. Effect of pH on the absorbance of a 3×10^{-5} M 2-methyl-7-hydroxychromone solution

Wavelength setting
○ 295 mμ
△ 335 mμ

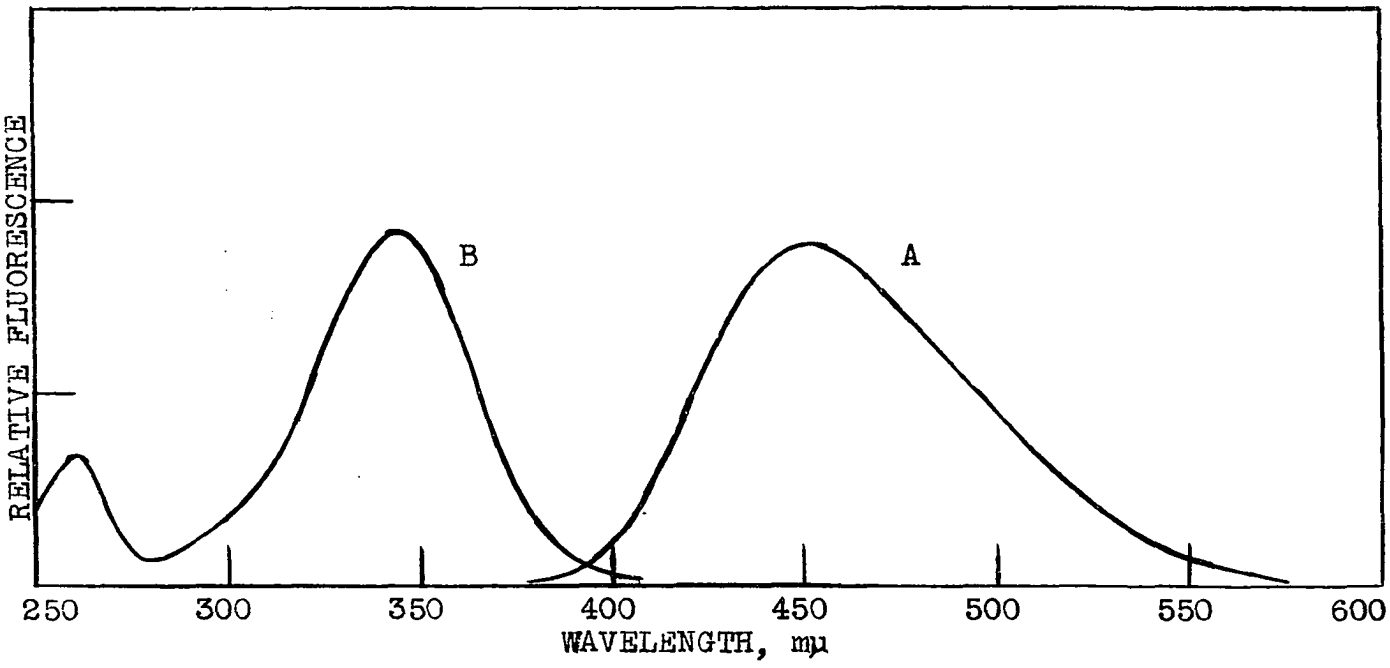


Figure 11. Fluorescence vs. wavelength for 2-methyl-7-hydroxychromone

A Fluorescent spectrum; excitation wavelength, 340 mμ; pH 10.15

B Excitation spectrum; fluorescent wavelength, 450 mμ; pH 10.15

Spectra of 7-hydroxyflavone

The absorption spectra of 2 x 10^{-5} M solutions of 7-hydroxyflavone at different acidities were obtained with a Carey Model 15 recording spectrophotometer, figure 12. One buffered solution was 0.01 M in citric acid and 0.01 M dihydrogen citrate ions to give a pH of 3.26, and the other solution was 0.05 M in potassium carbonate to give a pH of 10.80. Both solutions were 0.002 M in EDTA to sequester metal ions that might effect the spectra. The blank solutions contained the appropriate buffer and EDTA.

The effect of acidity of the solution on the absorbance of a 1 x 10^{-5} M solution of 7-hydroxyflavone is shown in figure 13. The data for absorbance versus pH at 315 and 360 mμ, wavelengths of maximum absorbance in figure 12, were obtained from the potentiometric titration of 100 ml. of a solution containing 1.00 ml. of 1 x 10^{-3} M 7-hydroxyflavone and the buffering agents described for the 2-methyl-7-hydroxychromone system, the titrant being 1 M hydrochloric acid. The pH was followed with a pH meter, and the absorbance was followed with a Beckmann Model DU spectrophotometer. Absorbance readings were corrected for the effect of the volume change resulting from the addition of titrant. The absorbance was less than desirable because of the limited solubility of the 7-hydroxyflavone

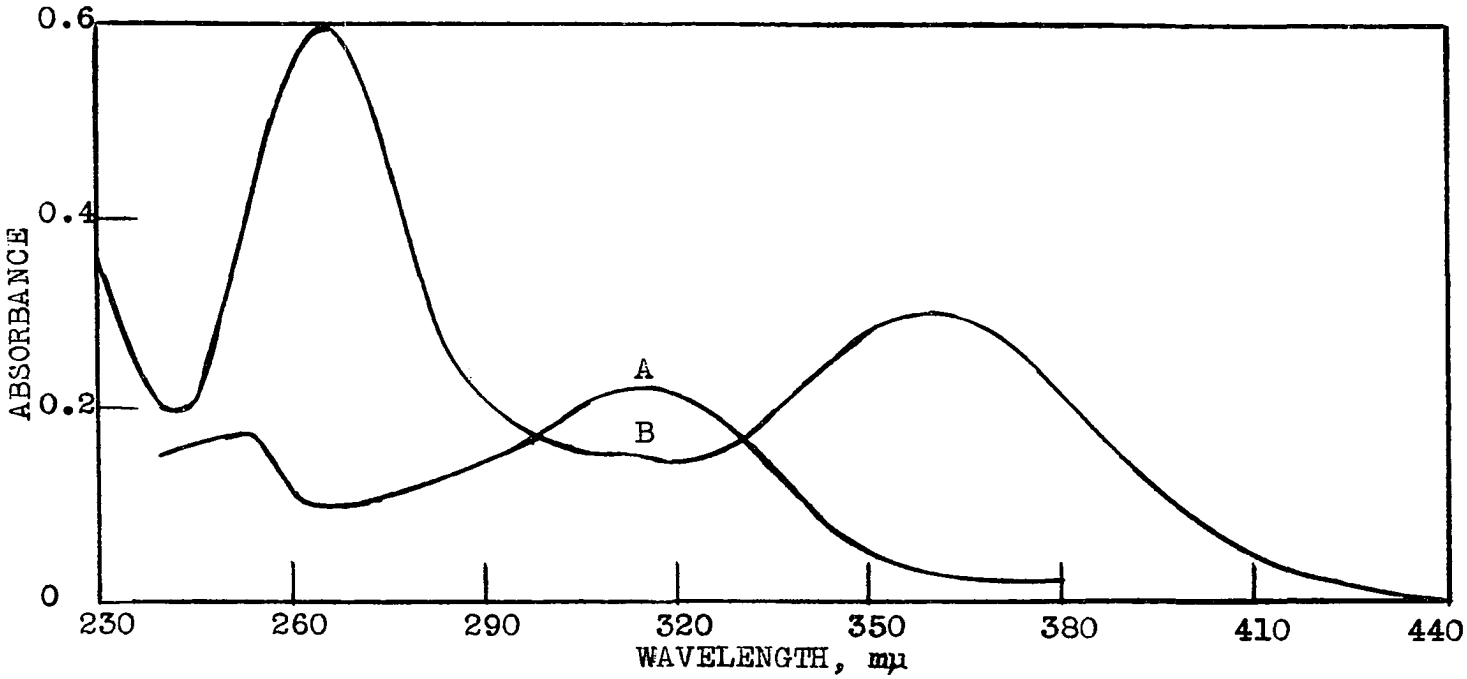


Figure 12. Absorption spectra of 7-hydroxyflavone

A pH 3.26

B pH 10.80

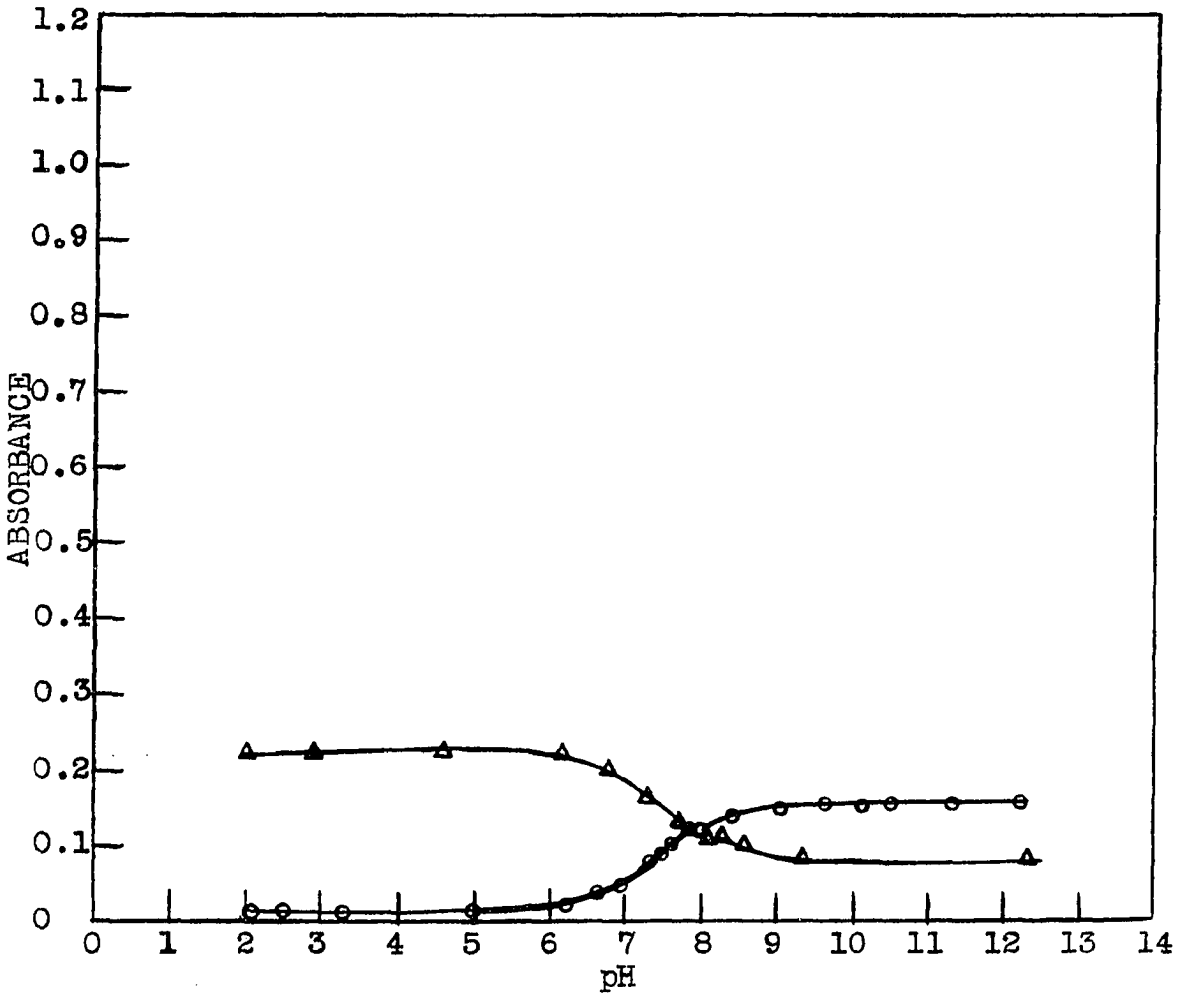


Figure 13. Effect of pH on the absorbance of a 1 x 10^{-5} M solution of 7-hydroxyflavone

Wavelength setting
△ 315 mμ
○ 360 mμ

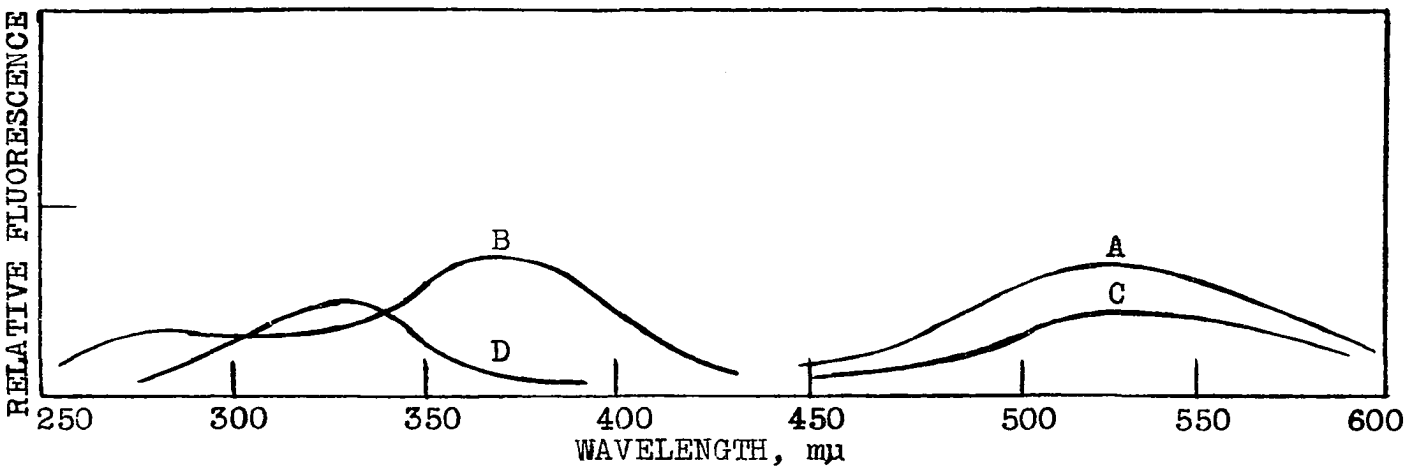


Figure 14. Fluorescence vs. wavelength for 7-hydroxyflavone

A Fluorescent spectrum; excitation wavelength, 360 mμ; pH 10.95

B Excitation spectrum; fluorescent wavelength, 520 mμ; pH 10.95

C Fluorescent spectrum; excitation wavelength, 320 mμ; pH 3.18

D Excitation spectrum; fluorescent wavelength, 525 mμ; pH 3.18

in acidic solutions. An acid dissociation constant, $10^{-7.38}$, was calculated from these data.

Fluorescent spectra, both fluorescence versus excitation wavelength and fluorescence versus emission wavelength, figure 14, for 8×10^{-6} M aqueous solutions of 7-hydroxyflavone at pH 10.95 and 3.18 were obtained for comparison with the spectra of its methyleneiminodiacetic acid derivative.

Spectra of 4'methoxy-7-hydroxyflavone

The absorption spectra of 2×10^{-5} M solutions of 4'-methoxy-7-hydroxyflavone at different acidities were obtained with a Carey Model 15 recording spectrophotometer, figure 15. The acidities of the solutions were pH 3.16 and pH 10.80 which were obtained with the same buffer systems described for the solutions of 2-methyl-7-hydroxychromone. Both solutions were 0.002 M in EDTA to sequester metal ions that might effect the spectra. The blank solutions contained the appropriate buffer and EDTA.

The effect of acidity of the solution on the absorbance of a 1×10^{-5} M solution of 4'methoxy-7-hydroxyflavone is shown in figure 16. The data for absorbance versus pH at 325 and 375 mμ wavelengths of maximum absorbance in figure 15, were obtained in the same manner described for 7-hydroxyflavone. As in the case of 7-hydroxyflavone the absorbance was less than desirable because of the limited solubility of

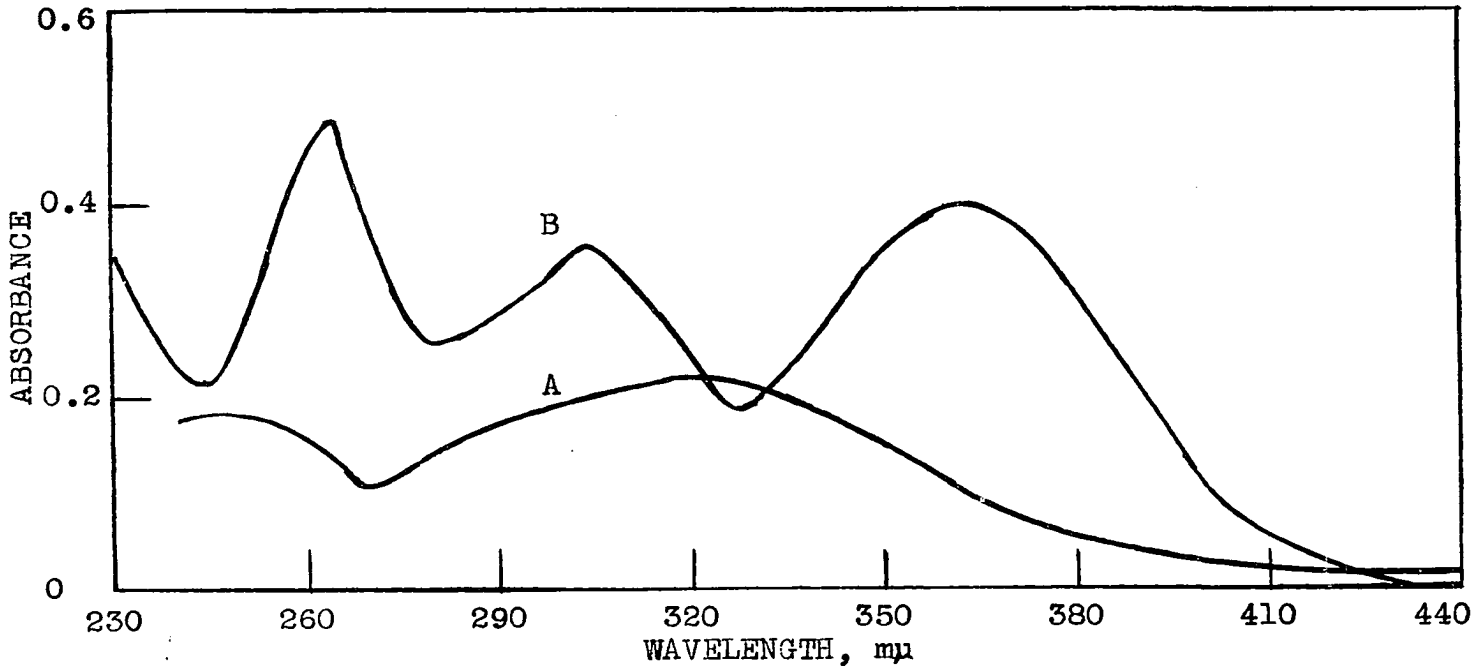


Figure 15. Absorption spectra of 4-methoxy-7-hydroxyflavone

A pH 3.16

B pH 10.80

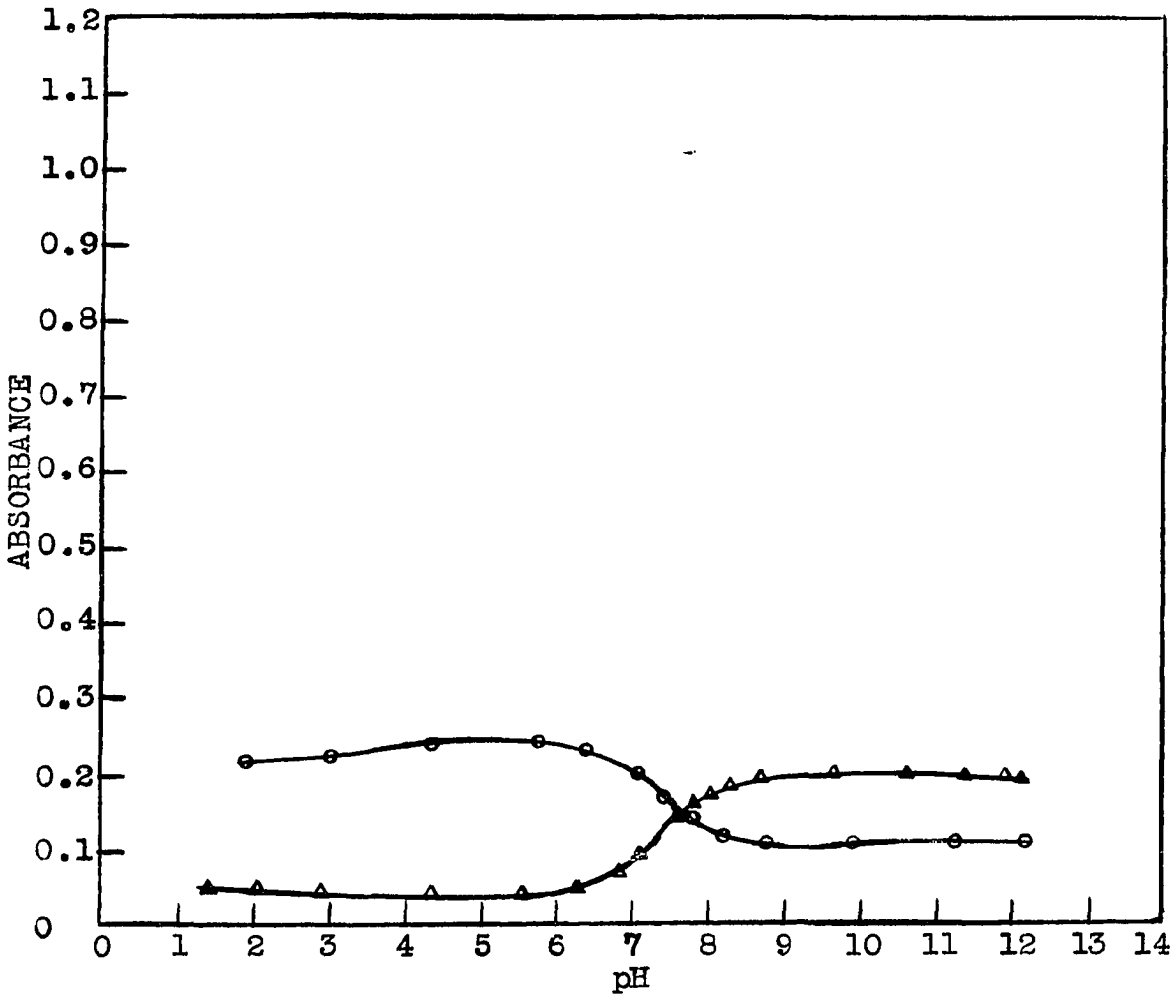


Figure 16. Effect of pH on the absorbance of a 1 x 10^{-5} M solution of 4'methoxyflavone

Wavelength setting
○ 325 mμ
△ 375 mμ

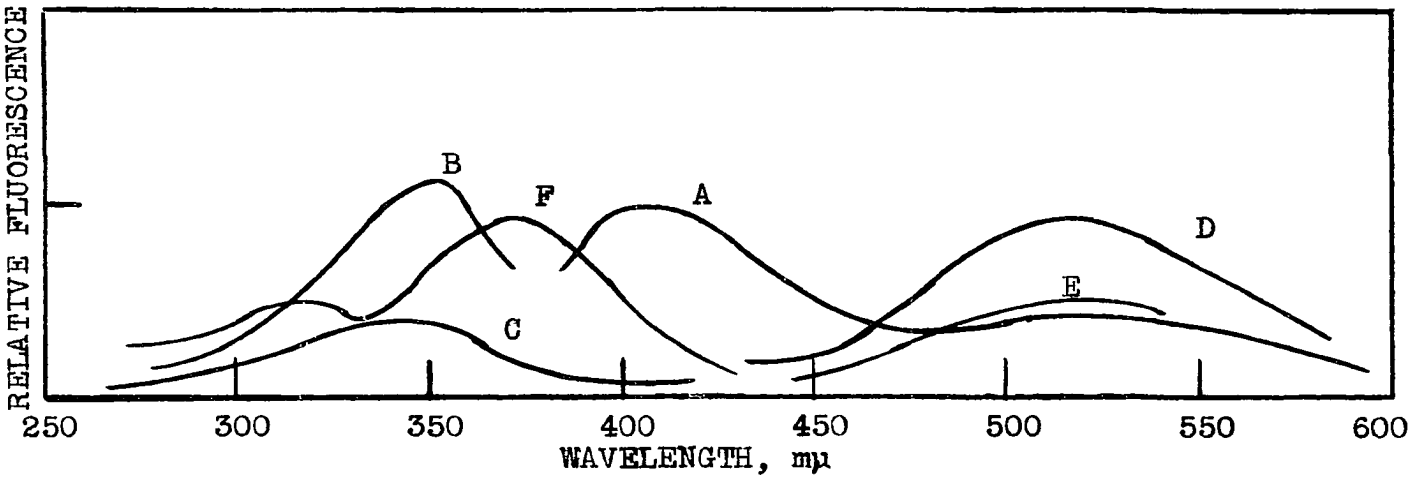


Figure 17. Fluorescence vs. wavelength for 4'methoxy-7-hydroxyflavone

A Fluorescent spectrum; excitation wavelength, 340 mμ; pH 3.20

B Excitation spectrum; fluorescent wavelength, 410 mμ; pH 3.20

C Excitation spectrum; fluorescent wavelength, 520 mμ; pH 3.20

D Fluorescent spectrum; excitation wavelength, 370 mμ; pH 11.05

E Fluorescent spectrum; excitation wavelength, 320 mμ; pH 11.05

F Excitation spectrum; fluorescent wavelength, 520 mμ; pH 11.05

4'-methoxy-7-hydroxyflavone in acidic solution. An acid dissociation constant, $10^{-7.27}$, was calculated from these data.

Fluorescent spectra, both fluoresnce versus excitation wavelength and fluorescence versus emission wavelength, figure 17, for a 8×10^{-6} M aqueous solution of 4'-methoxy-7-hydroxyflavone were obtained for comparison with the spectra of its methyleneiminodiacetic acid derivative. The acidities of the solutions were pH 3.20 and 11.05.

Spectra of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone

Absorption spectra of 2×10^{-5} M solutions of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone at different acidities were obtained with a Carey Model 15 recording spectrophotometer, figure 18. The buffers used and the resulting acidities were citric acid-potassium dihydrogen citrate for pH 3.23, dipotassium hydrogen citrate-tripotassium citrate for pH 6.10 and potassium carbonate for pH 10.80. All solutions including the blanks were 0.002 M in EDTA to sequester possible interferring metal ions. The blank solutions also contained the appropriate buffer.

The effect of the acidity of the solution on the absorbance of a 5×10^{-5} M solution of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone is shown in figure 19. The data for absorbance versus pH at 295 and 335 mμ, wavelengths of maximum absorbance in figure 18, were obtained by

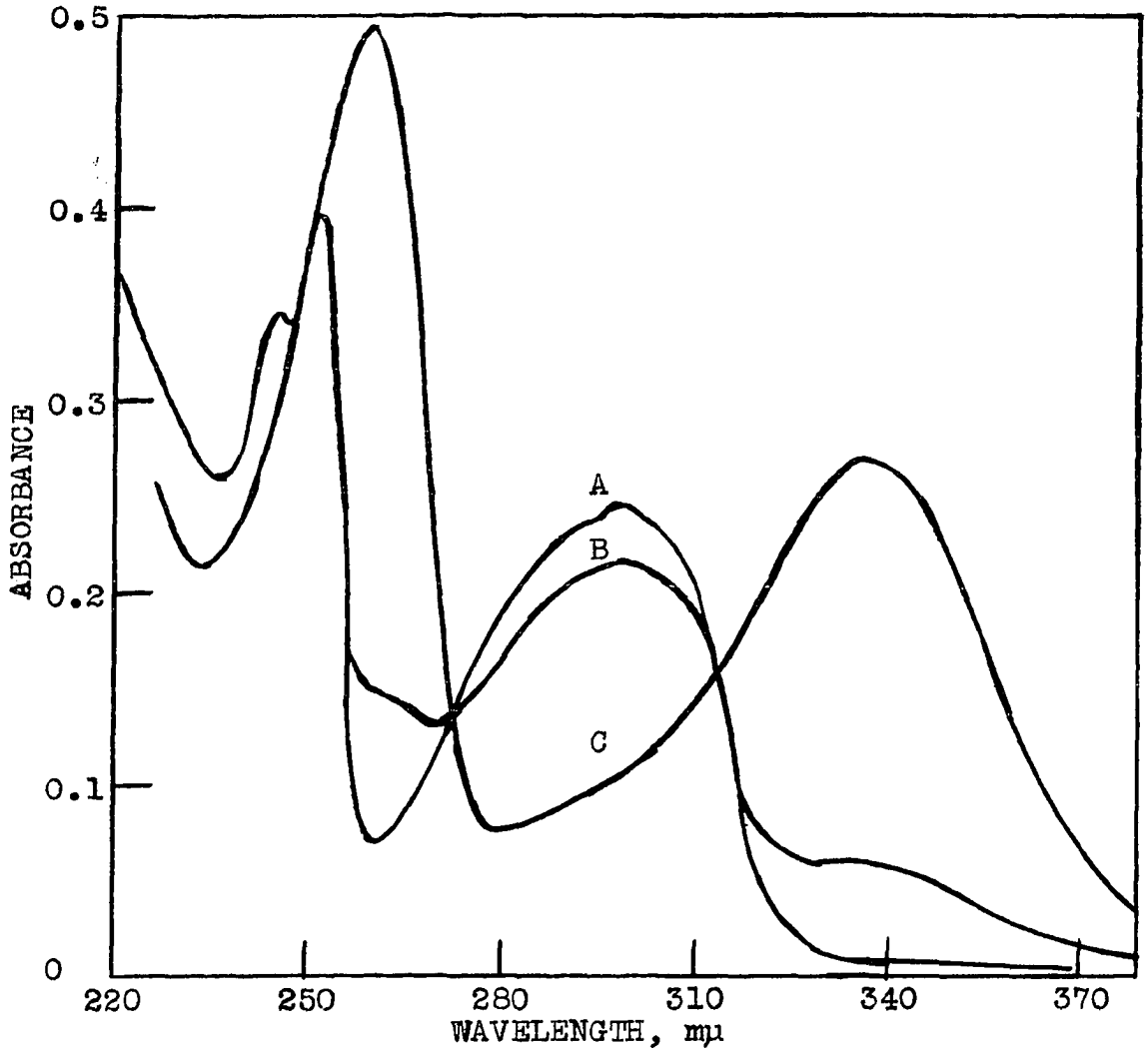


Figure 18. Absorption spectra of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone

A pH 3.23

B pH 6.10

C pH 10.80

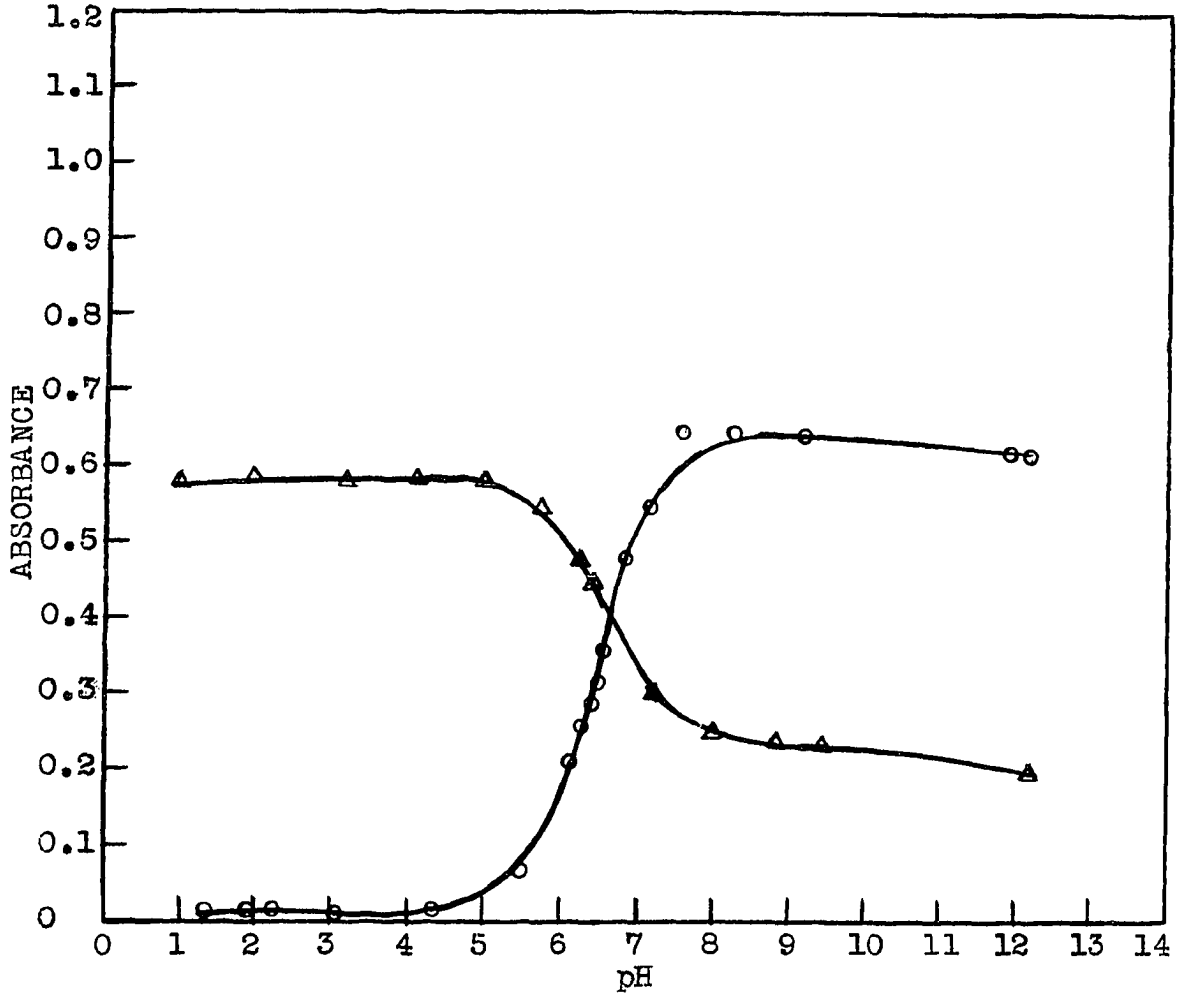


Figure 19. Effect of pH on the absorbance of a 5×10^{-5} M solution of 2-methyl-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)chromone

Wavelength setting
△ 295 mμ
○ 335 mμ

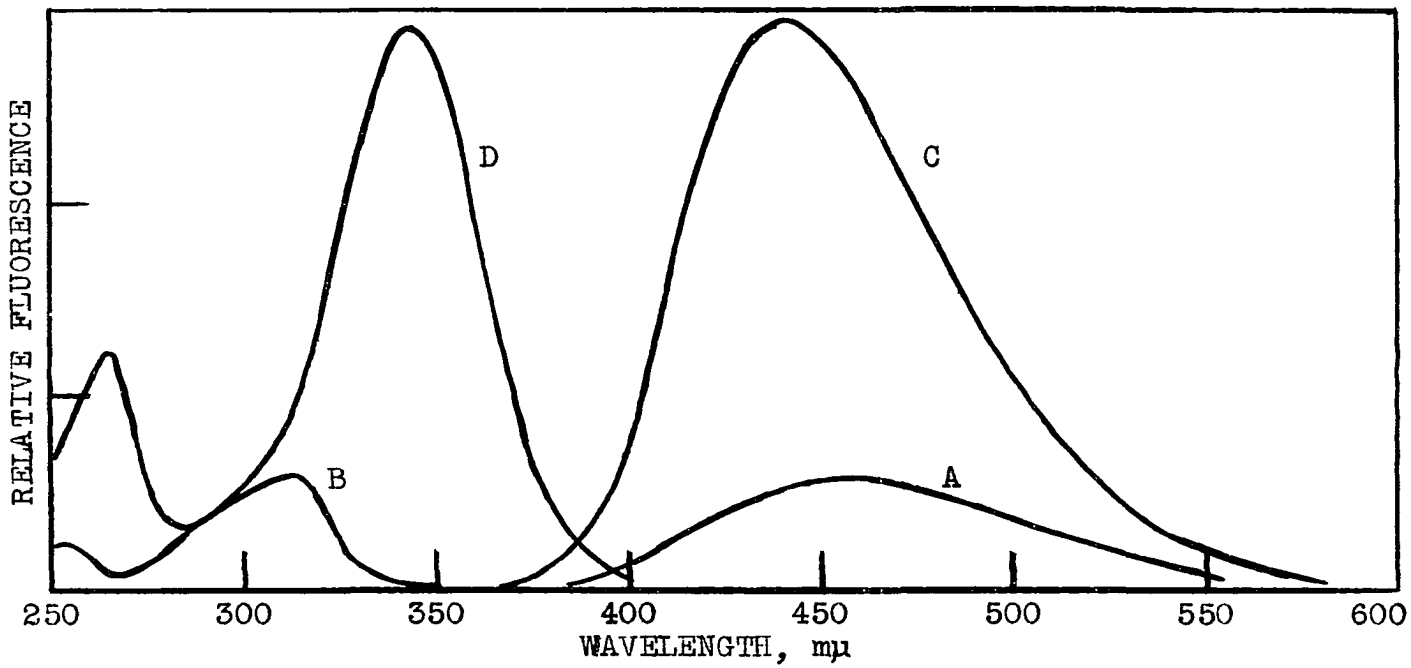


Figure 20. Fluorescence vs. wavelength for 2-methyl-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)chromone

A Fluorescent spectrum; excitation wavelength, 310 mμ; pH 3.40

B Excitation spectrum; fluorescent wavelength, 460 mμ; pH 3.40

C Fluorescent spectrum; excitation wavelength, 340 mμ; pH 10.50

D Excitation spectrum; fluorescent wavelength, 435 mμ; pH 10.50

a procedure identical to that described for 2-methyl-7-hydroxychromone. An acid dissocation constant, $10^{-6.50}$, was calculated from these data.

Fluorescent spectra, both fluorescence versus excitation wavelength and fluorescence versus emission wavelength, figure 20, were obtained for 2×10^{-6} M aqueous solutions of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone. The solutions were prepared by a ten fold dilution of the solutions used for the absorption spectra. Acidities of the solutions were pH 3.40 and 10.50.

The effect of the acidity of the solution on the fluorescence of a 4×10^{-6} M solution of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone is shown in figure 21. The selection of the wavelength settings was based on the information in figure 20. The fluorescent measurements were made on a series of buffered solutions. The buffers used were citric acid, trishydroxymethylaminomethane, boric acid and appropriate amounts of potassium hydroxide or hydrochloric acid. All solutions were 4×10^{-5} M in EDTA to sequester metal ions. The acidity was measured with a Leeds and Northrup pH meter, and the fluorescence was measured with an Aminco Bowman spectrofluorometer. The third ionization constant for 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone calculated from these data is $10^{-11.41}$.

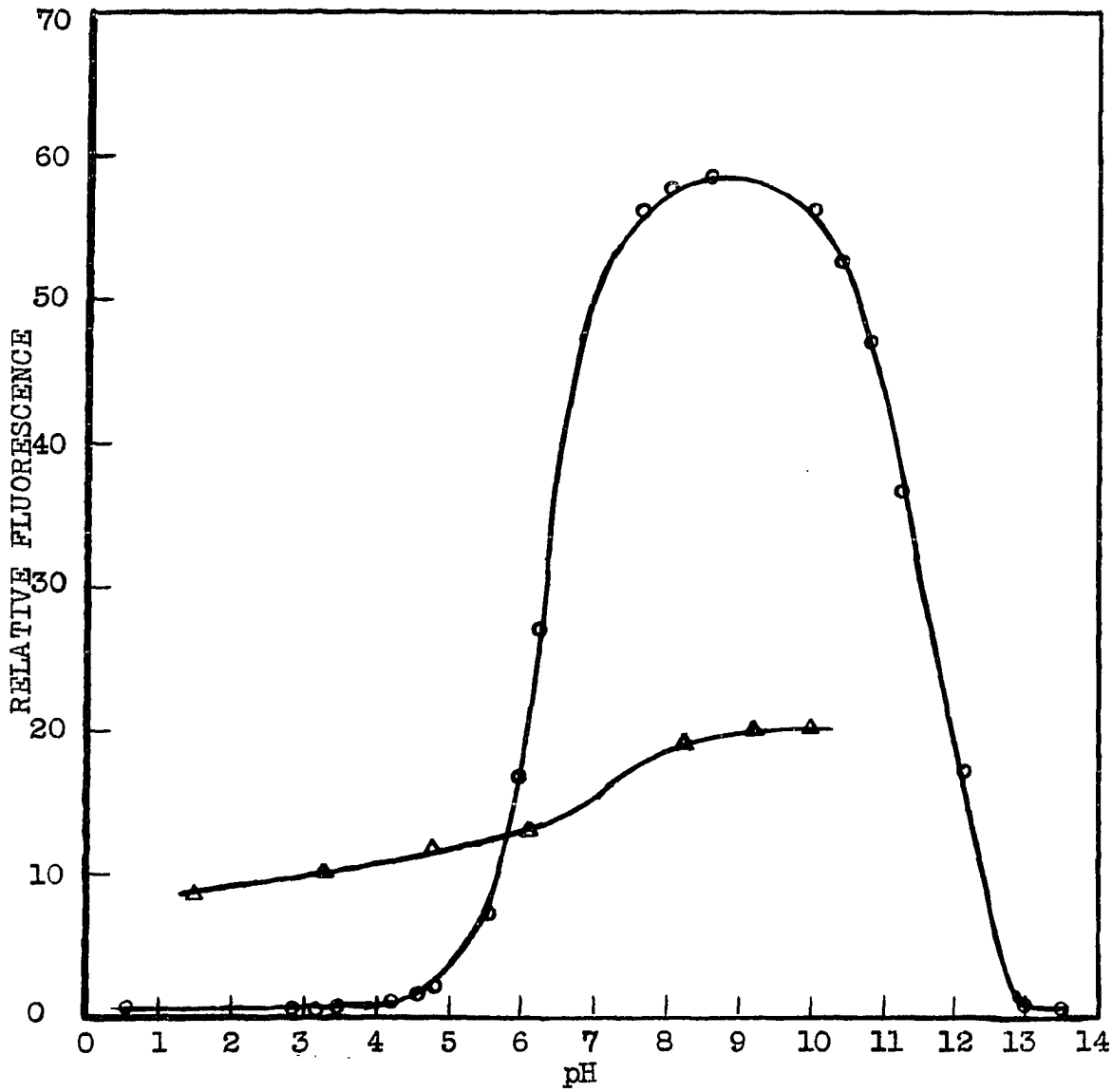


Figure 21. Effect of pH on the relative fluorescence of 4×10^{-6} M 2-methyl-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)chromone

Wavelength settings
○ excitation, 340 mμ; fluorescence, 440 mμ
△ excitation, 315 mμ; fluorescence, 460 mμ

Table 3. Wavelengths for maximum fluorescence of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone with metal ions present

pH	Metal ion	Excitation wavelength for maximum fluorescence	Emission wavelength for maximum fluorescence
3	None	310 mμ	460 mμ
10	None	340	435
4	Mg	310	455
8	Mg	340	432
12	Mg	337	430
4	Sr	312	455
8	Sr	340	440
12	Sr	347	447
4	Ba	312	452
8	Ba	342	442
12	Ba	350	452
4	Zn	312	450
8	Zn	335	425
12	Zn	333	440
3	Ca	310	460
8	Ca	340	440
13	Ca	338	443

Fluorescent spectra of 4×10^{-6} M solutions of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone with metal ions present were also obtained with an Aminco Bowman spectrofluorometer. The approximate pH of the solutions, the metal ion present, the excitation wavelength for maximum fluorescence and the emission wavelength for maximum fluorescence are given in table 3.

The effect of acidity on the fluorescence of 4×10^{-6} M solutions of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone in the presence of the divalent ions of

magnesium, zinc, calcium, barium, strontium or copper is shown in figure 22. The fluorescent measurements were made on a series of buffer solutions with an Aminco Bowman spectrofluorometer with wavelength settings of 340 mμ for excitation and 440 mμ for emission. The buffers used were the same as those used for the determination of the effect of acidity on the 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl) chromone. The solutions were 4×10^{-5} M in the appropriate metal ion. The solutions were also 1.6×10^{-5} M in EDTA to eliminate the interference of calcium impurities of the potassium hydroxide in the pH range above 12.

The fluorescence of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone as a function of concentration is shown in figure 23. The solutions were buffered at pH 8.1 with trishydroxymethylaminomethane and hydrochloric acid. EDTA was added to sequester metal ions. The fluorescence was measured with the Aminco Bowman spectrofluorometer with wavelength settings of 340 mμ for excitation and 440 mμ for emission.

The rate of decomposition of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone in 0.5 potassium hydroxide, both in the presence and absence of calcium ions, is shown in figure 24. The solution in which the decomposition occurred was 4×10^{-5} M in the chromone derivative, 2×10^{-4} M in EDTA and 0.5 M in potassium hydroxide. The solution containing calcium was identical except that it was 4×10^{-4} M in calcium.

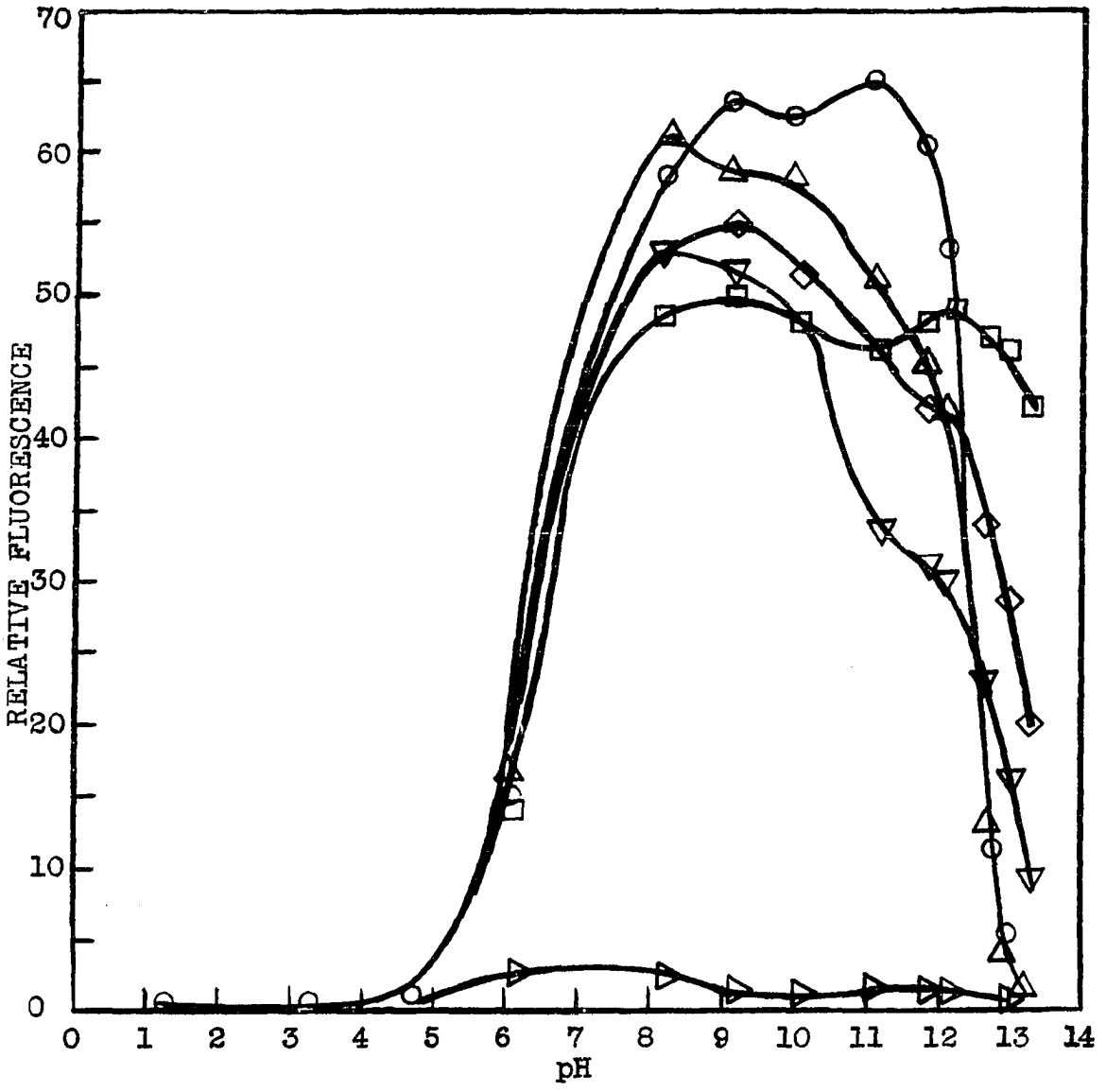


Figure 22. Effect of pH on the relative fluorescence of 4 x 10^{-6} M 2-methyl-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)chromone with metal ions present; wavelength settings, excitation, 340 mμ; fluorescence, 440 mμ

Metal present
○ Mg △ Zn □ Ca ▽ Ba ◇ Sr ▷ Cu

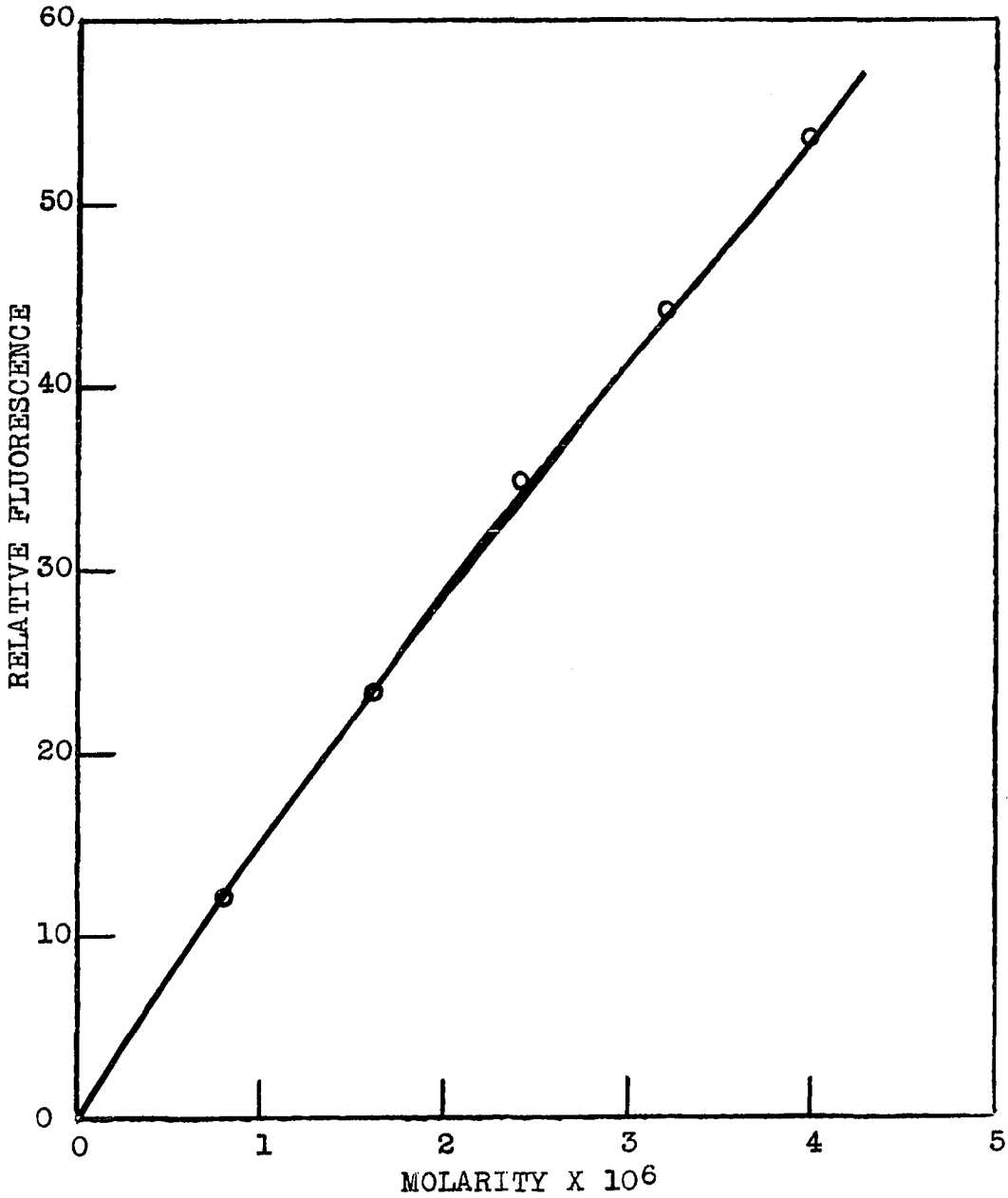


Figure 23. Effect of concentration on the fluorescence of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone

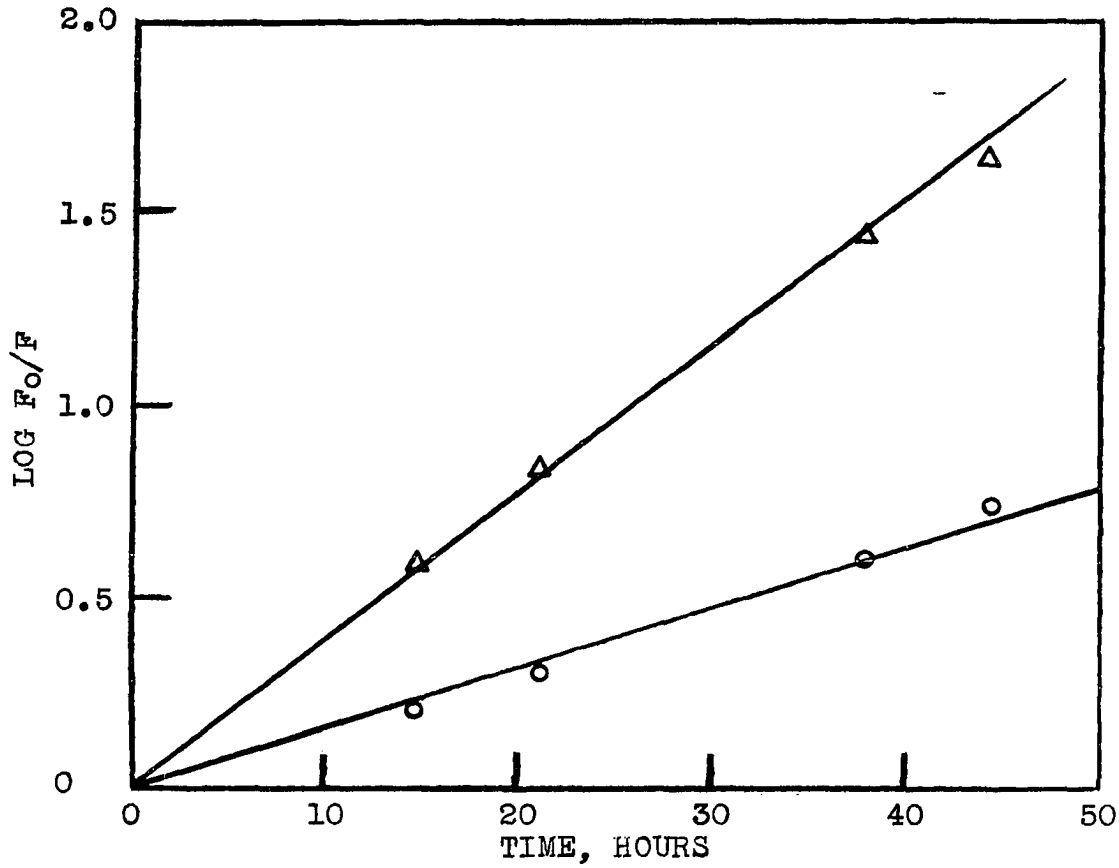


Figure 24. Decomposition of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone in 0.5 M potassium hydroxide

○ the substituted chromone only
△ the substituted chromone with calcium present

At various time intervals aliquots of the above solutions were diluted to ten times their volume with a trishydroxymethylaminomethane-hydrochloric acid buffer. The fluorescence was measured with an Aminco Bowman spectrofluorometer with wavelength settings of 340 and 440 mμ. The rate of decomposition was found to be first order with respect to the chromone derivative, the constants being 0.037 hr^{-1} without calcium present and 0.087 hr^{-1} with calcium present.

The increase of fluorescence upon addition of calcium chloride to a 10^{-4} M solution of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone in 0.5 M potassium hydroxide is shown in figure 25. The data were obtained from the titration of 100 ml. of the above solution with 10^{-4} M calcium chloride. The fluorescence was followed with a Turner Model 110 fluorometer with a Corning 7-60 filter as the primary filter and Wratten 2A and 47B filter plus a 10 per cent neutral density filter as the secondary filters. The fluorometer also had a flow through cell. The fluorescence was corrected for dilution resulting from the addition of titrant. The effect of calcium in 2 M potassium hydroxide and the effect of magnesium in 0.5 M, 1 M and 2 M potassium hydroxide solution on the fluorescence of 10^{-4} M 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone are also shown in figure 25. The data were obtained by the procedure used for effect of calcium on fluorescence in 0.5 M potassium hydroxide.

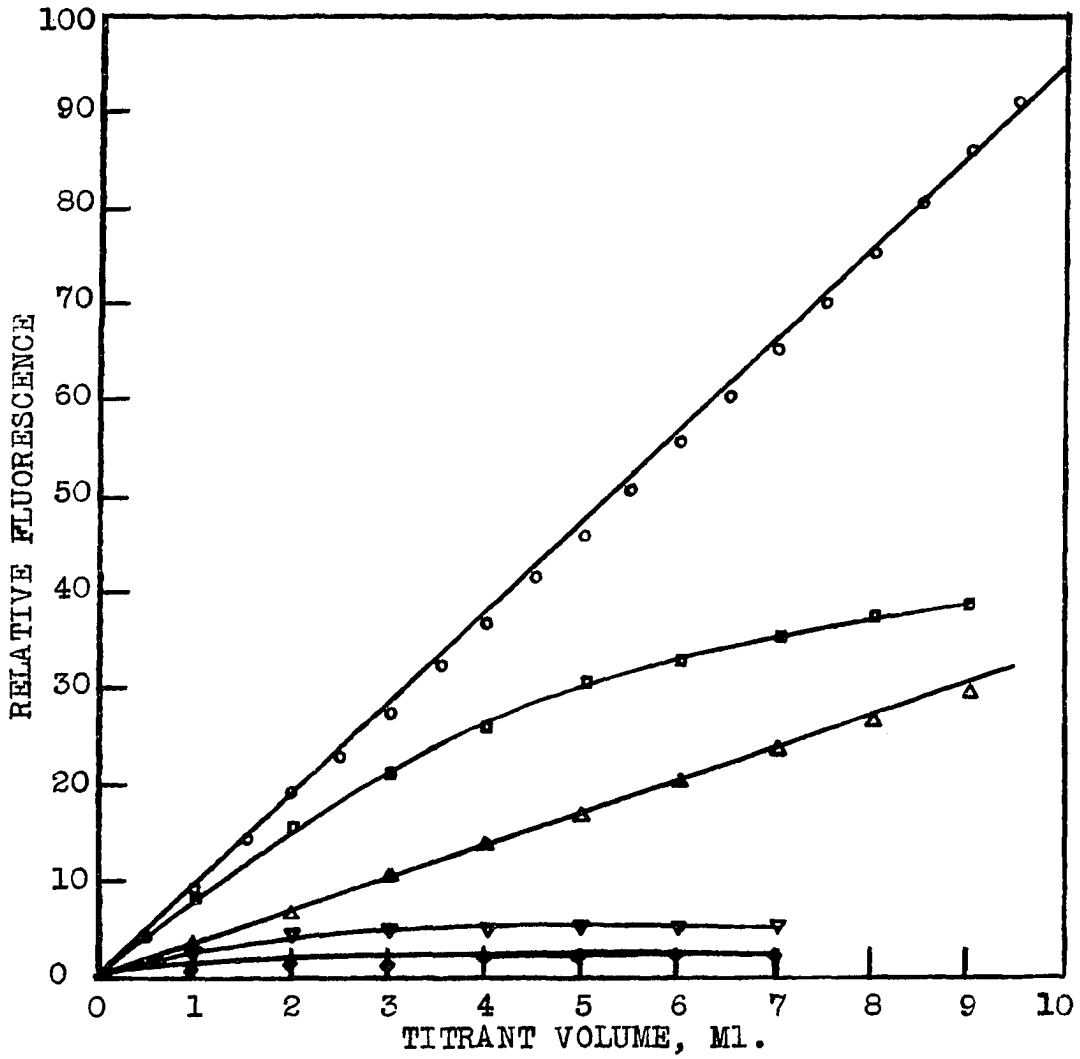


Figure 25. Fluorescence upon addition of 10^{-4} M calcium or magnesium chloride to 100 ml. of 10^{-4} M 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-chromone

○ solution is 0.5 M in KOH; titrant is $CaCl_2$

△ solution is 2.0 M in KOH; titrant is $CaCl_2$

□ solution is 0.5 M in KOH; titrant is $MgCl_2$

▽ solution is 1.0 M in KOH; titrant is $MgCl_2$

◇ solution is 2.0 M in KOH; titrant is $MgCl_2$

The effect of calcium on fluorescence of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone in a 0.5 M potassium hydroxide solution, where the amount of calcium ranged from a trace amount to two moles of calcium per mole of iminodiacetate derivative, is shown in figure 26. These data were obtained as described above except that the 10 per cent neutral density filter was replaced with a 1 per cent filter and the titrant was 2×10^{-3} M calcium chloride.

Spectra of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone

Absorption spectra, figure 27, of 2×10^{-5} M solutions of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone at various acidities, pH 3.20, pH 6.08, and pH 10.80, were obtained by a procedure identical to that used for the absorption spectra of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone.

The effect of the acidity of the solution on the absorbance of a 5×10^{-5} M solution of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone is shown in figure 28. The data for absorbance versus pH at 315 and 360 mμ, wavelengths of maximum absorbance in figure 27, were obtained by a procedure identical to that described for 2-methyl-7-hydroxychromone. An acid dissociation constant, $10^{-6.50}$, was calculated from these data.

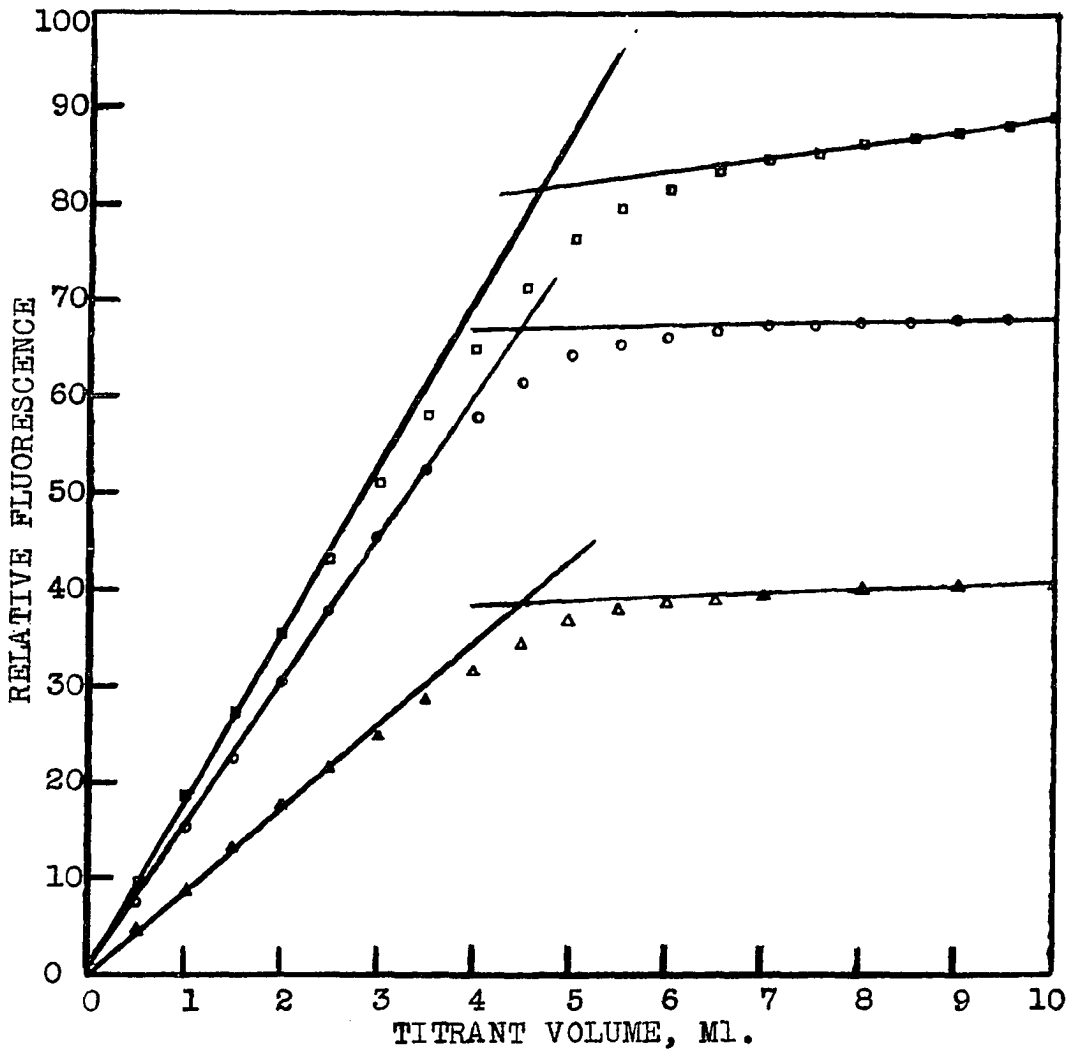


Figure 26. Titration of 10^{-5} mole of the methyleneiminodiacetate acid derivatives with 2 x 10^{-3} M calcium chloride solution

○ 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone

△ 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone

□ 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

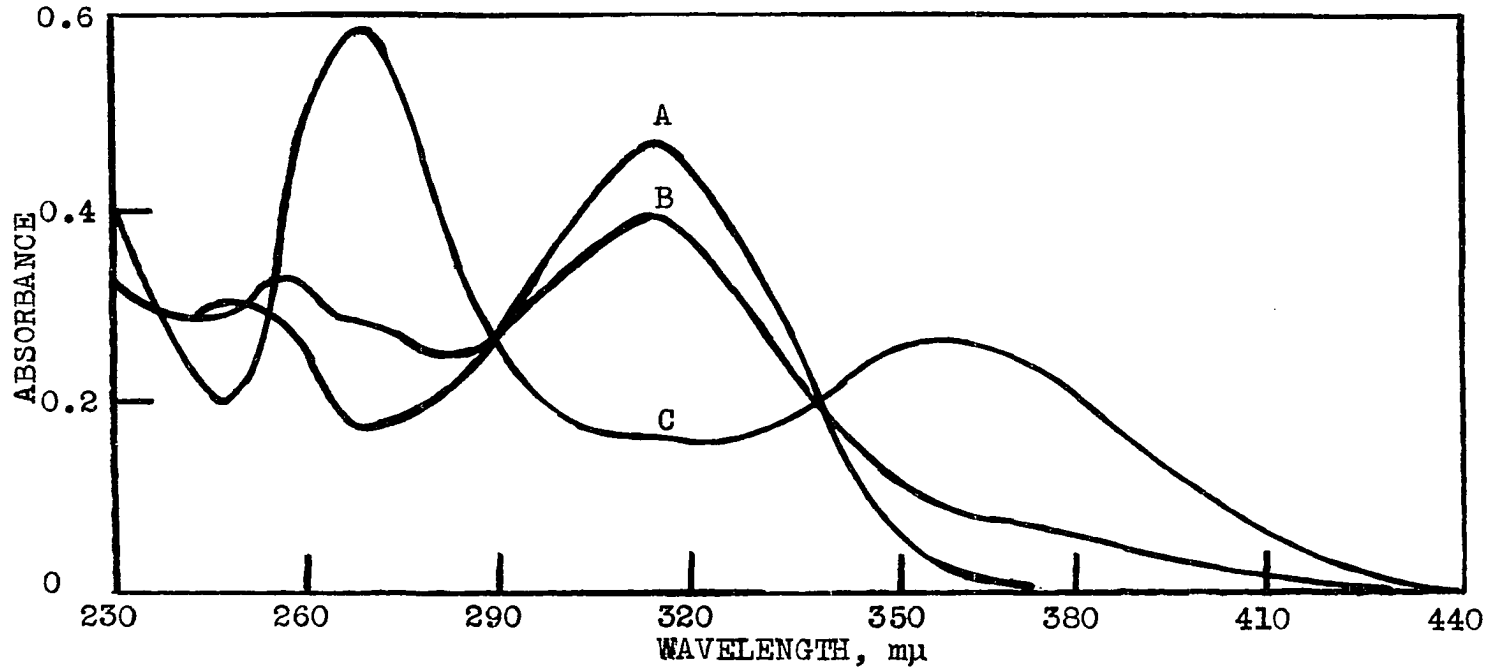


Figure 27. Absorption spectra of 7-hydroxy-8-(N,N biscarboxymethyl-aminomethyl)flavone

A pH 3.20

B pH 6.08

C pH 10.80

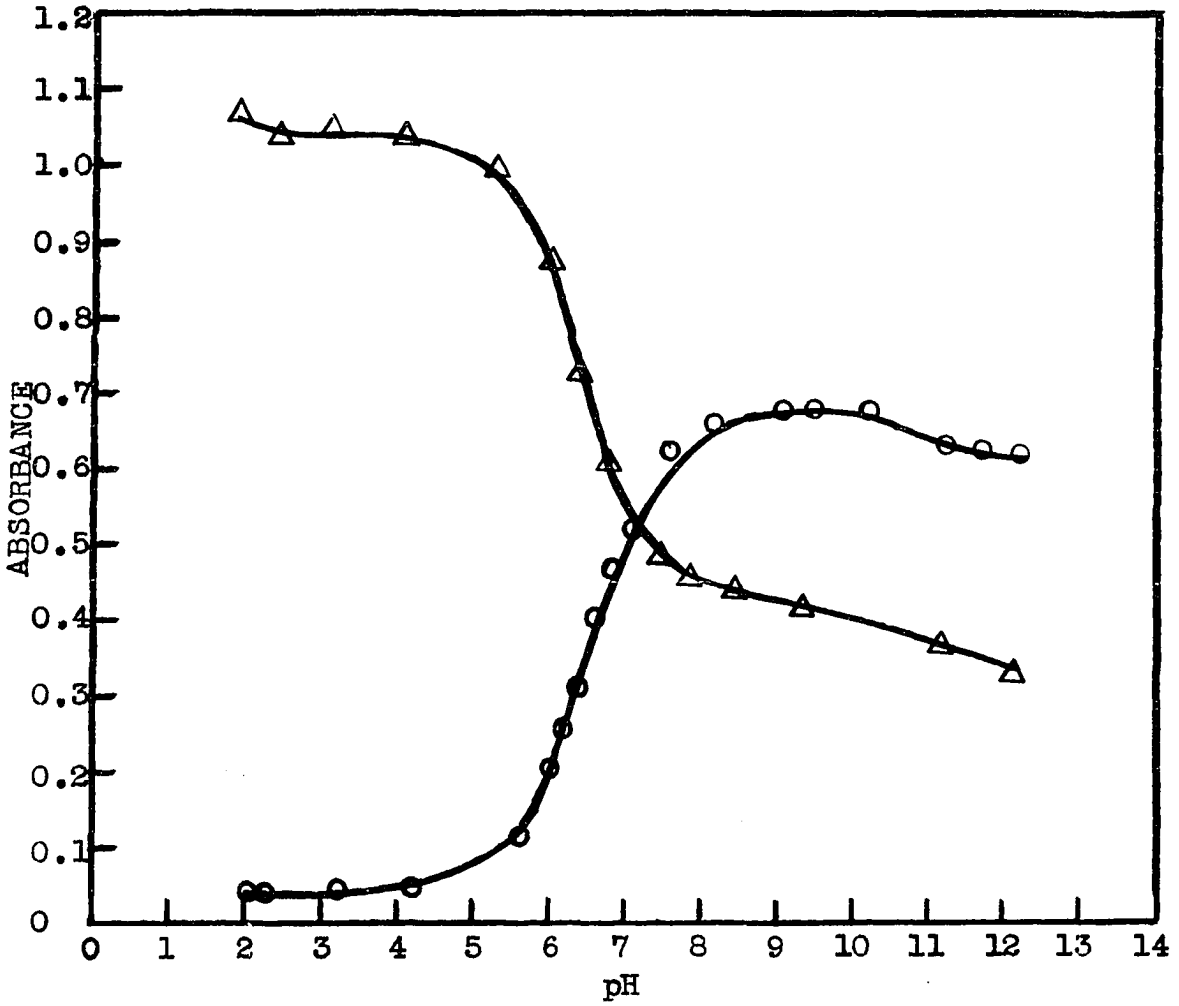


Figure 28. Effect of pH on the absorbance of a 5×10^{-5} M solution of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

Wavelength setting
○ 360 mμ
△ 315 mμ

Fluorescent spectra, both fluorescence versus excitation wavelength and fluorescence versus emission wavelength, figure 29, were obtained for 8 x 10^{-6} M aqueous solutions of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone. The solutions were prepared with the same buffer solutions used for the absorption spectra of this compound. The acidities of the solutions were pH 3.13 and 10.30.

The effect of the acidity of the solution on the fluorescence of a 2 x 10^{-5} M solution of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone is shown in figure 30. The selection of the wavelength settings was based on the information in figure 29. The instruments and buffers used were the same as those used for the same experiment with 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone. A third ionization constant, $10^{-11.28}$, for 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone was calculated from the fluorescent data.

Fluorescent spectra of 4 x 10^{-5} M solutions of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone with divalent metal ions present were also obtained with an Aminco Bowman spectrofluorometer. The approximate pH of the solutions, the metal ion present, the excitation wavelength for maximum fluorescence and the emission wavelength for maximum fluorence are given in table 4.

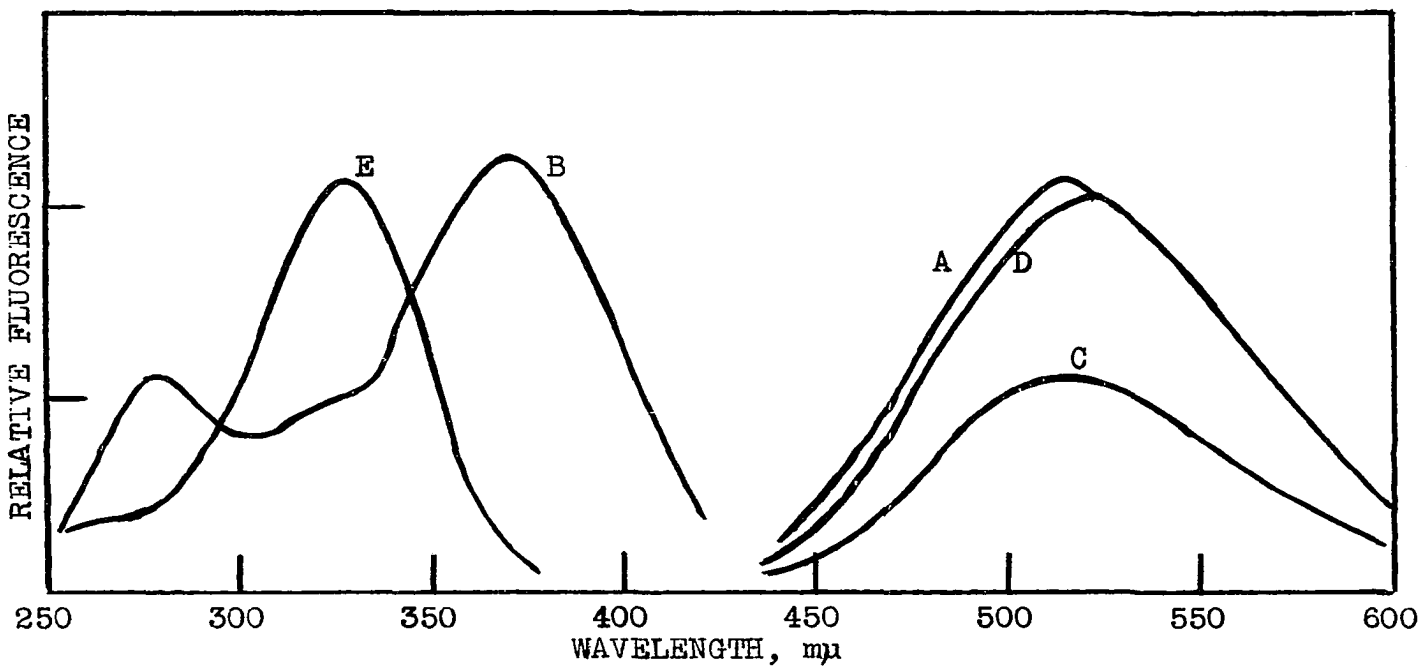


Figure 29. Fluorescence vs wavelength for 7-hydroxy-8-(N,N biscarboxymethyl-aminomethyl)flavone

A Fluorescent spectrum; excitation wavelength, 360 mμ; pH 10.30

B Excitation spectrum; fluorescent wavelength, 520 mμ; pH 10.30

C Fluorescent spectrum; excitation wavelength, 280 mμ; pH 10.30

D Fluorescent spectrum; excitation wavelength, 320 mμ; pH 3.13

E Excitation spectrum; fluorescent wavelength, 520 mμ; pH 3.13

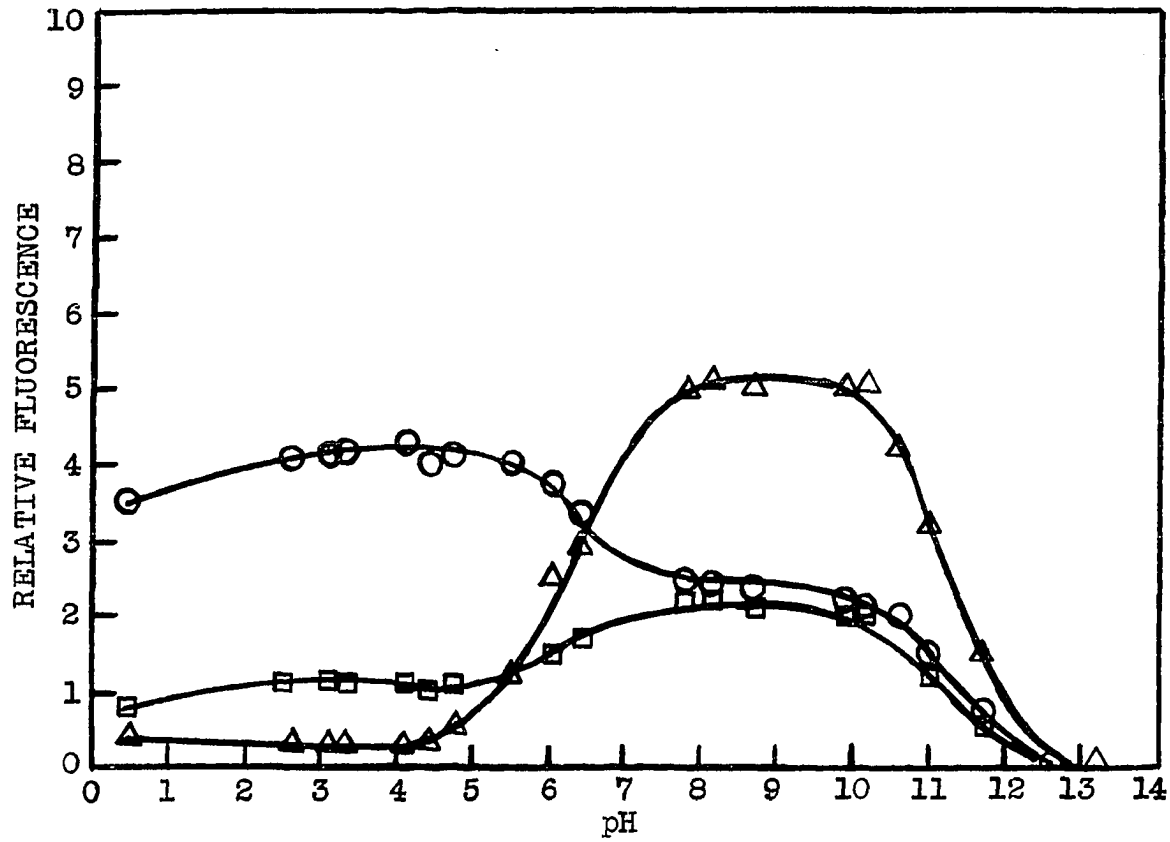


Figure 30. Effect of pH on the relative fluorescence of a 2 x 10^{-5} M solution of 7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)flavone

Wavelength settings
○ excitation, 325 mμ; fluorescence, 520 mμ

△ excitation, 370 mμ; fluorescence, 520 mμ

□ excitation, 280 mμ; fluorescence, 520 mμ

Table 4. Wavelengths for maximum fluorescence of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone with metal ions present

pH	Metal ion	Excitation wavelength for maximum fluorescence	Emission wavelength for maximum fluorescence
3	None	326 mμ	520 mμ
10	None	370	515
11	Mg	360	500
12	Zn	352	512
8	Sr	365	515
12	Sr	370	520
8	Ba	365	515
12	Ba	370	520
3	Ca	330	520
8	Ca	350	505
10	Ca	370	520
13	Ca	368	518

The effect of acidity on the fluorescence of 4×10^{-5} M solutions of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone in the presence of the divalent metal ions of magnesium, zinc, calcium, barium, strontium or copper is shown in figure 31. The fluorescent measurements were made on a series of buffer solutions with an Aminco Bowman spectrofluorometer with wavelength settings of 370 mμ for excitation and 520 mμ for emission. The buffers used were the same as those used for the determination of the effect of acidity on the fluorescence of 2-methyl-7-hydroxy-8-(N,N carboxymethylaminomethyl)chromone. The solutions were 2×10^{-4} M in the appropriate metal ion. The solutions were also 1×10^{-4} M in EDTA to eliminate the

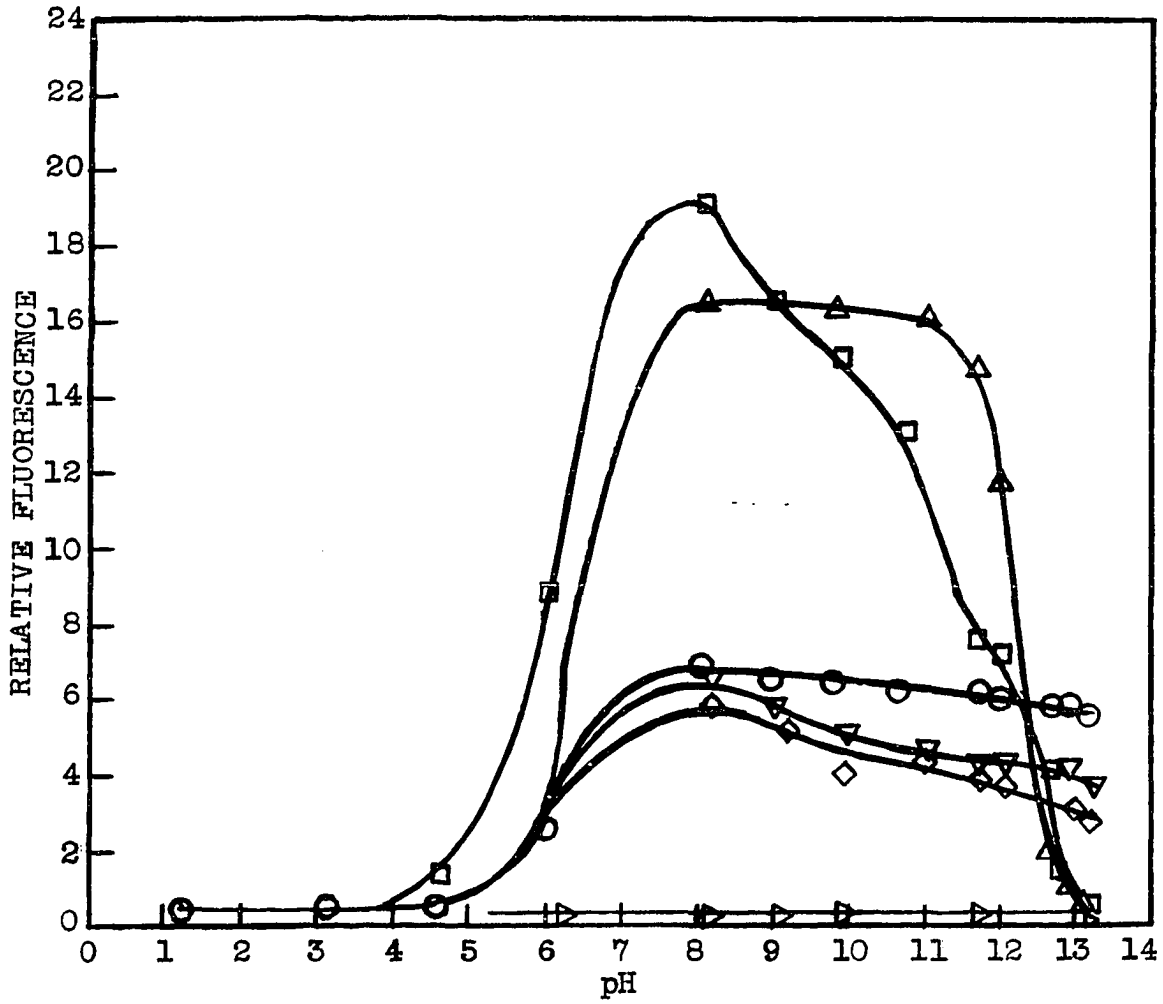


Figure 31. Effect of pH on the relative fluorescence of 4 x 10^{-5} M 7-hydroxy-8-(N,N biscarboxymethyl-aminomethyl)flavone with metal ions present; wavelength settings, excitation, 370 mμ; fluorescence, 520 mμ

Metal present
○ Ca □ Zn △ Mg ▷ Cu ▽ Sr ◇ Ba

interference of calcium impurities of the potassium hydroxide in the pH range above 12.

The fluorescence of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone as a function of concentration is shown in figure 32. The solutions were buffered at pH 8.1 with trishydroxymethylaminomethane and hydrochloric acid. EDTA was added to sequester metal ions. The fluorescence was measured with the Aminco Bowman spectrofluorometer with wavelength settings of 370 mμ for excitation and 520 mμ for emission.

The rate of decomposition of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone in 0.5 M potassium hydroxide, both in the presence and absence of calcium ions, is shown in figure 33. The solution with calcium present was 2×10^{-4} M in the derivative of the flavone, 4×10^{-4} M in EDTA, 10^{-3} M in calcium chloride, and 0.5 M in potassium hydroxide. The solution without calcium was identical in all other respects. At various time intervals aliquots of the above solutions were diluted to ten times their volume with a trishydroxymethylaminomethane-hydrochloric acid buffer. The fluorescence was measured with the Aminco Bowman spectrofluorometer with wavelength settings of 370 and 520 mμ. The rate of decomposition was found to be first order with respect to derivative, the constants being 0.0060 hr^{-1} without calcium and 0.017 hr^{-1} with calcium present.

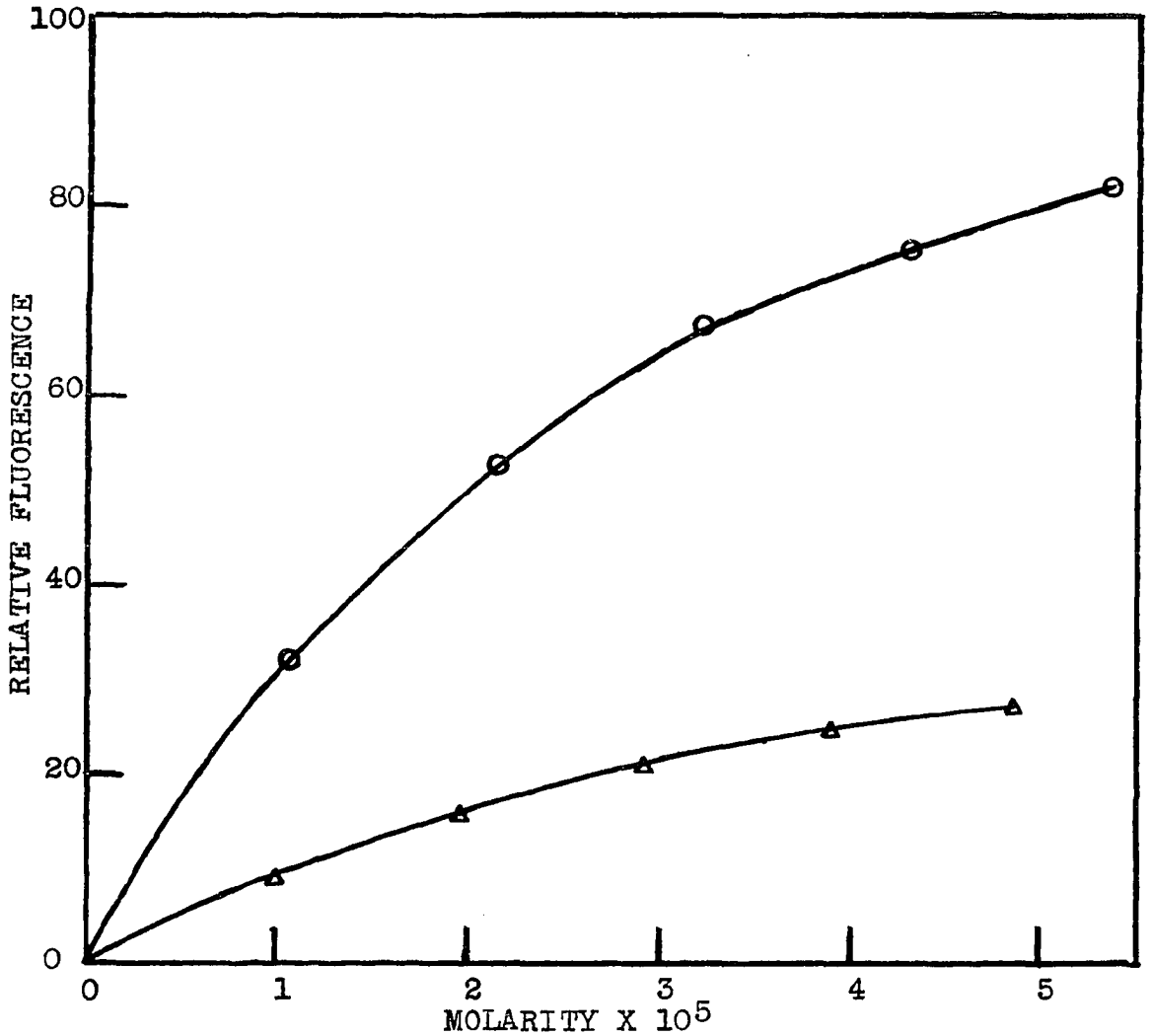


Figure 32. Effect of concentration on fluorescence

○ 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethyl-aminomethyl)flavone; wavelength settings, excitation, 375 mμ; fluorescence, 520 mμ; pH 8.1

△ 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone; wavelength settings, excitation 370 mμ; fluorescence 520 mμ; pH 8.1

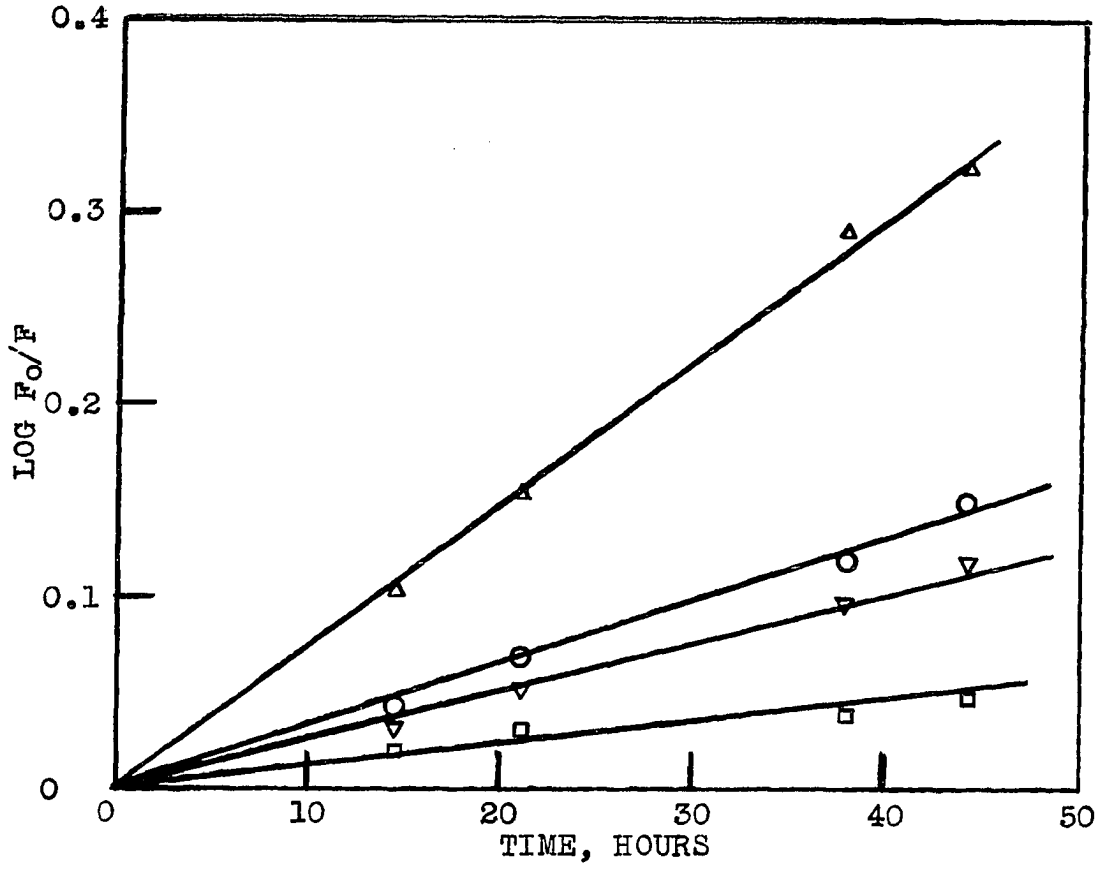


Figure 33. Decomposition of substituted flavones in 0.5 M potassium hydroxide

▽ 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

△ 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone with calcium present

□ 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

○ 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone with calcium present

The increase in fluorescence upon addition of calcium chloride to a 10^{-4} M solution of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone in 0.5 M potassium hydroxide is shown in figure 34. The data were obtained from the titration of 100 ml. of the above solution with 10^{-4} M calcium chloride. The fluorescence was followed with a Turner Model fluorometer with a Corning 7-60 filter as the primary filter and a Wratten 2A-12 and a 10 per cent neutral density filter as the secondary filters. The fluorescence of the solution was measured in a flow through pyrex cell. The fluorescence was corrected for dilution resulting from the addition of titrant. The effect of calcium in 2 M potassium hydroxide and the effect of magnesium in 0.5 M, 1 M, and 2 M potassium hydroxide solutions on the fluorescence of 10^{-4} M 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone are also shown in figure 34. The data were obtained by the procedure used for the effect of calcium on fluorescence in 0.5 M potassium hydroxide solution, where the amount of calcium ranged from a trace to two moles of calcium per mole of iminodiacetate derivative, is shown in figure 26. These data were obtained as described above except that the 10 per cent neutral density filter was replaced with a one per cent filter and the titrant was 2×10^{-3} M calcium chloride.

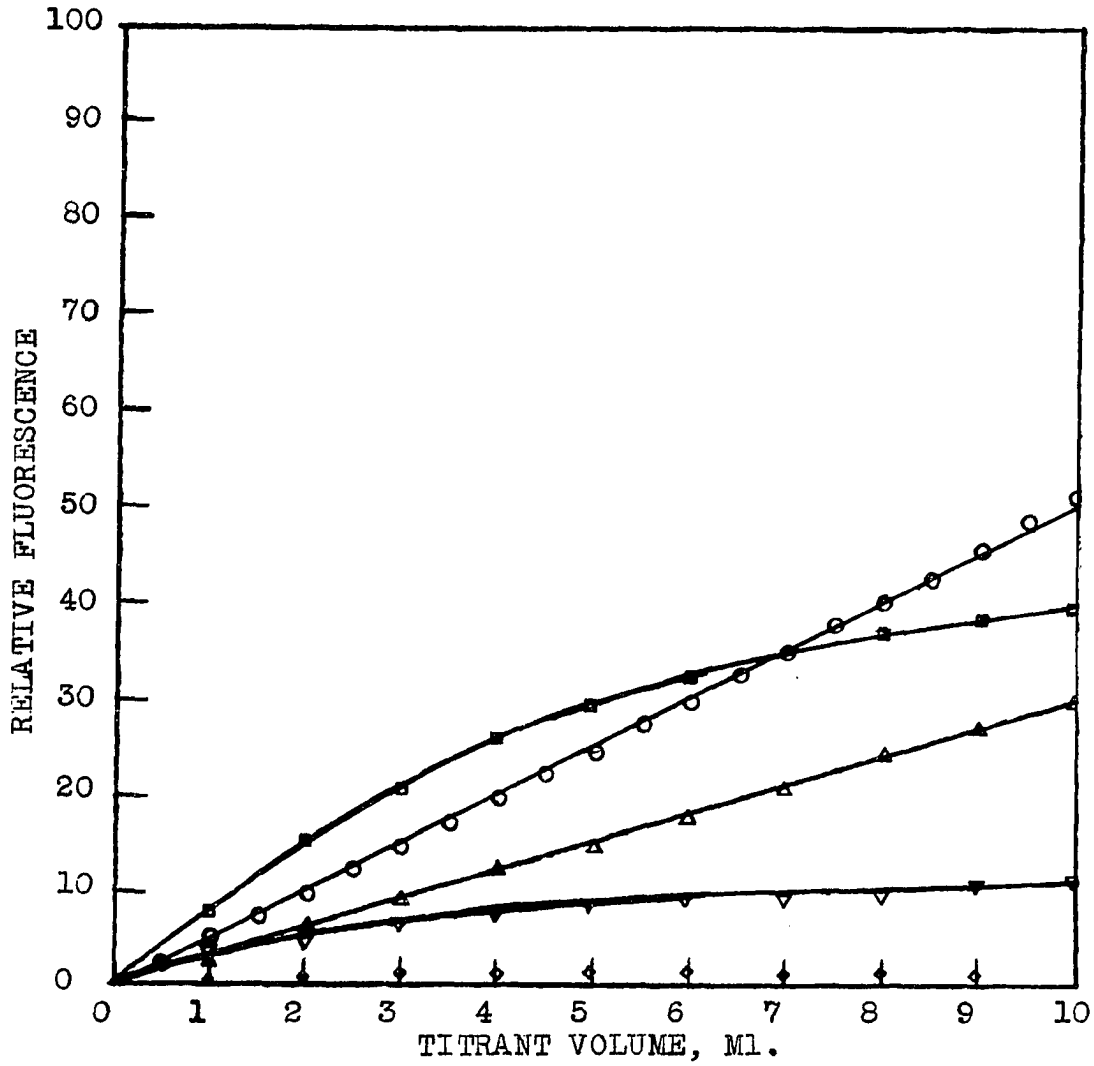


Figure 34. Fluorescence upon addition of 10^{-4} M calcium or magnesium chloride to 100 ml. of 10^{-4} M 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone

○ solution is 0.5 M in KOH; titrant is $CaCl_2$

△ solution is 2.0 M in KOH; titrant is $CaCl_2$

□ solution is 0.5 M in KOH; titrant is $MgCl_2$

▽ solution is 1.0 M in KOH; titrant is $MgCl_2$

◇ solution is 2.0 M in KOH; titrant is $MgCl_2$

Spectra of 4'methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

Absorption spectra, figure 35, of 2 x 10^{-5} M solutions of 4'methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone at various acidities, pH 3.30, pH 6.10 and pH 10.80, were obtained by a procedure identical to that used for the absorption spectra of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone.

The effect of the acidity of the solution on the absorbance of a 3 x 10^{-5} solution of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone is shown in figure 36. The data for absorbance versus pH at 335 and 360 mμ, wavelengths of maximum absorbance in figure 35, were obtained by a procedure identical to that described for 2-methyl-7-hydroxychromone. An acid dissociation constant, $10^{-6.58}$, was calculated from these data.

Fluorescent spectra, both fluorescence versus excitation wavelength and fluorescence versus emission wavelength, figure 37, were obtained for 8 x 10^{-6} M aqueous solutions of 4-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone. The solutions were prepared with the same buffer solutions used for the absorption spectra of this compound. The acidities of the solution were pH 3.18 and 10.33.

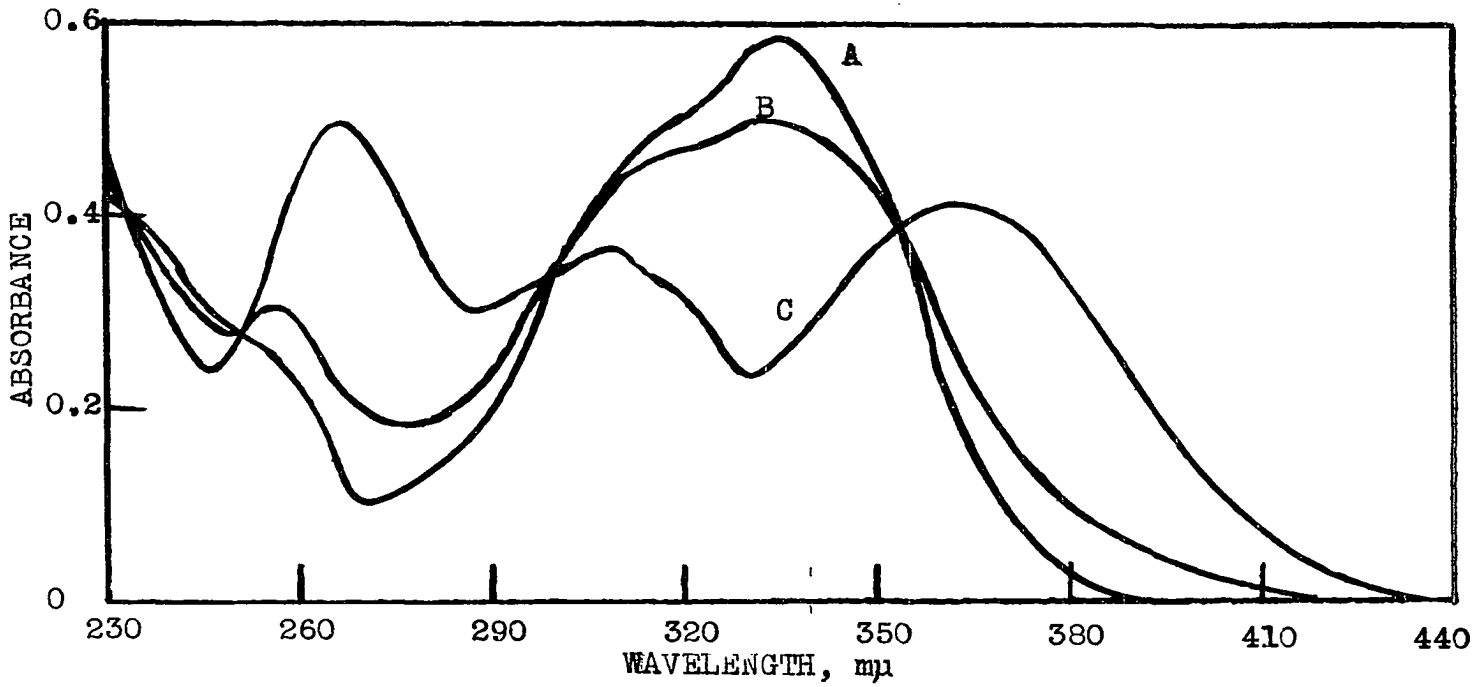


Figure 35. Absorption spectra of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

A pH 3.30

B pH 6.10

C pH 10.80

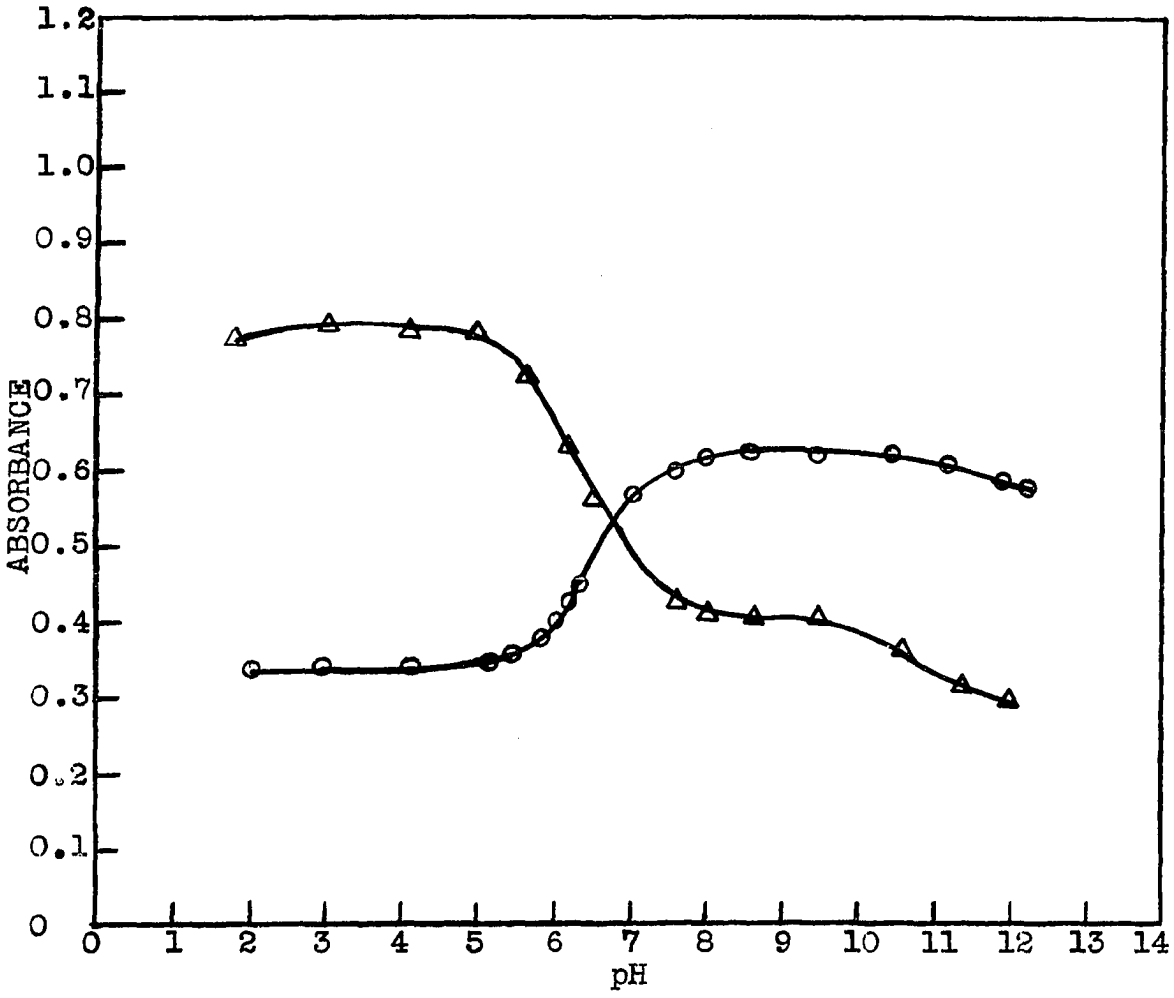


Figure 36. Effect of pH on the absorbance of a 3 x 10^{-5} M solution of 4'-methoxy-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)flavone

Wavelength setting
○360 mμ
△335 mμ

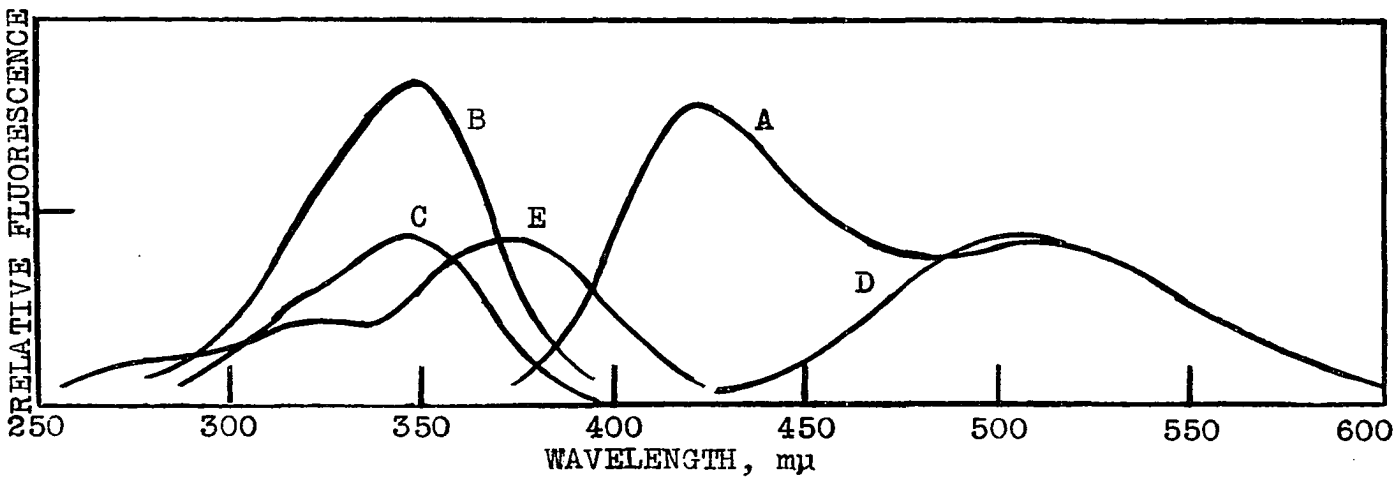


Figure 37. Fluorescence vs. wavelength for 4-methoxy-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)flavone

A Fluorescent spectrum; excitation wavelength, 340 mμ; pH 3.18

B Excitation spectrum; fluorescent wavelength, 425 mμ; pH 3.18

C Excitation spectrum; fluorescent wavelength, 520 mμ; pH 3.18

D Fluorescent spectrum; excitation wavelength, 365 mμ; pH 10.33

E Excitation spectrum; fluorescent wavelength, 510 mμ; pH 10.33

The effect of the acidity of the solution on the fluorescence of a 2 x 10^{-5} M solution of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone is shown in figure 38. The instruments and buffers used were the same as those used for the same experiment with 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone. A third ionization constant, $10^{-11.31}$ for 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone was calculated from the fluorescent data.

Fluorescent spectra of 4 x 10^{-5} M solution of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone with divalent metal ions present were also obtained with an Aminco Bowman spectrofluorometer. The approximate pH of the solutions, the metal ion present, the excitation wavelength for maximum fluorescence and the emission wavelength are given in table 5.

The effect of acidity on the fluorescence of 4 x 10^{-6} M solutions of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone in the presence of the divalent metal ions of magnesium, zinc, calcium, barium, strontium or copper is shown in figure 39. The fluorescent measurements were made on a series of buffer solutions with an Aminco Bowman spectrofluorometer with wavelength settings of 375 mμ for excitation and 520 mμ for emission. The buffers used were the same as those used for the determination of the effect of acidity on the fluorescence of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone. The solutions were 4 x 10^{-5} M in the

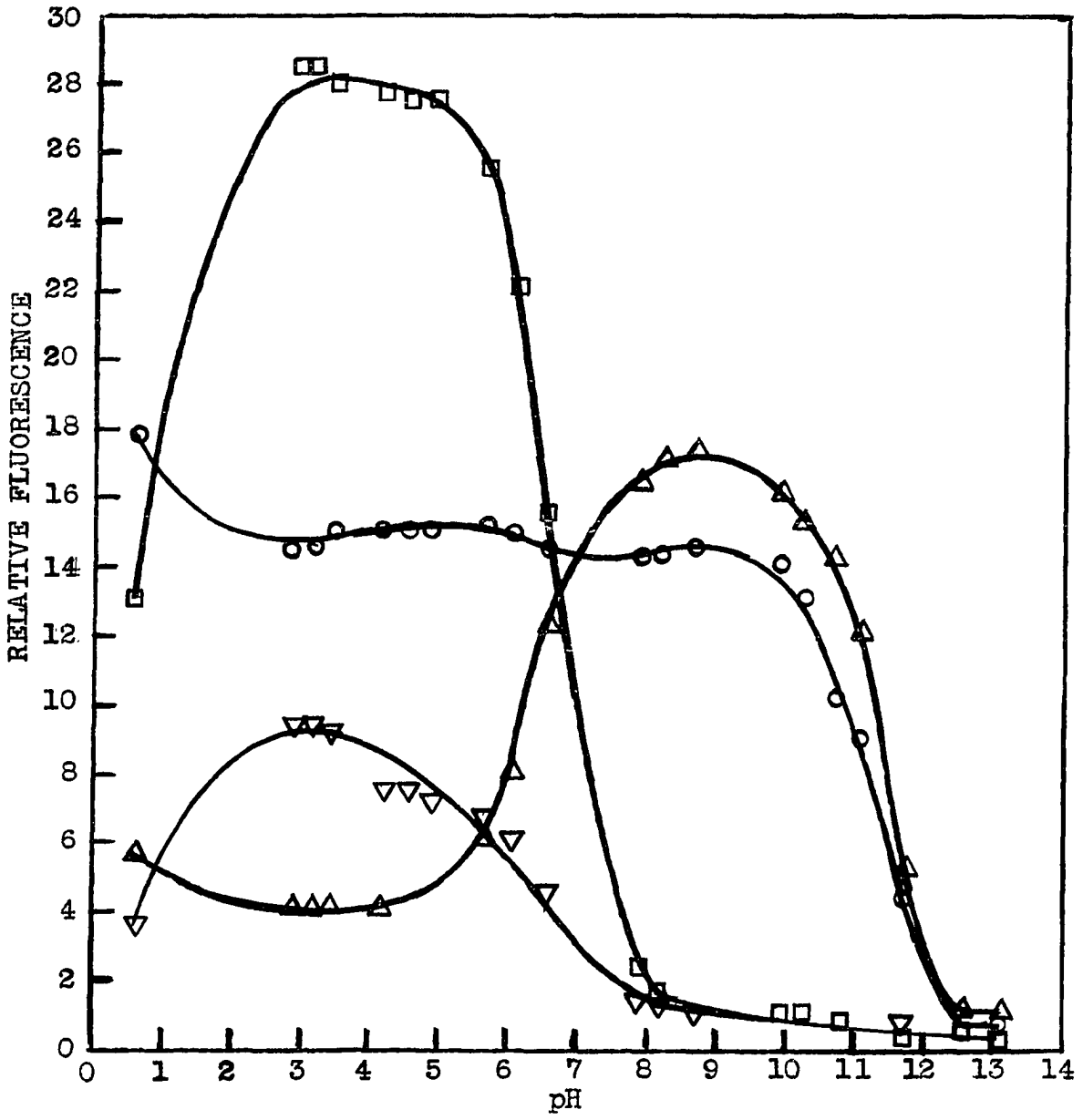


Figure 38. Effect of pH on the relative fluorescence of a 2×10^{-5} M solution of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

Wavelength settings
- ○ excitation, 350 mμ; fluorescence, 520 mμ
- □ excitation, 350 mμ; fluorescence, 425 mμ
- △ excitation, 375 mμ; fluorescence, 520 mμ
- ▽ excitation, 375 mμ; fluorescence, 425 mμ

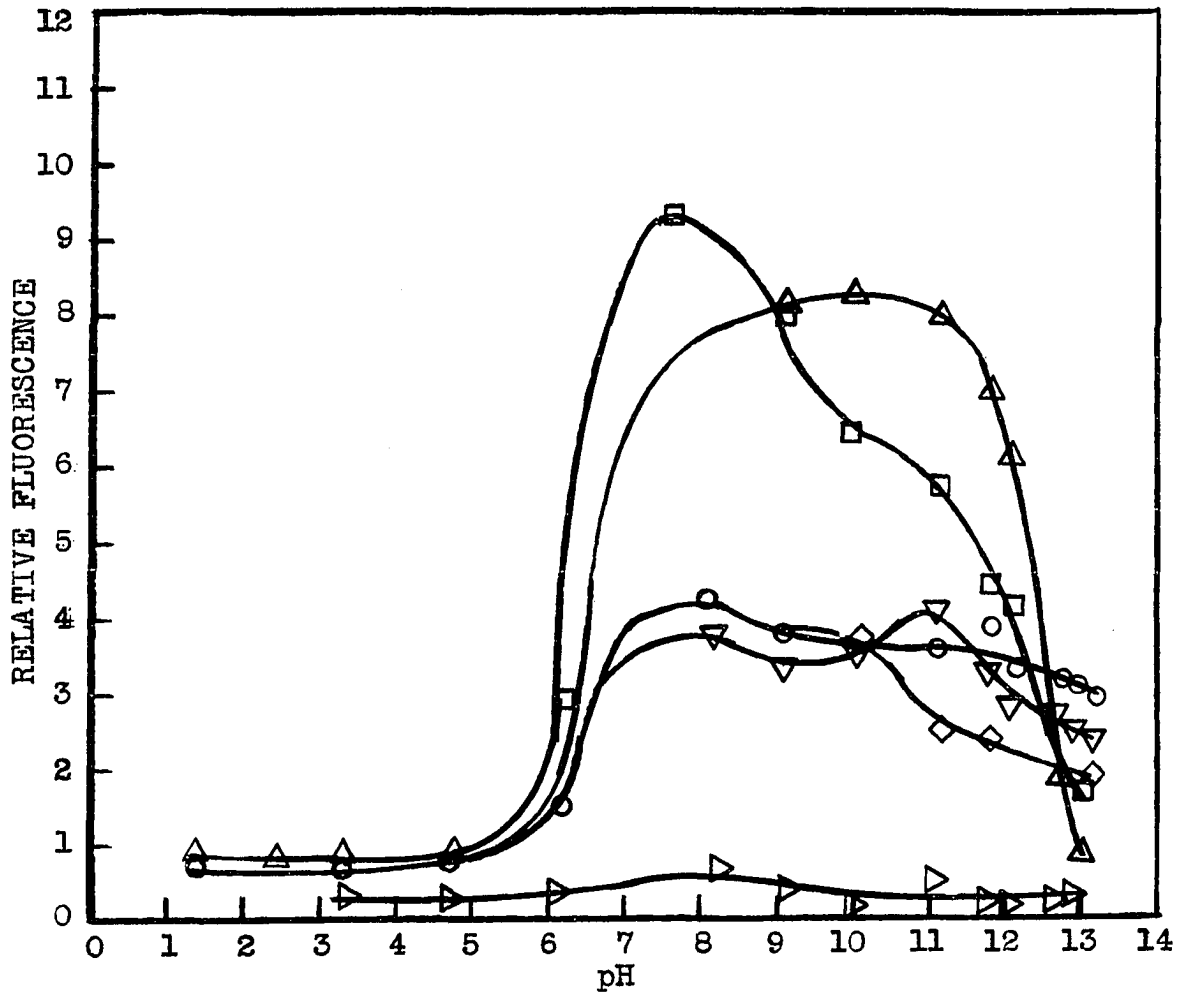


Figure 39. Effect of pH on the relative fluorescence of 4×10^{-6} M 4'-methoxy-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)flavone with metal ions present; wavelength settings, excitation, 375 mμ; fluorescence, 520 mμ

Metal present
○ Ca □ Zn △ Mg ▷ Cu ▽ Sr ◇ Ba

Table 5. Wavelengths for maximum fluorescence of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone with metal ions present

pH	Metal ion	Excitation wavelength for maximum fluorescence	Emission wavelength for maximum fluorescence
3.2	None	348 mμ	425 mμ
10.3	None	372	510
2.5	Mg	348	420
12.7	Mg	370	495
3.3	Zn	348	420
12.7	Zn	365	500
12.1	Sr	372	510
8.2	Sr	348	520
3.3	Sr	303	418
3.4	Ba	345	415
8.1	Ba	353	468
12.6	Ba	380	510
3.0	Ca	350	422
10.0	Ca	375	505
13.0	Ca	373	507

appropriate metal ion. The solution was also 1.6×10^{-5} M in EDTA to eliminate the interference of calcium impurities of the potassium hydroxide in the pH range above 12.

The effect of the concentration on the fluorescence of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone is shown in figure 32. The solutions were buffered at pH 8.1 with trishydroxymethylaminomethane and hydrochloric acid. EDTA was added to sequester metal ions. The fluorescence was measured with an Aminco Bowman spectrofluorometer with wavelength settings of 375 mμ for excitation and 520 mμ for emission.

The rate of decomposition of 4'methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone in 0.5 M potassium hydroxide, both in the presence and absence of calcium ions, is shown in figure 33. The solution with calcium present was 2×10^{-4} M in the derivative of the 4'-methoxy-7-hydroxy-flavone, 4×10^{-4} M in EDTA, 10^{-3} M in calcium chloride, and 0.5 M in potassium hydroxide. The solution without calcium was identical in all other respects. At various time intervals aliquots of the above solutions were diluted to ten times their volume with a trishydroxymethylaminomethane-hydrochloric acid buffer. The fluorescence was measured with the Aminco Bowman spectrofluorometer with wavelength settings of 375 and 520 mμ. The rate of decomposition was found to be first order with respect to derivative, the constants being 0.0028 hr^{-1} without calcium present and 0.0078 hr^{-1} with calcium present.

The increase in the fluorescence upon the addition of calcium chloride or magenesium chloride to a 10^{-4} M solution of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone in 0.5 M, 1 M, or 2 M potassium hydroxide is shown in figure 40. The procedures and instruments used were identical to those described for the same experiment with 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone. The effect of calcium on the fluorescence of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone in a 0.5 M potassium

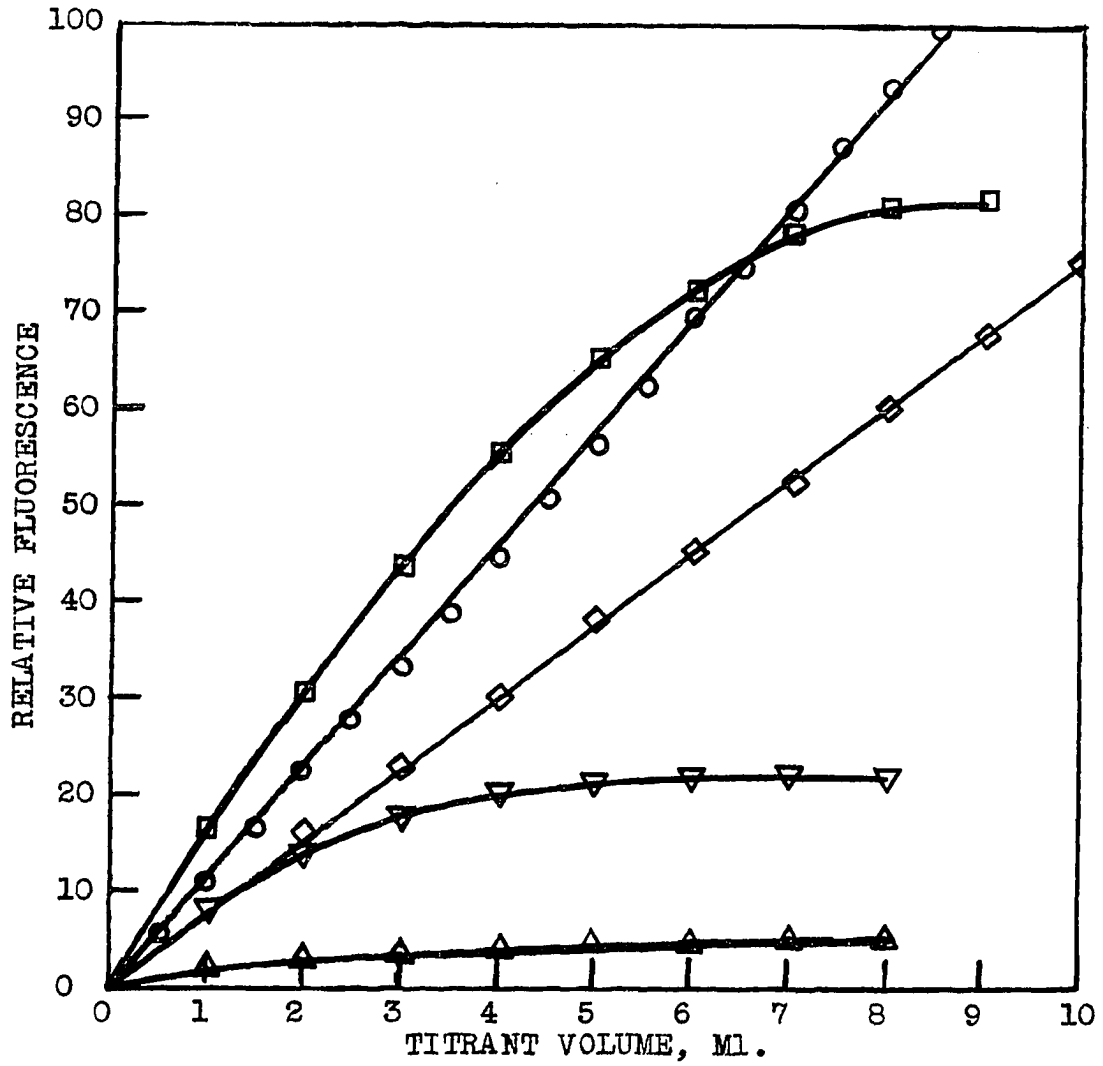


Figure 40. Fluorescence upon addition of 10^{-4} M calcium or magnesium chloride to 100 ml. of 10^{-4} M 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone

○ solution is 0.5 M in KOH; titrant is $CaCl_2$

◇ solution is 2.0 M in KOH; titrant is $CaCl_2$

□ solution is 0.5 M in KOH; titrant is $MgCl_2$

▽ solution is 1.0 M in KOH; titrant is $MgCl_2$

△ solution is 2.0 M in KOH; titrant is $MgCl_2$

hydroxide solution, where the amount of calcium ranged from a trace amount to two moles of calcium per mole of the compound, is shown in figure 26.

EDTA as a sequestering agent

The decrease in fluorescence of 100 ml. of a 10^{-4} M solution of each of the three reagents, 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone, 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone and 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone, in 0.5 M potassium hydroxide upon addition of 0.868 x 10^{-4} M dipotassium dihydrogen ethylenediaminetetraacetate is shown in figure 41. The result of a similar fluorometric titration of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone is also shown in figure 41. The fluorescent measurements were taken with the Turner fluorometer using the appropriate filters previously described for each compound.

Apparatus

Fluorescent spectra were taken with an Aminco Bowman spectrofluorometer equipped with a D.C. arc, xenon discharge tube. The cell holder was situated in such a way that the fluorescence was emitted through the same face that the activating light entered. The cells used were P-E-CO Photovolt 10 m/m silica cells. Several fluorometric titrations

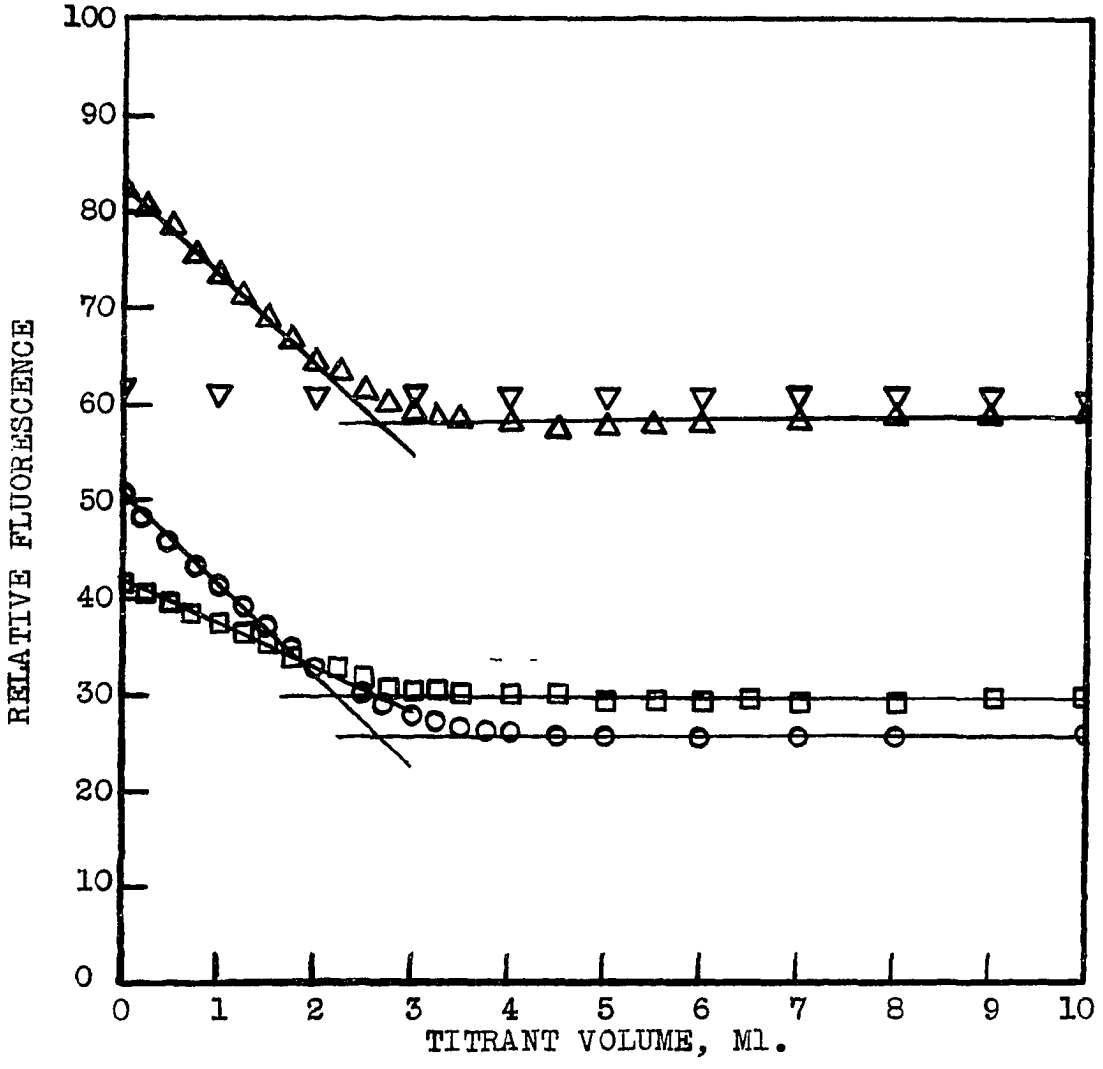


Figure 41. Fluorometric titrations with 8.68×10^{-5} M EDTA, K_2H_2Y

△ 100 ml. of 10^{-4} M 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone; 0.5 M KOH

▽ 100 ml. of 10^{-4} M 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone; 0.3 M tetrabutylammonium hydroxide

□ 100 ml. of 10^{-4} M 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone; 0.5 M KOH

○ 100 ml. of 10^{-4} M 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone; 0.5 M KOH

were performed using a Turner Model 110 fluorometer fitted with a pyrex flow through cell.

Absorption spectra were taken with a Carey Model 15 recording spectrophometer. Other absorption measurements were taken with a Beckman Model DU spectrophotometer. The Beckman was fitted with a flow-through pyrex cell for spectrophotometric titrations when the absorbed wavelength was greater than 330 mu. At other times the P-E-CO photovolt 10 m/m silica cells were used.

Nuclear magnetic resonance spectra of 2-methyl-7-hydroxychromone and its methyleneiminodiacetic acid derivative were obtained with a Varian Associates Model A 60 spectrometer. The spectra of the methyleneiminodiacetic acid derivatives of 7-hydroxyflavone and 4'-methoxy-7-hydroxyflavone were obtained with a Varian Associates Model H R 60 spectrometer. The work was performed by Dr. Roy King's department at Iowa State University.

DISCUSSION

2-Methyl-7-hydroxychromone was synthesized by a method similar to that described by Blaise (5) for the preparation of 7-hydroxychromone. The procedure was simple and required only readily available materials, resorcinol, ethylacetoacetate and nitrobenzene. The yield of the crude produce was about 40 per cent of the theoretical yield. Recrystallization of the red-brown material from ethanol gave a produce which did not noticeably differ from the crude material. Treatment with activated charcoal in hot ethanol was effective in removing much of the dark color; however, there was a considerable loss of material. The partially decolorized material was recrystallized from glacial acetic acid and washed with ethanol, giving a slightly yellow product which melted at 254-255° C. The overall yield was 5 per cent. Geissman (14) reported a melting point of 253-254° C. The calculated percentages of carbon and hydrogen in 2-methyl-7-hydroxychromone are 68.18 and 4.56 per cent respectively, and those found by elemental analysis were 68.08 and 4.44 per cent.

Some 7-hydroxyflavone was synthesized by the method of Blaise (5) using the starting materials, ethyl benzoylacetate and resorcinol in nitrobenzene. The yield of crude produce was about 40 per cent of the theoretical. However, the purification of the 7-hydroxyflavone proved to be even more difficult than the purification of 2-methyl-7-hydroxychromone.

After decolorization with charcoal and repeated recrystallization from ethanol, the silky needle crystals discolored at 235° C. and melted at 241-242° C. Blaise (5) reported a melting point of 240° C.

7-Hydroxyflavone was prepared by the method described by Baker (4). The starting materials in this procedure were 2', 4'-dihydroxyacetophenone and benzoyl chloride. Although this procedure involved two intermediates, the overall yield was 17 per cent of the theoretical yield. After a single recrystallization from ethanol the 7-hydroxyflavone had less coloration than that obtained by the method of Blaise after decolorization with charcoal and several recrystallizations. The melting point was 242-243° C. while that reported by Baker was 240° C.

The method of Baker was also used for the preparation of 4'-methoxy-7-hydroxyflavone starting with 2',4'-dihydroxyacetophenone and p-anisoyl chloride. The melting point of the silky needle crystals of 4'-methoxy-7-hydroxyflavone was 268-269° C. Baker reported a melting point of 263-264° C. with rapid heating.

2-Methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-chromone was prepared via the Mannich reaction. Because of the similarity between 2-methyl-7-hydroxychromone and 4-methyl-7-hydroxycoumarin, the conditions selected for the condensation were the same as those found to be most suitable

for the preparation of the methyleneiminodiacetic acid derivative of 4-methyl-7-hydroxycoumarin by Huitink (16). The conditions were a 3:2:3 mole ratio of disodium iminodiacetic acid monohydrate, 2-methyl-7-hydroxychromone and formaldehyde in glacial acetic acid at 70° C. The crude product isolated from the reaction mixture was dissolved in dilute sodium hydroxide and reprecipitated with hydrochloric acid. The resulting white crystalline material was dried at 100° C. The melting point was 155-178° C. with decomposition.

A neutralization equivalent weight, 177.6 g. per equivalent, was determined by a potentiometric titration with sodium hydroxide. Assuming two replaceable hydrogens the theoretical neutralization equivalent weight is 160.6 g. per equivalent. Increasing the drying time and temperature caused a decrease in the equivalent weight; however, the compound began to discolor before the theoretical equivalent weight was reached. Thermogravimetric analysis showed that the rate of weight loss increased steadily as the temperature changed from 70° C. to 170° C. Beyond 170° C. the rate of weight loss became much more rapid. Therefore, it appears that there is no optimum drying condition to yield a compound of definite composition.

The amount of water present in different samples of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone was determined by Karl Fischer titrations. Shown in table 1 is a comparison of the amount of water found by Karl Fischer

titrations and that calculated from the difference between the experimental and theoretical equivalent weights. In all cases there is reasonable agreement which supports the premise that water is the cause of the deviation from the theoretical equivalent weight.

Elemental analysis of two samples of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone showed the compound to be 51.28 per cent carbon, 5.16 percent hydrogen, and 4.97 per cent nitrogen for one sample and 50.93 per cent carbon, 5.40 per cent hydrogen, and 4.17 per cent nitrogen for the other sample. The analysis of the former corresponds to the formula $C_{15}H_{19.2}N_{1.25}O_{8.48}$ and the later corresponds to $C_{15}H_{19.1}N_{1.06}O_{8.74}$. The excess hydrogen and oxygen in these formulas compared to the theoretical formula of $C_{15}H_{15}NO_7$ is $H_{3.2}O_{1.5}$ in one case and $H_{4.1}O_{1.7}$ in the other. If excess water is calculated from the excess hydrogen, ignoring the excess oxygen because it reflects the errors of all analyses, the apparent molecular weight of the first sample is 350.0 compared to 353.4 which is twice the neutralization equivalent weight of the same sample. For the second sample the apparent molecular weight is 358.1 compared to 358.2 which is twice the neutralization equivalent weight of that sample.

Because of the large discrepency in the amount of nitrogen in the results of one of the above elemental analyses, various samples of the compound were analyzed for nitrogen by the

Kjeldahl method. The results in table 2 show the per cent nitrogen found to be within one per cent relative to the amount calculated by assuming one-half nitrogen per neutralization equivalent.

The above analyses support the premise that there is one methyleneiminodiacetic acid group per 2-methyl-7-hydroxychromone molecule and that water is the only significant impurity.

Figure 2 is a representation of the nuclear magnetic resonance spectrum of 2-methyl-7-hydroxychromone. The large singlet at 2.18 PPM which integrated as three protons was attributed to the protons of the methyl group in the 2 position. The singlet at 5.80 PPM which integrated as one proton was attributed to the hydrogen in the 3 position. The doublet at 6.30 and 6.35 PPM which integrated as one proton was attributed to the hydrogen in the 8 position. The cause of the splitting of 2.4 cycles per second, which is a reasonable amount for a meta hydrogen, was assigned to the hydrogen in the 6 position. The quartet at 6.58, 6.63, 6.73 and 6.76 PPM which integrated as one proton was attributed to the hydrogen in the 6 position. The cause of the splitting of nine cycles per second was assigned to the proton in the 5 position, and the cause of the splitting of two cycles per second was assigned to the hydrogen in the 8 position. The doublet at 7.67 and 7.53 PPM which also integrated as one proton was attributed to the hydrogen in the 5 position. The cause of the splitting of nine cycles per second was assigned to the hydrogen in the 6 position. In

the spectrum of the methyleneiminodiacetic acid derivative, figure 3, the peak and the proton or protons to which it was attributed are as follows: 2.40 PPM three hydrogens of the two methyl group; 3.75 PPM, four hydrogens of the two methylene groups between the nitrogen and carboxy groups; 4.43 PPM, two hydrogens of the methylene group between the nitrogen and the chromone ring; 6.03 PPM, hydrogen in 3 position; doublet at 6.62 and 6.77 PPM, hydrogen in 6 position; doublet at 7.62 and 7.77 PPM, hydrogen in 5 position. In the above spectrum there was no splitting attributed to hydrogens meta to one another; therefore, it was concluded that the hydrogen in either the 6 or 8 position was replaced. Because the splitting attributed to hydrogens ortho to one another was still present, the methyleneiminodiacetic acid group was assigned to the 8 position.

Two acid dissociation constants were determined from the potentiometric titration of 2-methyl-7-hydroxy-8-(N,N bis-carboxymethylaminomethyl)chromone dissolved in water and a small excess of sodium hydroxide using hydrochloric acid as titrant, figure 1. The constants obtained were $10^{-2.9}$ and $10^{-6.45}$.

Evidence of the reaction of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone with calcium ions was observed when the derivative was titrated potentiometrically with sodium hydroxide in the presence of calcium, figure 4.

An approximate formation constant of $10^{6.6}$ was calculated from these data. This is an average of three values, $10^{6.4}$, $10^{6.6}$, and $10^{6.8}$, which were found at three different acidities. The calculations were made with the assumption that the moles of calcium-derivative complex ions were equal to the moles of sodium hydroxide added which were in excess of the amount that would have been required to reach the second end point if calcium had not been present. It was also assumed that the moles of calcium ions were equal to the total calcium minus the moles of complex ions. The 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone species assumed to react with calcium was the anion with three hydrogens replaced. The amount of this ion present was calculated from the ratio of total derivative less the amount combined with calcium to the amount of the anion present. The ratio was calculated from the acid dissociation constants and the hydrogen ion concentrations.

7-Hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone was also prepared via the Mannich reaction. The conditions were a 3:2:3 mole ratio of disodium iminodiacetic acid to 7-hydroxyflavone to formaldehyde in glacial acetic acid at 70° C. The crude product dissolved in dilute sodium hydroxide solution and reprecipitated with hydrochloric acid was yellow-green in color and gelatinous. After drying at 70° C. the solid was yellow and caked. The material shows no melting point. Decomposition starts at 150° C.

A neutralization equivalent weight of 197.7 g. per equivalent was determined by potentiometric titration with sodium hydroxide, figure 5. If two replaceable hydrogens are assumed, the theoretical neutralization equivalent weight is 191.6 g. per equivalent.

Elemental analysis of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone showed carbon 60.41 per cent, hydrogen 4.61 per cent and nitrogen 3.60 per cent which corresponds to a compound of $C_{20}H_{18.43}N_{1.02}O_{7.80}$. The difference between this formula and the theoretical formula, $C_{20}H_{17}NO_7$, is $H_{1.43}O_{0.80}$. If water present is calculated from the excess hydrogen, the apparent molecular weight is 396.1 compared to 395.4 which is twice the neutralization equivalent weight.

Analyses of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone for water by a Karl Fischer titration showed the compound to be 5.95 per cent water. This was considerably higher than the three per cent calculated from neutralization equivalent weight titration. Because the 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone was found to contain variable amounts of water, it was suspected that the flavone derivative had absorbed moisture. The per cent water calculated from the data obtained from a different potentiometric titration performed shortly before the Karl Fischer titration was 6.12 per cent which is in good agreement with 5.95 per cent found by the Karl Fischer titration.

Shown in figure 6 is a representation of the nuclear magnetic resonance spectrum of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone. It can not be compared to the spectrum of 7-hydroxyflavone because 7-hydroxyflavone was not sufficiently soluble in deuterium oxide and potassium carbonate to obtain a spectrum. However, the premise that the methyleneiminodiacetic acid group is in the 8 position is supported by much the same argument used for 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone, namely that there is no splitting attributed to hydrogens meta to one another, but there is splitting attributed to hydrogen ortho to one another. The peak at 3.65 PPM integrated as four protons and was attributed to the hydrogens of the methylene group between the nitrogen and the carboxy group. The peak at 4.13 PPM integreated as two protons and was attributed to the methylene group between the nitrogen and the ring. The singlet at 6.23 PPM integrated as a single proton and was attributed to the hydrogen in the 3 position. The doublet at 6.61 and 6.76 PPM integrated as a single proton and was attributed to the proton in the 6 position with the splitting of nine cycles per second attributed to the hydrogen in the 5 position. The broad peak at 7.43 PPM integrated as 6 protons. The spikes at 7.70 and 7.54 PPM were attributed to the hydrogen in the 5 position with the splitting attributed to the hydrogen in the 6 position. The remainder of peak was attributed to the five hydrogens of the phenyl group.

Two acid dissociation constants were determined from the potentiometric titration of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone dissolved in water and a small excess of sodium hydroxide using hydrochloric acid as titrant, figure 5. The constants obtained were $10^{-2.8}$ and $10^{-6.37}$.

The synthesis of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone was identical to that of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone except that the starting flavone was 4'-methoxy-7-hydroxyflavone. The physical appearance of the two compounds was also the same; however, the decomposition temperature, 190° C., was 40° C. higher for the 4'-methoxy derivative.

The neutralization equivalent weight, 215.1 g. per equivalent, was determined by a potentiometric titration of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone with sodium hydroxide, figure 7. The theoretical equivalent weight, assuming two replaceable hydrogens is 206.7 g. per equivalent.

Percentage composition from elemental analysis was 59.18 per cent carbon, 4.90 per cent hydrogen and 3.30 per cent nitrogen which corresponds to a compound with the formula $C_{21}H_{20.9}N_{1.01}O_{8.69}$. If the difference between the amount of hydrogen in this formula and the theoretical formula, $C_{21}H_{19}NO_8$, is assumed to be present as water and the water is added to the theoretical molecular weight, the apparent

molecular weight is 430.5 compared to 430.2 which is twice the neutralization equivalent weight. The amount of water found by a Karl Fischer titration was 3.93 per cent compared to 3.88 per cent calculated from the difference in the experimental and theoretical equivalent weights.

The nuclear magnetic resonance spectrum, figure 8, of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone is compatible with the proposed structure. The peak at 3.54 PPM was attributed to the four hydrogens of the methylene groups next to the carboxy groups, and the peak at 3.69 PPM was attributed to the three hydrogens of the methoxy group. The two peaks combined integrated as 7 protons. The peak at 4.26 PPM integrated as 2 protons and was attributed to the hydrogens of the methylene group next to the ring. The singlet at 6.07 PPM integrated as one proton and was attributed to the proton in the 3 position. The three peaks at 6.69, 6.57 and 6.42 PPM integrated as 3 protons. It is believed that these three peaks are the result of two doublets with one-half of each superimposed on one another. These were attributed to the 6, 3' and 5' hydrogens. The four peaks at 7.61, 7.47, 7.40 and 7.25 PPM integrated as 3 protons. These are believed to be a doublet at 7.61 and 7.47 PPM attributed to the hydrogen in the 5 position, and a doublet at 7.40 and 7.25 PPM attributed to the hydrogens in the 2' and 6' positions.

Two acid dissociation constants, $10^{-3.0}$ and $10^{-6.37}$, were determined from the potentiometric titration of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone dissolved in water and a small excess of sodium hydroxide with hydrochloric acid, figure 7.

Again the above analyses support the premise that the compound is 4'-methoxy-7-hydroxy-8-(N,N carboxymethylaminomethyl)-flavone.

Absorption and Fluorescence

Absorption spectra of 2-methyl-7-hydroxychromone are shown in figure 9. The wavelength of maximum absorbance is 295 mμ for an acidic solution and 335 mμ for a basic solution. The effect of the acidity of the solution on the absorbance at these wavelengths is shown in figure 10. An acid dissociation constant for 2-methyl-7-hydroxychromone calculated from these data is $10^{-7.36}$.

Absorption spectra of 7-hydroxyflavone are shown in figure 12. The wavelength of maximum absorbance is 315 mμ for an acidic solution and 360 mμ for a basic solution. The effect of the acidity of the solution on the absorbance at these wavelengths is shown in figure 13. An acid dissociation constant for 7-hydroxyflavone calculated from these data is $10^{-7.38}$.

Absorption spectra of 4'-methoxy-7-hydroxyflavone are shown in figure 15. The wavelength of maximum absorbance is

325 mμ for an acidic solution and 375 mμ for a basic solution. The effect of acidity on the absorbance of 4'-methoxy-7-hydroxyflavone at these wavelengths is shown in figure 16. An acid dissociation constant, $10^{-7.27}$, was calculated from these data.

Absorption spectra of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone are shown in figure 18. The wavelength of maximum absorbance is 295 mμ for an acidic solution and 335 mμ for a basic solution. These wavelengths are the same as those found for 2-methyl-7-hydroxychromone. The effect of acidity on the absorbance of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone is shown in figure 19. The acid dissociation constant, $10^{-6.50}$, calculated from these data is slightly smaller than the $10^{-6.45}$ calculated from potentiometric data. In either case the constant is about eight times greater than the $10^{-7.36}$ for the 2-methyl-7-hydroxychromone which is about the shift that would be predicted upon substitution of a methyleneiminodiacetic acid group ortho to a phenolic group (30).

Fluorescent spectra of 2-methyl-7-hydrxoy-8-(N,N biscarboxymethylaminomethyl)chromone are shown in figure 20. The difference between these spectra and spectra of 2-methyl-7-hydroxychromone, figure 11, is that the intensity of the fluorescence of the derivative is about 1.5 times as great, the wavelength of emitted light at maximum fluorescence is

10 mμ less for the derivative, and the fluorescence at pH 3 is significant for the derivative but not for the 2-methyl-7-hydroxychromone.

The effect of acidity on the fluorescence of aqueous solution of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone is shown in figure 21. A third acid dissociation constant calculated from these data is $10^{-11.41}$. This value is also in agreement with the findings of Schwarzenbach (30). That is, the proton of the ammonium group is weaker than normal, $10^{-9.89}$ for iminodiacetic acid, when the methylene iminodiacetic acid group is adjacent to a hydroxy group.

The effect of metal ions on the wavelengths of maximum fluorescence is given in table 3. Although some metal ions do cause a shift in wavelength, the magnitude of the shift is not great enough to use this as the basis for the analysis of a specific metal ion.

The effect of acidity on the fluorescence of aqueous solutions of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone in the presence of various divalent metal ions is shown in figure 22. From the information obtained from figure 22 it appears that 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone can be used as a reagent for calcium in the presence of magnesium in strongly alkine solutions. As expected, barium and strontium would interfer. The increased fluorescence caused by zinc and magnesium in

an acidity range of about pH 7 to 8 might also be used in some analytical scheme.

As seen in figure 23, the fluorescence of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone is not a linear function of concentration. The slope decreases as the concentration increases.

The rate constants for the decomposition of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone in 0.5 M potassium hydroxide, in the absence and presence of calcium, were found to be first order with respect to derivative concentration, figure 24. The constant was 0.037 hr^{-1} with calcium absent and 0.087 hr^{-1} with calcium present. The time required for one-half the compound to decompose with calcium absent is 18.5 hours compared to 1.3 hours found by Eggers (10) for Calcein Blue under similar conditions. In the presence of calcium eight hours are required for one-half decomposition compared to 0.6 hours for Calcein Blue. Eggers' explanation of the more rapid decomposition in the presence of metals which form complex ions with coumarin derivatives appears to be applicable to the chromone derivatives. That is, the positive charge of the metal ion attracts the negative charge of the phenolate making the carbon adjacent to the ring oxygen more susceptible to nucleophilic attack.

The increase in fluorescence of solutions of 2-methyl-7-hydroxy-8-(N,N carboxymethylaminomethyl)chromone upon addition

of calcium or magnesium chloride is shown in figure 25. As seen in the graph the response of fluorescence to calcium concentration is linear. If the hydroxide ion concentration is increased, the intensity of the fluorescence is less, but it is still linear with respect to calcium concentration. The increased fluorescence upon addition of magniesum to the chromone derivative in 0.5 M potassium hydroxide is as great as that for calcium for low concentrations of the metals; however, it becomes less than that for calcium at higher concentrations. In two molar potassium hydroxide there is no significant change in fluorescence upon addition of magnesium.

The data for the fluorometric titration of 2-methyl-7-hydroxy-8-(N,N carboxymethylaminomethyl)chromone, figure 26, were used to calculate a conditional equilibrium constant for the formation of the calcium-chromone derivative complex ion in 0.5 M potassium hydroxide. The method used was a modification of that used by Wallach and Steck (35). The calculation was made as described below.

The intersection of the extrapolated linear portions of the curve occurs at a ratio of 1.1 moles of chromone derivative to 1.0 moles of calcium added. Therefore, it is assumed that the complex ion formed is of a one to one ratio. The constant is expressed as

$$(1) \quad K = \frac{(CaA)}{(Ca)(A)} \quad ,$$

where A is the free anion of the chromone derivative, CaA is the complex ion, and Ca is all calcium that is not combined with A. It was assumed that (CaA) was proportional to the fluorescence, f, and that (A) was proportional to the f_{max}, where all A is combined with Ca, minus f. Consequently,

$$(2) \quad K = \frac{f}{(Ca)(f_{max}-f)} .$$

It was also assumed that the uncombined calcium was equal to the total calcium added times $(f_{ex}-f)/f_{ex}$ where f_{ex} is the fluorescence that the solution would have if the all calcium were combined with A, found by extrapolating the linear portion of the curve. Hence,

$$(3) \quad K = \frac{f}{(Ca_t)\left(\frac{f_{ex}-f}{f_{ex}}\right)(f_{max}-f)} .$$

Rearrangement gives

$$(4) \quad \frac{1}{f} = \frac{1}{f_{max}} + \frac{1}{K(f_{max})(Ca_t)\left(\frac{f_{ex}-f}{f_{ex}}\right)}$$

Plotting 1/f vs. $\frac{1}{(Ca_t)\left(\frac{f_{ex}-f}{f_{ex}}\right)}$ gives a slope of $\frac{1}{K(f_{max})}$ and an intercept of $\frac{1}{f_{max}}$. The constant obtained, figure 42, for calcium and 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone is $10^{5.9}$. The explanation offered to account difference between this and $10^{6.6}$ found potentiometrically is that calcium is present as a species other than the simple hydated ion or the chromone derivative-calcium complex ion.

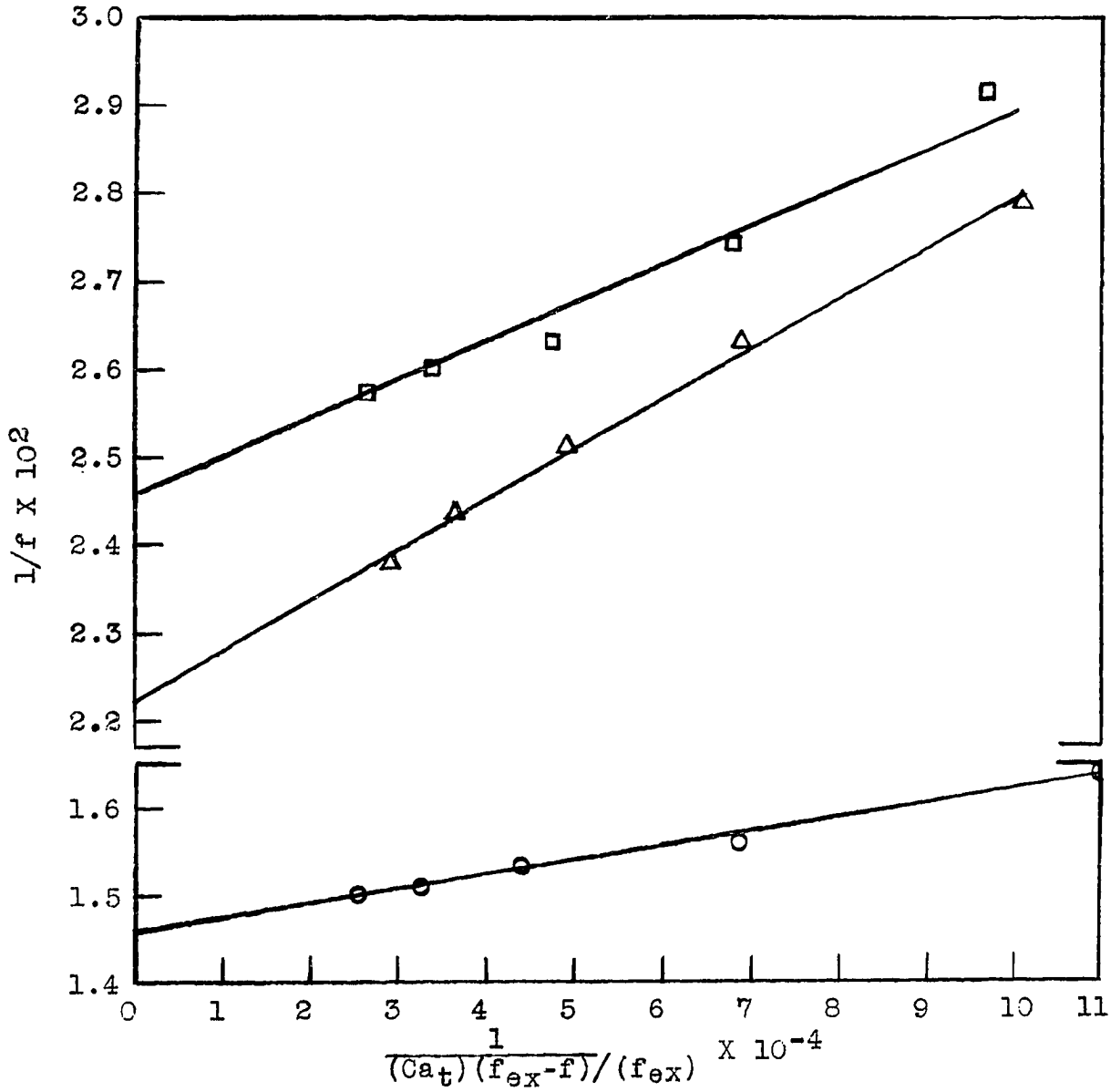


Figure 42. Determination of formation constants

slope, $(1/f_{max})(1/K)$; intercept, $1/f_{max}$

○ 2-methyl-7-hydroxy-8-(N,N biscarboxymethyl-aminomethyl)chromone

□ 7-hydroxy-8-(N,N biscarboxymethylaminomethyl) flavone

△ 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethyl-aminomethyl)flavone

Such a species could also explain the decrease in intensity of the fluorescence of the compound and calcium at higher potassium hydroxide concentration. The decomposition of the chromone derivative during the titration could also account for a lower formation constant. As seen in equation 3 a decrease in fluorescence which would occur with decomposition of the chromone derivative reduces the size of the constant in three ways. That is the numerator would be smaller and two factors of the denominator would be larger. This could also account for the fact that the ratio of chromone to calcium is not exactly one.

The absorption spectra of solutions of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone are shown in figure 27. The wavelengths of maximum absorbance are the same as those for 7-hydroxyflavone, which are 315 mμ for an acidic solution and 360 mμ for a basic solution. The effect of acidity on the absorbance of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl) flavone is shown in figure 28. The acid dissociation constant, $10^{-6.50}$, calculated from these data is smaller than the $10^{-6.37}$ calculated from the potentiometric data. As in the case of the chromone derivative either of these constants for the flavone derivative is from 8 to 10 times the $10^{-7.38}$ calculated from absorption data for 7-hydroxyflavone.

Fluorescent spectra of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone are shown in figure 29. The comparison

of these spectra to those of 7-hydroxyflavone, figure 14, leads to the same observations found in the comparison of the spectra of the chromone and its derivative.

The effect of the acidity of the solution on the fluorescence of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone is shown in figure 30. A third acid dissociation constant calculated from these data is $10^{-11.28}$ compared to $10^{-11.41}$ for the analogous chromone derivative.

Again as in the case of the chromone derivative, the effect of metal ions on the wavelengths of maximum fluorescence, table 4, is not sufficient to be used for any analytical purposes.

The effect of acidity on the fluorescence of aqueous solution of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone in the presence of various divalent metal ions is shown in figure 31. The effect is the same as that found for 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone.

The fluorescence of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone as a function of concentration, figure 32, deviates from a straight line even more than that of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone.

The rate constants for the decomposition of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone in 0.5 M potassium hydroxide, in the absence and presence of calcium, were found to be first order with respect to derivative concentration,

figure 33. The constant was 0.0060 hr^{-1} with calcium absent and 0.017 hr^{-1} with calcium present. The time required for one-half the compound to decompose with calcium absent is 115 hours compared to 18.5 hours for 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone and 1.3 hours for Calcein Blue (10). In the presence of calcium 40 hours are required for one-half decomposition compared to 8 hours for the analogous chromone derivative and 0.6 hours for Calcein Blue.

The increase in fluorescence of solutions of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone upon addition of calcium or magnesium ions is shown in figure 34. The results are the same as those discussed for 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone with the exception that in 0.5 M potassium hydroxide and low concentrations of calcium or magnesium the fluorescence was greater for the magnesium complex ion.

The data for the fluorometric titration of 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone, figure 26, were used to calculate a conditional equilibrium constant for the formation of a calcium-flavone derivative complex ion in 0.5 M potassium hydroxide. The method used was identical to that described for 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone, figure 42. The constant found was $10^{5.8}$.

The absorption spectra of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone are shown in figure 35.

The spectrum for the basic solution is almost identical to that of 4'-methoxy-7-hydroxyflavone, figure 15. The spectra of the acidic solutions are similar, however, the 4'-methoxy-7-hydroxyflavone was not sufficiently soluble at pH 3 to give a good spectrum. The effect of acidity on the absorbance of 4'-methoxy-7-hydroxy-8-(N,N carboxymethylaminomethyl)flavone is shown in figure 36. The acid dissociation constant calculated from these data is $10^{-6.58}$ which is considerably smaller than the $10^{-6.37}$ calculated from potentiometric data. However, both of the above constants are much larger than the $10^{-7.27}$ found for 4'-methoxy-7-hydroxyflavone.

The fluorescent spectra of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone has about the same relation to 4'-methoxy-7-hydroxyflavone as the 7-hydroxyflavone and the chromone derivatives have to their parent molecules. One feature found with the 4'-methoxy compounds in acidic solutions that was not found for the other compounds is that part of the light emitted, when the excitation wavelength is 340 mμ, is of a wavelength suitable for activating the compound. The result is a fluorescent curve with a broad shoulder.

As shown in figure 38 the effect of acidity on the fluorescence of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone is much more complicated than in the case of the other compounds. However, in the region above pH 10,

the region with which this work is primarily concerned, the behavior is the same as that of 2-methyl-7-hydroxychromone and 7-hydroxyflavone derivatives. A third acid dissociation constant for 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone calculated from the above data is $10^{-11.31}$ compared to $10^{-11.41}$ and $10^{-11.28}$ for 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone and 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone respectively.

The effect of metal ion on the wavelengths of maximum fluorescence, table 5, is not sufficient for analytical purposes.

The effect of acidity on the fluorescence of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone in the presence of divalent metal ions is shown in figure 39. Again the effect is esentially the same as that found for 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone.

The fluorescence of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone as a function of concentration, figure 32, is not a linear function. The deviation from linearity is greater at the higher concentrations.

The rate of decomposition of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone in 0.5 M potassium hydroxide, in the absence and presence of calcium, were found to be first order with respect to derivative. With calcium absent the constant is 0.0028 hr^{-1} and with calcium present the

constant is 0.0078 hr^{-1}. The time required for one-half the compound to decompose would be 236 hours without calcium present and 88 hours with calcium present.

The increase in fluorescence of solutions of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone upon addition of calcium or magnesium is shown in figure 40. The results are identical to those discussed for 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone.

The data for the fluorometric titration of 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone with calcium chloride, figure 26, were used to calculate a conditional equilibrium constant for the formation of a calcium-4'-methoxyflavone derivative complex ion in 0.5 M potassium hydroxide. The method used was identical to that used for 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone, figure 42. The constant found was 105.6.

EDTA as a sequestering agent

Whenever any of the three methyleneiminodiacetic acid derivatives were placed in potassium hydroxide a fluorescence which could be quenched by adding a small amount of EDTA was observed. In order to find the magnitude of this fluorescence each of the three reagents were titrated fluorometrically in 0.5 M potassium hydroxide with EDTA, figure 41. Because the results were similar for all three derivatives, it was suspected that the potassium hydroxide was a source of impurity.

A fluorometric titration of the chromone derivative in 0.3 M tetrabutylammonium hydroxide with EDTA did not show a decrease in fluorescence. This supports the premise that potassium hydroxide is a source of impurity and that EDTA is an effective sequestering agent. Quantitatively, the per cent impurity in the potassium hydroxide calculated as per cent calcium was 0.001 per cent.

Effect of substitution in the 2 position

Because the iminodiacetic acid derivatives of 2-methyl-7-hydroxychromone, 7-hydroxyflavone, and 4'-methoxy-7-hydroxyflavone differ only in the group at the 2 position, any difference in properties can be attributed to that group. As might be expected there is very little if any difference in their acid dissociation constants, in their chemical purity, or their reactions with metal ions. There is a difference in their physical properties in as much as the chromone derivative forms as a crystalline precipitate and dries pure white while both flavone derivatives form greenish gelatinous precipitates which dry to a yellow caked material. The solids of all three derivatives contain water in variable amounts with no apparent optimum drying conditions. All three also show large melting with decomposition ranges.

The fluorescence of basic solutions of the 2-methyl-7-hydroxychromone derivative with calcium present is blue while that of the flavone derivatives is yellow. The relative

Table 6. Relative intensity of the fluorescence of the methyleneiminodiacetic acid derivatives of benzo-γ-pyrones

Pyrone	Conditions	Derivative concentration x 10^6	Total calcium concentration x 10^6	Relative intensity
Chromone	pH 10	4	0	560[a]
Flavone	pH 10	20	0	50[a]
4'-Methoxyflavone	pH 10	20	0	160[a]
Chromone	0.5 M KOH	4	40	420[a]
Flavone	0.5 M KOH	40	200	56[a]
4'-Methoxyflavone	0.5 M KOH	4	40	30[a]
Chromone	0.5 M KOH	100	100	213[b]
Flavone	0.5 M KOH	100	100	36[b]
4'-Methoxyflavone	0.5 M KOH	100	100	76[b]

[a]Measured with a spectrofluorometer.

[b]Measured with a filter instrument.

intensity of the fluorescence of solutions of the derivatives under various conditions are shown in table 6. As can be seen in the table the fluorescence of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone is much more intense than that of the derivatives of either of the flavones. The substitution of a methoxy group into the 4' position greatly

Table 7. Rate of decomposition of methyleneiminodiacetic acid derivatives of benzopyrones in 0.5 M potassium hydroxide

Benzopyrone	Time for one-half calcium absent (hours)	Decomposition calcium present (hours)
2-methyl-7-hydroxychromone	18.5	8.0
7-hydroxychomone	11.5	40.0
4'-methoxy-7-hydroxyflavone	236.0	88.0
4-methyl umbelliferone[a]	1.3	0.6

[a]Reported by Eggers (10).

increased the fluorescence of the flavone derivative. Similar results were reported by Jatkar and Mattoo (18). Although the fluorescence of the chromone derivative is about 500 times that of the flavone derivative when measured with the spectrofluorometer, the fluorescence of the calcium-chromone complex ion is only about six times that of calcium-flavone complex ion when measured with a filter instrument.

Shown in table 7 is the time required for one-half the decomposition of the methyleneiminodiacetic acid derivatives of four benzopyrones in 0.5 M potassium hydroxide. The results support the initial premise that the benzo-γ-pyrones would be more stable is strong base than the benzo-α-pyrones.

SUMMARY

The 8-(N,N biscarboxymethylaminomethyl) derivatives of 2-methyl-7-hydroxychromone, 7-hydroxychromone and 4'-methoxy-7-hydroxyflavone have been prepared by condensing the appropriate benzopyrone with disodium iminodiacetate and formaldehyde in glacial acetic acid. The 7-hydroxyflavone and 4'-methoxy-7-hydroxyflavone used in the condensations were prepared by established procedures (4, 5). The method of Blaise (5) for 7-hydroxyflavone was successfully used for the preparation of 2-methyl-7-hydroxychromone. The procedure involves the reaction of resorcinol and ethyl acetoacetate in nitrobenzene.

It was shown by potentiometric titrations of the 8-(N,N biscarboxymethylaminomethyl) derivatives that their neutralization equivalent weights were higher than their theoretical equivalent weights. However, it was shown by elemental analyses and Karl Fischer titrations that the only significant impurity was water. The position of the 8-(N,N biscarboxymethylaminomethyl) group was established by nuclear magnetic resonance.

Three acid dissociation constants are reported for each of the three benzopyrone derivatives. The first was obtained from potentiometric data, the second from both potentiometric data and absorption data, and the third constant was obtained from fluorescence data. The value of the constants are consistent with Schwarzenbach's work (30). Specifically, the

first constant for each is about 10^{-3}, that of the phenolic hydrogens is about $10^{-6.5}$ or ten times greater than those of the 7-hydroxybenzo-γ-pyrones without the adjacent methyleneiminodiacetic acid groups and the third constants are about $10^{-11.5}$ which is considerably lower than that for iminodiacetic acid.

Absorption spectra for benzo-γ-pyrone derivatives are essentially the same as those of their parent molecules. The wavelength of activating light for maximum fluorescence is the same for benzo-γ-pyrones and their derivatives while the wavelength of the emitted light is about 10 mμ shorter for the derivative. Although some divalent metal ions cause a change in wavelengths of maximum fluorescence, the change is not large enough for analytical purposes.

Each of the three 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)benzo-γ-pyrones shows a quenching of fluorescence as the pH changes from 11 to 12. The divalent ions of calcium, magnesium, barium, strontium, and zinc restore the fluorescence at pH 12; however, further increase in hydroxide ion concentration to one molar or greater causes the fluorescence with zinc and magnesium to disappear. This makes the derivatives suitable analytical reagents for calcium in the presence of magnesium. Copper II quenches the fluorescence of the derivatives in the pH range from 6 to 11. Zinc ions cause a significant increase in fluorescence at pH 7 to 9.

The formation constant for the calcium derivative of 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone calculated from potentiometric data is $10^{6.6}$. Formation constants calculated from fluorometric data for the calcium derivative in 0.5 M potassium hydroxide are $10^{5.9}$ for 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone, $10^{5.8}$ for 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone and $10^{5.6}$ for 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone. These constants have a suitable magnitude for using these compounds as indicators for the EDTA titration of calcium in strongly alkaline solutions.

The initial premise that the 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)benzo-γ-pyrones would decompose slower than Calcein Blue, a benzo-α-pyrone, in strongly alkaline solutions has been demonstrated as shown in table 7.

Although 2-methyl-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)chromone decomposes in strongly alkaline solution faster than the two flavone compounds, it is the best reagent from the standpoint of fluorescent intensity and ease of preparation. If a reagent with yellow-green fluorescence is more desirable than one with blue fluorescence, the reagent of choice would be 4'-methoxy-7-hydroxy-8-(N,N biscarboxymethylaminomethyl)flavone. It has greater fluorescent intensity, but the 7-hydroxy-8-(N,N biscarboxymethylaminomethyl)-flavone is somewhat easier to prepare.

Further work in this area might include determining whether or not decomposition in alkaline media is effected by light, especially light of wavelengths which activate the compound for fluorescence. It might also include the synthesis of 4',7-dihydroxy-8-(N,N biscarboxymethylaminomethyl)flavone to determine if a 4'-hydroxy group would further reduce the rate of decomposition. The possibility that a 4'-hydroxy group would reduce the rate of decompositions is quite likely in view of the discussion of decomposition of Calcein Blue by Eggers (10).

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