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Fluorescence spectra of alkaline earth derivatives of azo compounds

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FLUORESCENCE SPECTRA OF ALKALINE
EARTH DERIVATIVES OF AZO COMPOUNDS.

Iowa State University of Science and Technology
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FLUORESCENCE SPECTRA OF ALKALINE EARTH
DERIVATIVES OF AZO COMPOUNDS

by

Rodney Louis Olsen

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Analytical Chemistry

Approved:

Signature was redacted for privacy.

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1962

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I. INTRODUCTION

Mason, in an article, Analytical Problems in Biology and Medicine, points out that "The apparent discrepancy between the biological significance of these elements (calcium and magnesium) and the relative infrequency of their determination reflects the absence of good analytical procedures applicable to biological samples. Magnesium," he states, "is seldom determined except for research purposes." (14). Calcium is generally present in materials to be analyzed for magnesium and, not infrequently, in much higher concentration. Several procedures are based on the EDTA titration of calcium plus magnesium (4) followed by a similar titration for calcium only (2). In these, magnesium is reported as the difference. Of the spectrophotometric methods, some are based on various azo compounds, usually commercially available dyes. One or more of several problems seems to be invariably encountered. The reagent may not differentiate between calcium and magnesium satisfactorily or it may absorb seriously at the wave length of maximum absorbance of the magnesium derivative. A method proposed by Mann and Yoe (12) requires the presence of 80% alcohol in the final solution for satisfactory discrimination against calcium and is critical with respect to time. Several methods are based on the formation of a lake with magnesium hydroxide (10,15). In addition to the problems usually encountered with lakes the presence of large amounts of calcium interferes and not predictably. A very sensitive fluorometric method has been proposed by White and Cuttitta (16). It requires the use of anhydrous dimethylformamide as a solvent

and the complete exclusion of water. The authors state that the reagent does not fluoresce in the presence of calcium but say nothing of the influence of calcium on the fluorescence of the magnesium derivative. Flame photometry has also been used for the determination of magnesium. According to Manna et al. (13) 1 to 6 micrograms can be determined in 80% acetone using a very sensitive instrument such as a Beckman DU spectrophotometer with a photomultiplier attachment.

A magnesium reagent which would differentiate against calcium and operate in aqueous medium would appear to be very useful. *o,o'*-Dihydroxyazobenzene, for the fluorometric determination of magnesium, has been shown to be such a reagent.

In 1956, Ellingboe and Diehl (3,7) determined the minimum structural requirements for an azo compound to be capable of combining with calcium or magnesium. Two hydroxy groups, *o*- and *o'*- to the azo group, were found necessary and sufficient; the combining ratio of metal and azo compound was invariably one to one. See also Diehl and Lindstrom (5,11). For the *o,o'*-dihydroxyazo compounds studied the formation constant toward magnesium was greater than that toward calcium, the ratio of the two being in the range of 50 to 1000. One compound alone of these compounds stood out, *o,o'*-dihydroxyazobenzene, the simplest member of the group. For this compound Ellingboe found no reaction with calcium and a formation constant toward magnesium of $10^{4.85}$, a startling difference, greater by far than displayed by any other compound of any type and sufficient to cause doubt that the organic compound was actually the material advertised.

We have now found that the yellow material, melting point 174°C. , first described by Willstätter (17) and more recently synthesized by another method by Freeman and White (8) is indeed *o,o'*-dihydroxyazobenzene; that it is indeed a reagent which will differentiate magnesium from calcium. Moreover, its magnesium derivative exhibits a strong fluorescence (9) but the free azo compound, alone or in the presence of calcium, does not. The magnesium derivative can be extracted into isoamyl alcohol, too, and this makes for some interesting variations in utilizing the compound in chemical analysis.

II. FLUORESCENCE SPECTRA

It was decided that the fluorescent properties of the magnesium derivative of *o,o'*-dihydroxyazobenzene should be studied in some detail, particularly with respect to the effects of the other alkaline earths, and that the study should be extended to several other azo compounds known to form calcium or magnesium derivatives.

Experimental Work

Apparatus

Spectra were obtained on an Aminco-Kiers spectrophotofluorometer. This instrument makes it possible to scan both the exciting and the fluorescent light. The instrument was equipped with an Osram xenon lamp as the source and a 1P28 photomultiplier as the detector. The slit arrangement was as follows: excitation beam 3, 2 and 3 mm. in order; fluorescent beam 3, 2, 3 and 2 mm. This arrangement was a compromise between the one permitting greatest sensitivity and greatest resolution, 10 mm., square, quartz cells being used. Spectra were recorded on a Moseley Model 2-S X-Y recorder. Measurements of pH were made with a Beckman Model G pH meter and Beckman No. 40495 electrode.

Reagents

Compounds designated B-1 and B-3 through B-7 were prepared by John Ellingboe (3,7). *o,o'*-Dihydroxyazobenzene was prepared as described in Part III. The compound F-241 was prepared by Fred Lindstrom (5). The compound Calmagite was obtained from the G. F. Smith Chemical

Co., Columbus, Ohio. It was assumed to be about 85% pure (11). The pH 11.4 buffer was prepared by partially neutralizing 670 ml. of anhydrous redistilled ethylenediamine and 200 ml. of water with 120 ml. of 2N hydrochloric acid. The pH 10 buffer was prepared from ammonium hydroxide and ammonium chloride as described by Diehl and Smith (6). Inorganic chemicals used were of reagent grade quality. All water used was distilled and deionized by passage through Amberlite MB-1 ion-exchange resin.

Stock solutions of each of the azo compounds was prepared within a few hours before use. Small quantities of alcohol and potassium hydroxide were used to facilitate solution. Solutions on which the spectra were run were prepared from stock solutions of the azo compounds, potassium chloride and buffer. Each was 2×10^{-5} M in the azo compound, 10^{-4} M in magnesium or calcium (if desired), 0.1 M in potassium chloride and contained 1 ml. of buffer per 50 ml. of solution. Except in the case of B-4 the alcohol content did not exceed one per cent. The pH of each of the solutions was checked and found to be within 0.05 of the value reported.

Fluorescent properties of the compounds and their calcium and magnesium derivatives

B-1. 1-(2'-Hydroxy-1'-benzeneazo)-2-hydroxynaphthalene. This compound itself shows moderate fluorescence at around 412 m μ . At 578 m μ the magnesium derivative only is appreciably fluorescent. The intensity is a little greater at pH 11.4 than at pH 10.2. The spectra are given in Figures 1 and 2 respectively.

B-2. 1-(2'-Hydroxy-1'-benzeneazo)-2-hydroxybenzene. This compound is later referred to as o,o'-dihydroxyazobenzene or DHAB. The compound itself shows no significant fluorescence; however, the magnesium derivative exhibits moderate fluorescence at around 580 m μ . The intensity is about the same whether at pH 11.4 or 10.2. The pertinent spectra are given in Figure 3.

B-3. 1-(2'-Hydroxy-1'-benzeneazo)-2,4-dihydroxybenzene. This compound itself shows no fluorescence. The magnesium derivative does fluoresce, however, at about 558 m μ . The intensity is less at pH 10.2 than at pH 11.4. In either case very little fluorescence is caused by the presence of calcium. This is illustrated in Figure 4.

B-4. 1-(2'-Carboxy-1'-benzeneazo)-2-hydroxynaphthalene.

B-5. 1-(2'-Carboxy-1'-benzeneazo)-2-hydroxy-5-methylbenzene.

No fluorescence characteristic of either of these compounds or of their magnesium or calcium derivatives was observed at either pH 10.2 or 11.4.

B-6. 1-(2',4'-Dihydroxy-1'-benzeneazo)-2-hydroxy-5-phenylbenzene. The compound itself is moderately fluorescent at 404 m μ . The excitation and fluorescence spectra are shown in Figure 5. At pH 11.4 both the calcium and magnesium derivatives fluoresce at 548 m μ , as is shown in Figure 6. The nature of the fluorescence changes with time. After several hours the calcium derivative becomes the more fluorescent and there is a decrease in wave length of both maximum excitation and maximum fluorescence. The spectra of the same solutions twenty-four hours later is shown in Figure 7. At pH 10.2 the magnesium derivative fluoresces at 580 m μ . This fluorescence is weaker than that at pH 11.4.

It is stable over a period of several hours. It is also interesting that at pH 10.2 the calcium derivative is virtually non-fluorescent. Refer to Figure 8.

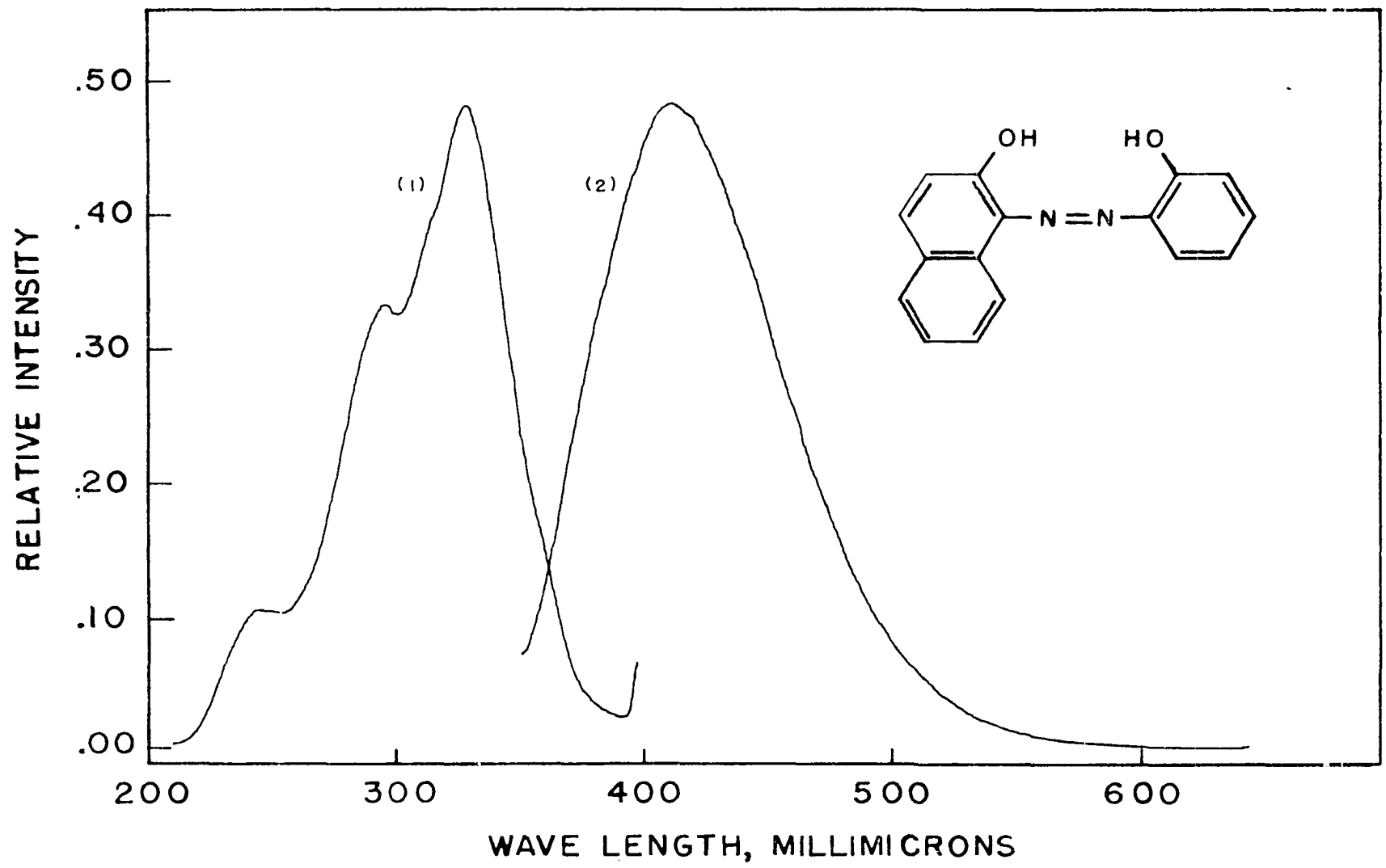
B-7. 1-(2'-Hydroxy-1'-naphthylazo)-2-hydroxy-5-phenylbenzene. This compound is strongly fluorescent at 412 $m\mu$. The spectra are shown in Figure 9. At pH 11.4 there is very little fluorescence characteristic of the calcium or magnesium derivatives if the solutions are freshly prepared. On standing for a day fluorescence develops at 540 $m\mu$. Refer to Figure 10. At pH 10.2 freshly prepared solutions of the calcium or magnesium derivatives exhibit no fluorescence except, of course, that of the compound itself. On standing overnight the compound precipitated.

Calmagite. 1-(2'-Hydroxy-5'-methyl-1'-benzeneazo)-2-naphthol-4-sulfonic acid. The compound exhibits maximum fluorescence at either 424 $m\mu$ or 440 $m\mu$, depending on the excitation wave length. Refer to Figure 11. The magnesium derivative exhibits a weak fluorescence having its maximum intensity at 595 $m\mu$. At this wave length the presence of calcium causes very little fluorescence. This is illustrated by the spectra shown in Figure 12. The intensity is about the same whether at pH 10.2 or 11.4 and is fairly stable with respect to time.

F-241. 1-(Hydroxy-2-naphthylazo)-2-nitro-2-naphthol-4-sulfonic acid. This compound exhibits no significant fluorescence nor do its calcium or magnesium derivatives.

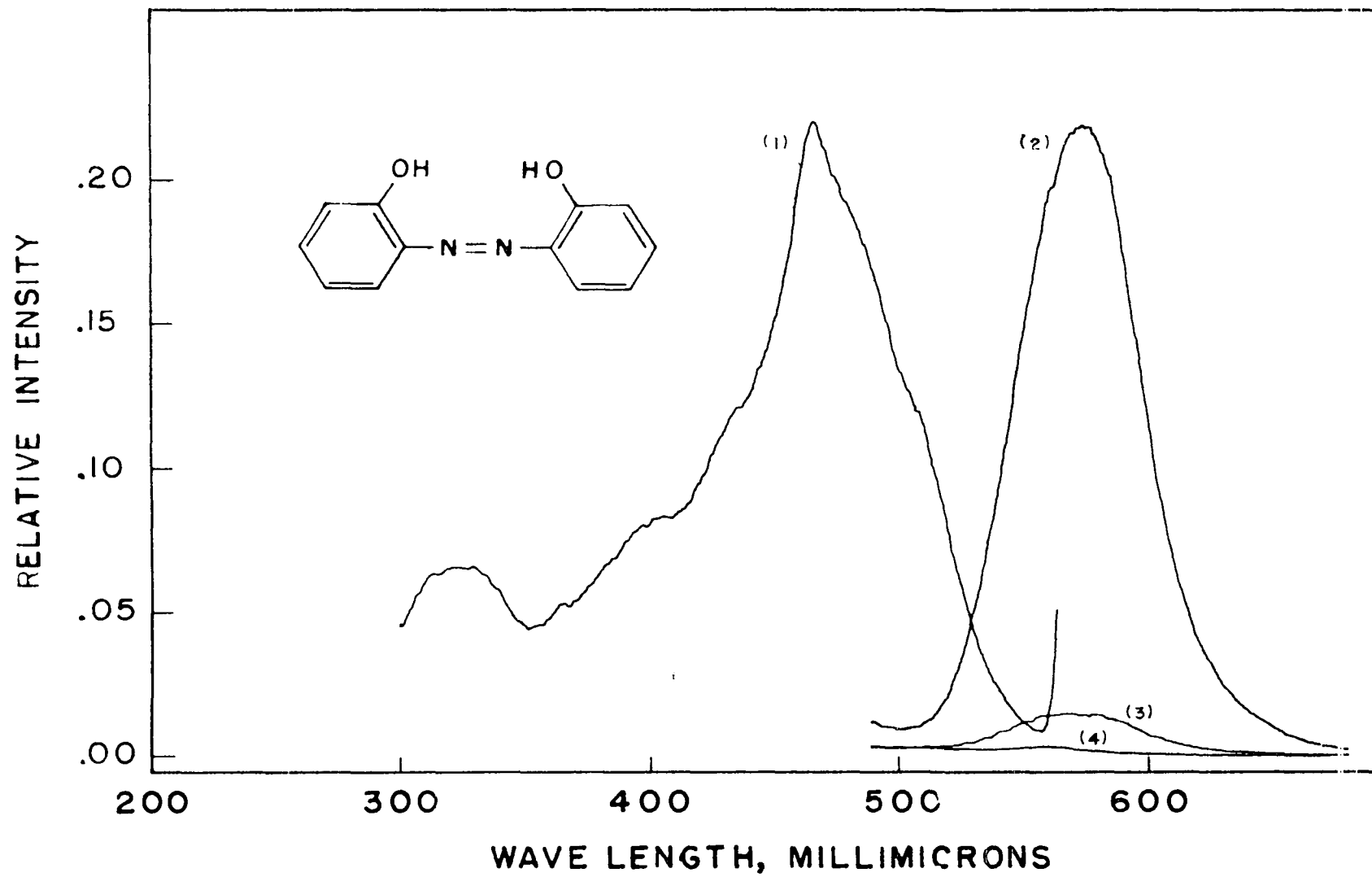
The wave lengths of maximum excitation and fluorescence and the relative intensities of the various compounds are summarized in Tables 1 and 2.

Figure 1. Fluorescence spectra of B-1 at pH 11.4
(1) Excitation, fluorescence monochromator set at
412 $m\mu$.
(2) Fluorescence, excitation set at 328 $m\mu$.



- Figure 2. Fluorescence spectra of B-1 at pH 11.4
- (1) Excitation of magnesium derivative, fluorescence monochromator set at $578 \text{ m}\mu$.
 - (2) Fluorescence of magnesium derivative, excitation set at $480 \text{ m}\mu$.
 - (3) Fluorescence of calcium derivative, excitation set at $480 \text{ m}\mu$.
 - (4) Fluorescence of the compound itself, excitation set at $480 \text{ m}\mu$.

- Figure 3. Fluorescence spectra of B-2 at pH 11.4
- (1) Excitation of magnesium derivative, fluorescence monochromator set at 580 m μ .
 - (2) Fluorescence of magnesium derivative, excitation set at 468 m μ .
 - (3) Fluorescence of the compound in the presence of calcium, excitation set at 468 m μ .
 - (4) Fluorescence of the compound itself, excitation set at 468 m μ .



- Figure 4. Fluorescence spectra of B-3 at pH 11.4
- (1) Excitation of magnesium derivative, fluorescence monochromator set at 558 m μ .
 - (2) Fluorescence of magnesium derivative, excitation set at 466 m μ .
 - (3) Fluorescence of calcium derivative, excitation set at 466 m μ .
 - (4) Fluorescence of the compound itself, excitation set at 466 m μ .

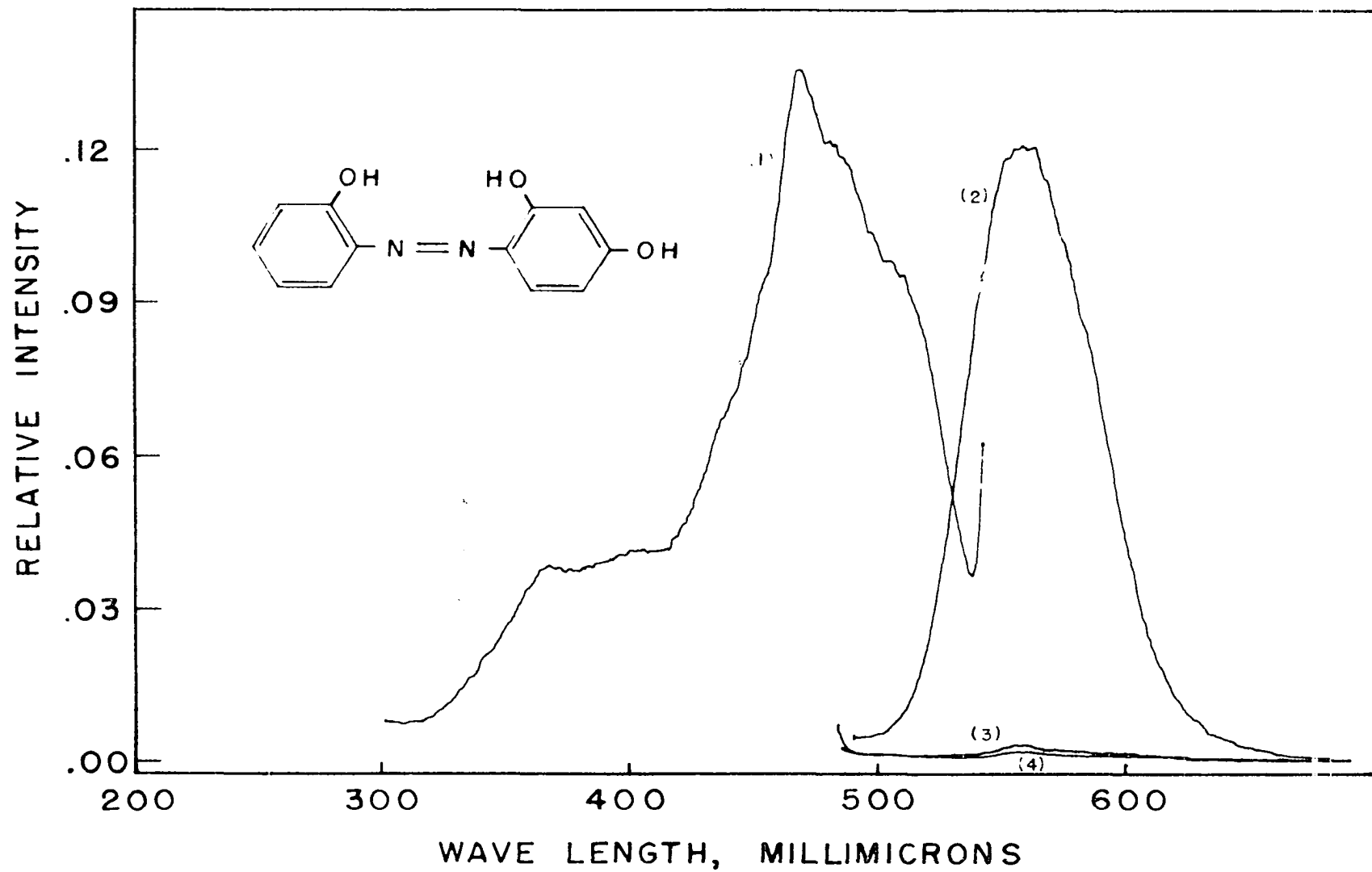
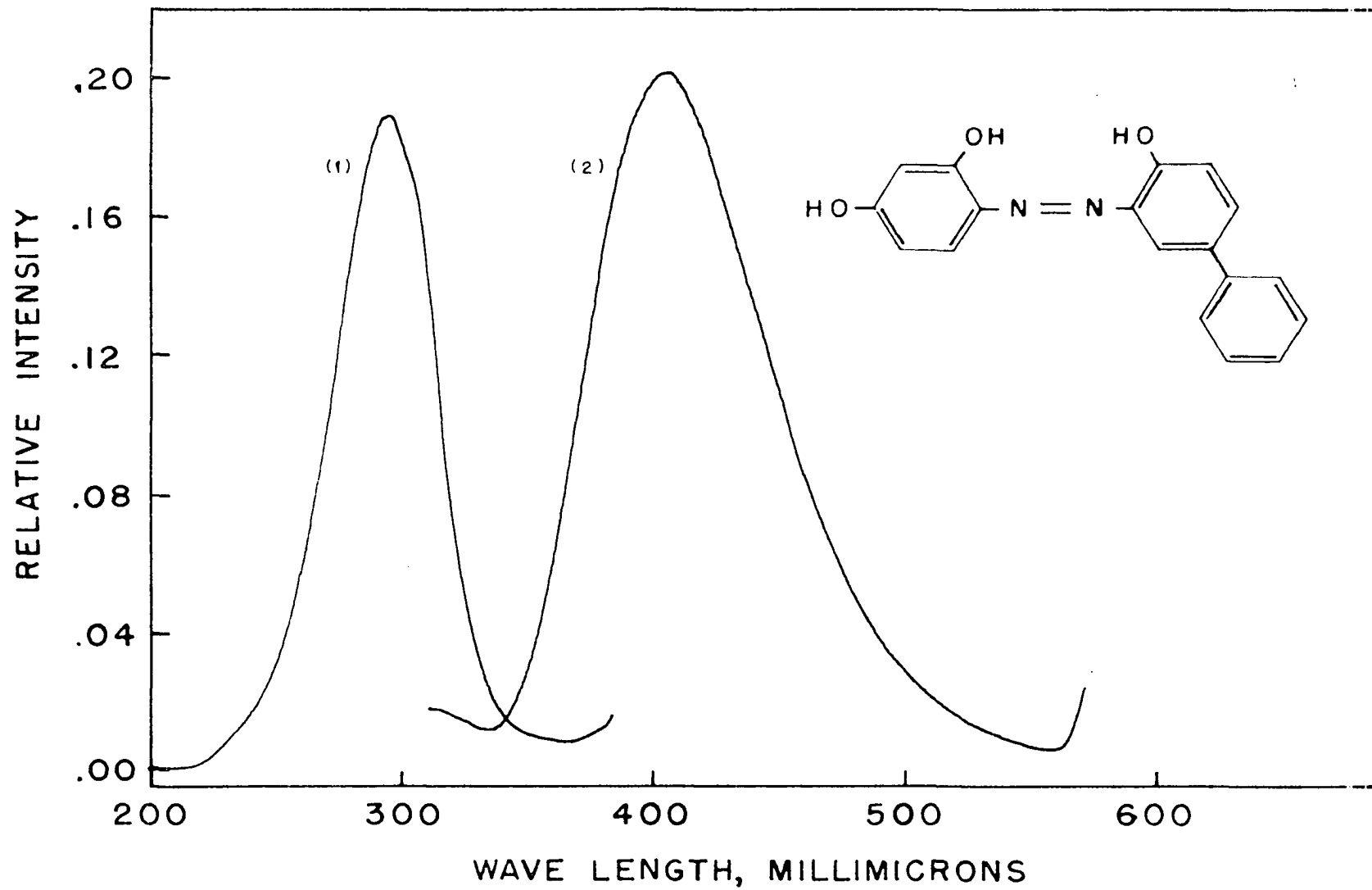
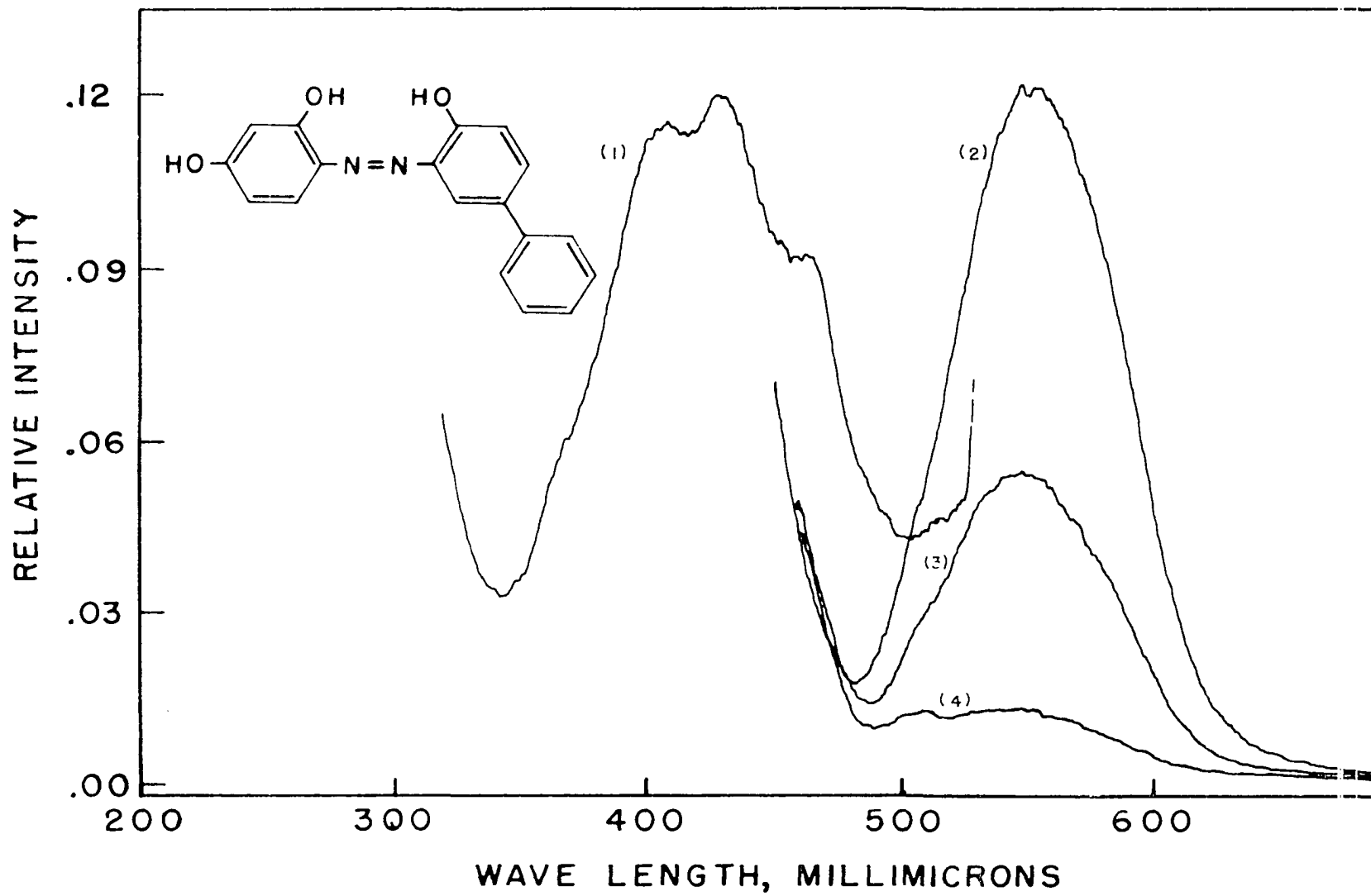


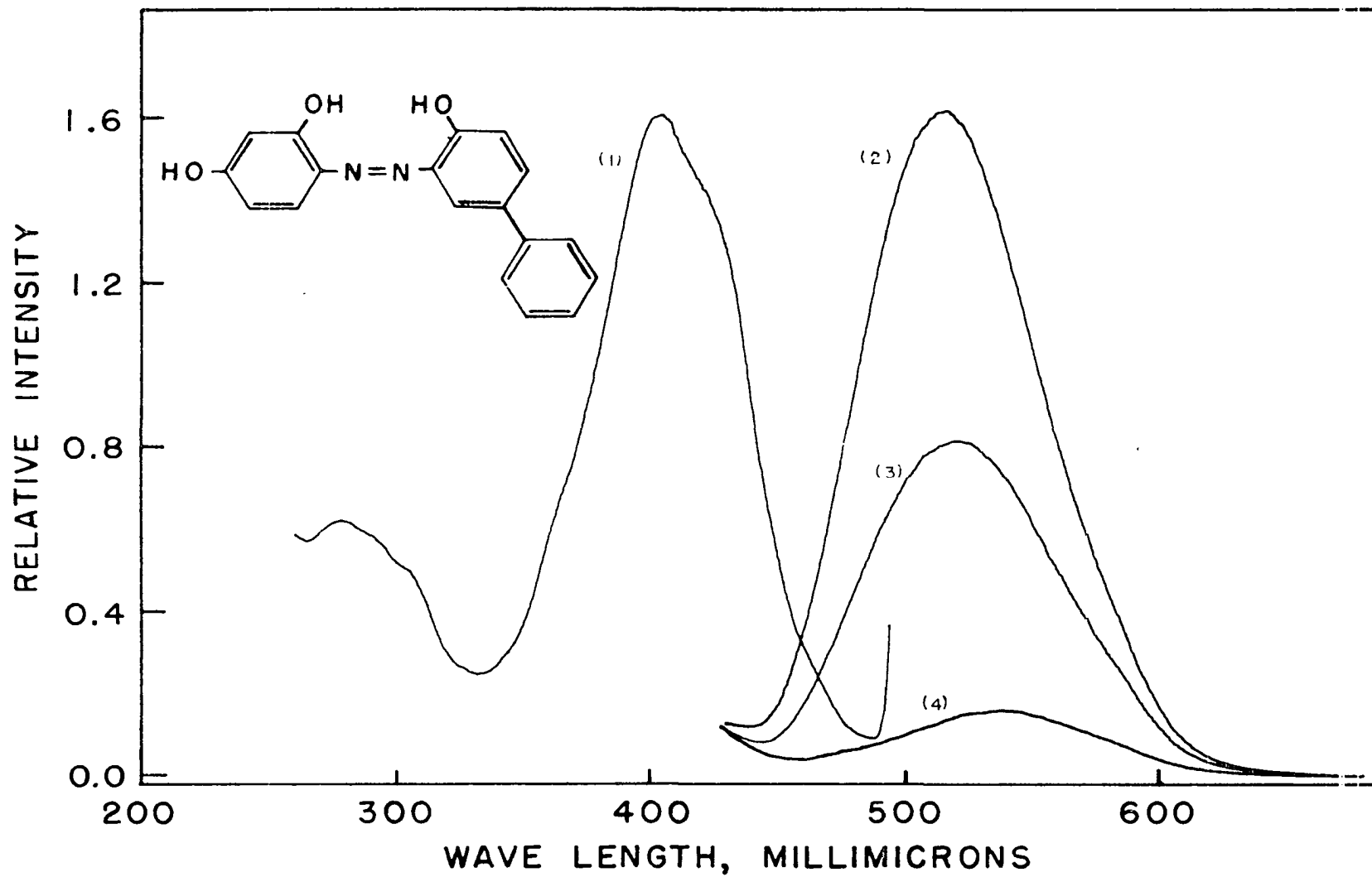
Figure 5. Fluorescence spectra of B-6 at pH 11.4
(1) Excitation, fluorescence monochromator set at
404 $m\mu$.
(2) Fluorescence, excitation set at 288 $m\mu$.



- Figure 6. Fluorescence spectra of B-6 at pH 11.4
- (1) Excitation of magnesium derivative, fluorescence monochromator set at 548 m μ .
 - (2) Fluorescence of magnesium derivative, excitation set at 428 m μ .
 - (3) Fluorescence of calcium derivative, excitation set at 428 m μ .
 - (4) Fluorescence of the compound itself, excitation set at 428 m μ .



- Figure 7. Fluorescence spectra of B-6 pH 11.4 (Taken 24 hours after solutions prepared)
- (1) Excitation of calcium derivative, fluorescence monochromator set at 512 m μ .
 - (2) Fluorescence of calcium derivative, excitation set at 402 m μ .
 - (3) Fluorescence of magnesium derivative, excitation set at 402 m μ .
 - (4) Fluorescence of the compound itself, excitation set at 402 m μ .



- Figure 8. Fluorescence spectra of B-6 at pH 10.2
- (1) Excitation of magnesium derivative, fluorescence monochromator set at 580 m μ .
 - (2) Fluorescence of magnesium derivative, excitation set at 472 m μ .
 - (3) Fluorescence of calcium derivative, excitation set at 472 m μ .
 - (4) Fluorescence of the compound itself, excitation set at 472 m μ .

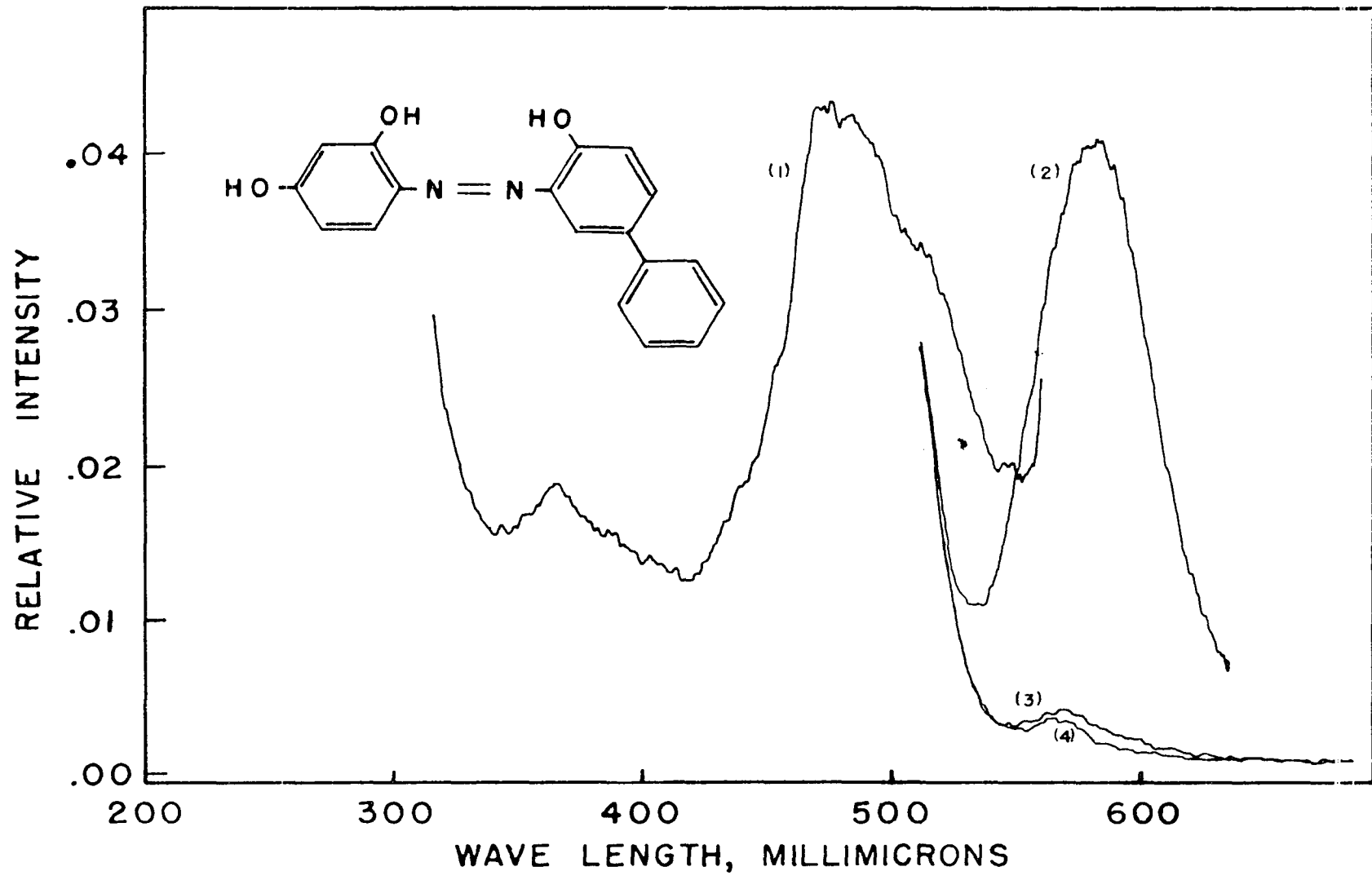
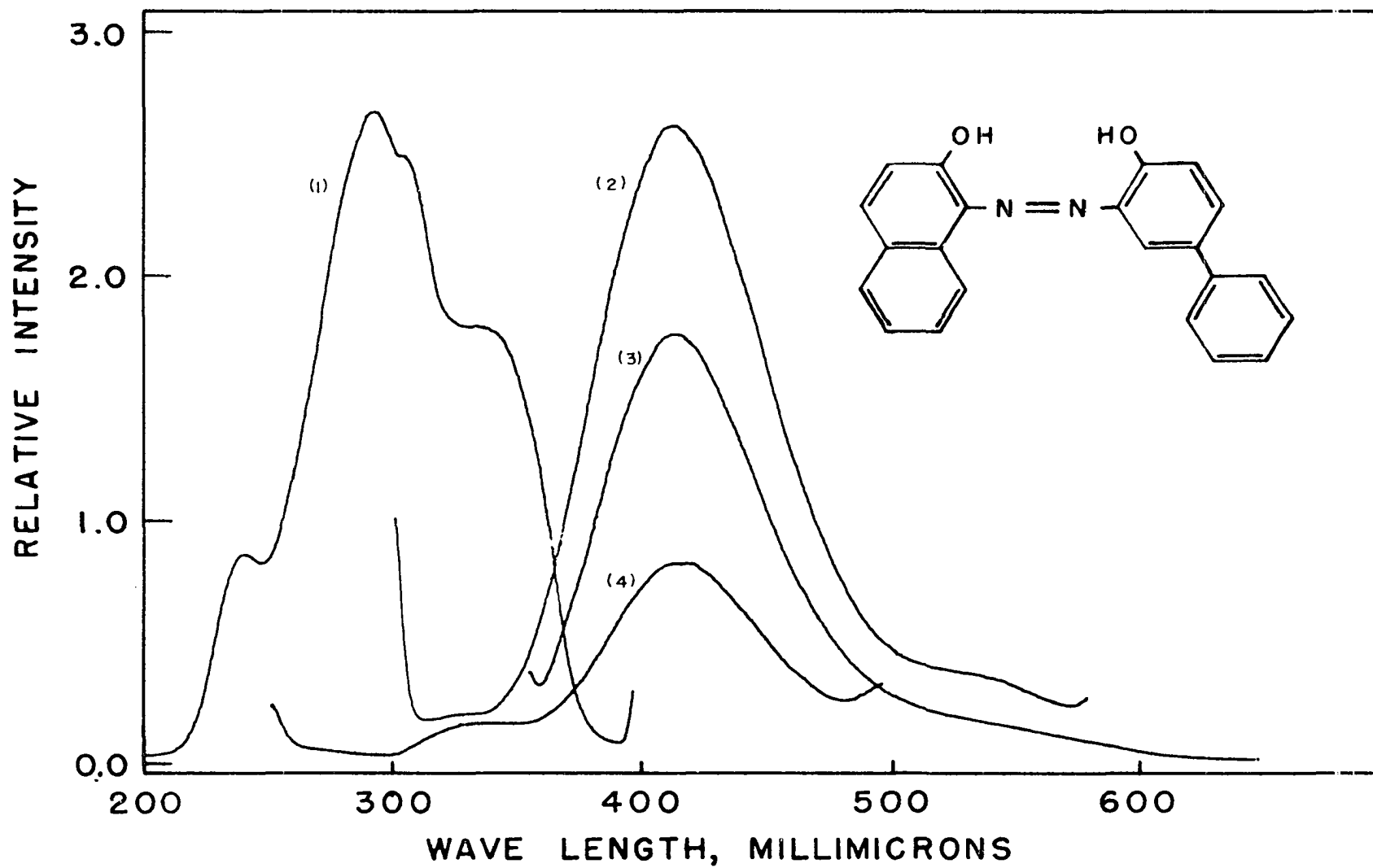


Figure 9. Fluorescence spectra of B-7 at pH 11.4
(1) Excitation, fluorescence monochromator set at
412 m μ .
(2,3,4) Fluorescence, excitation set at 290 m μ .,
336 m μ , and 241 m μ ., respectively



- Figure 10. Fluorescence spectra of B-7 at pH 11.4 (Taken 24 hours after solutions prepared)
- (1) Excitation of calcium derivative, fluorescence monochromator set at 540 m μ .
 - (2) Fluorescence of calcium derivative, excitation set at 404 m μ .
 - (3) Fluorescence of magnesium derivative, excitation set at 404 m μ .
 - (4) Fluorescence of the compound itself, excitation set at 404 m μ .

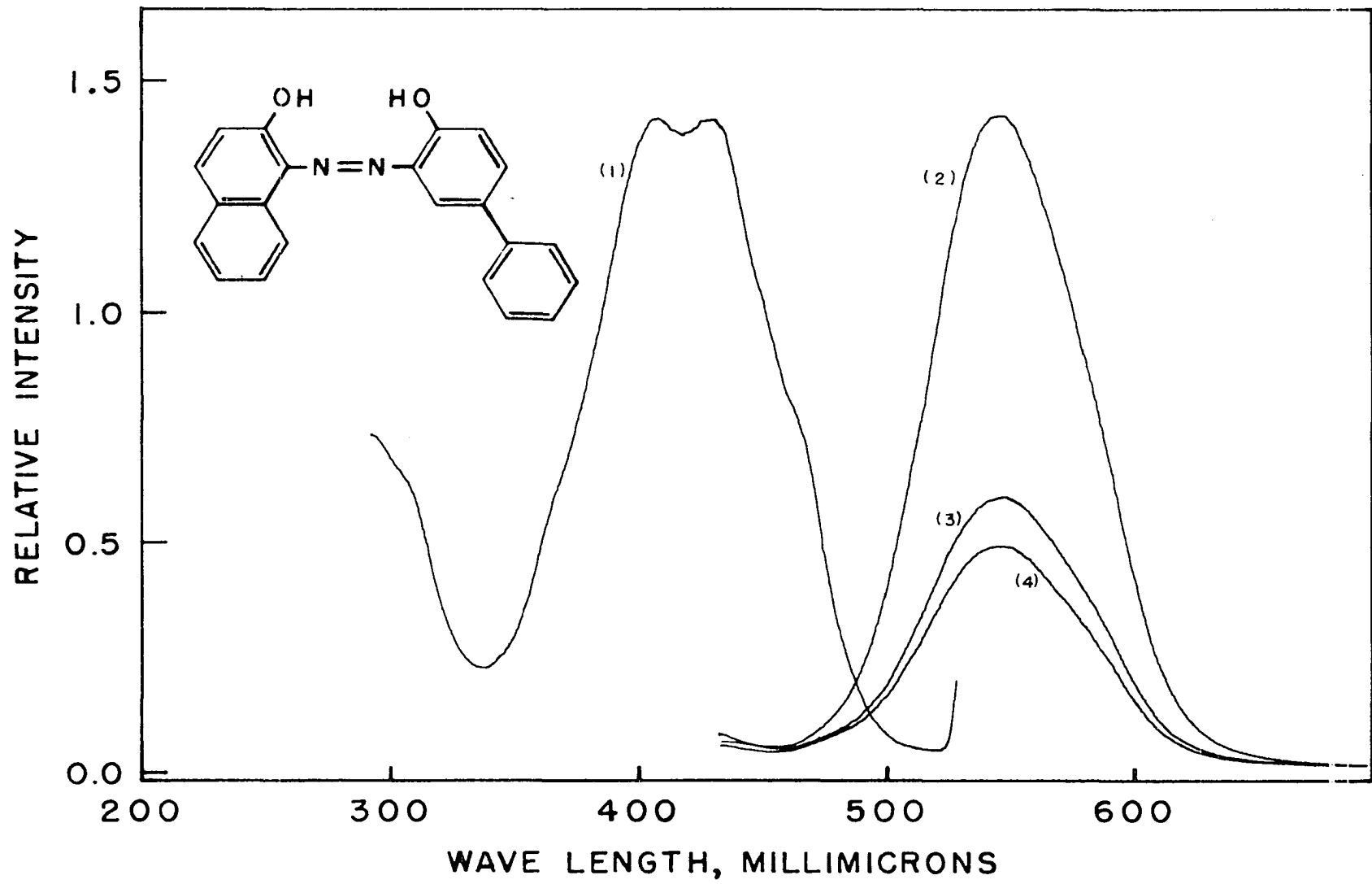
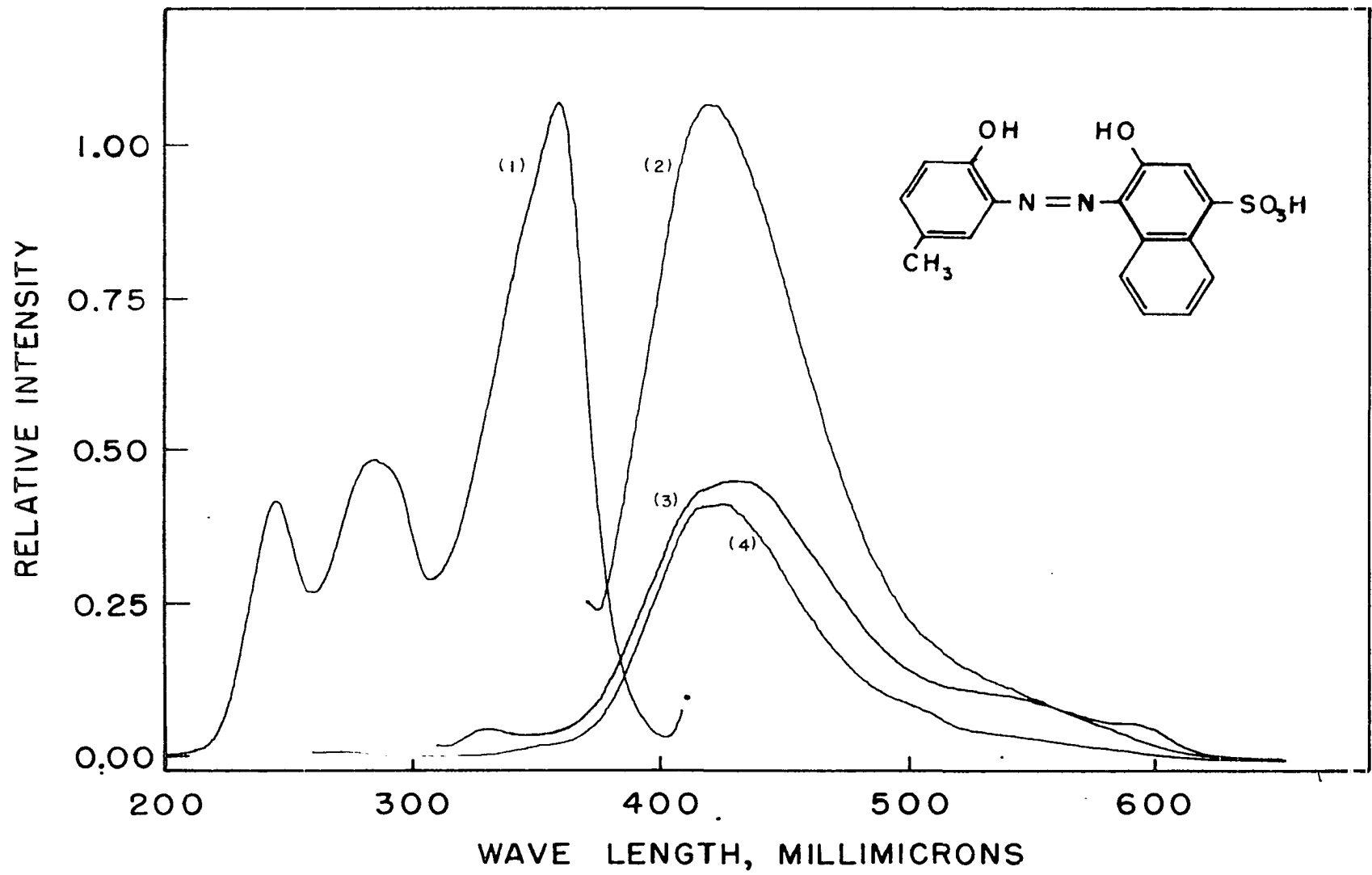


Figure 11. Fluorescence spectra of Calmagite at pH 11.4
(1) Excitation, fluorescence monochromator set at
424 $m\mu$.
(2,3,4) Fluorescence, excitation set at 360 $m\mu$.,
292 $m\mu$., and 246 $m\mu$., respectively



- Figure 12. Fluorescence spectra of Calmagite at pH 11.4
- (1) Excitation of magnesium derivative, fluorescence monochromator set at 596 m μ .
 - (2) Fluorescence of magnesium derivative, excitation set at 520 m μ .
 - (3) Fluorescence of calcium derivative, excitation set at 520 m μ .
 - (4) Fluorescence of Calmagite itself, excitation set at 520 m μ .

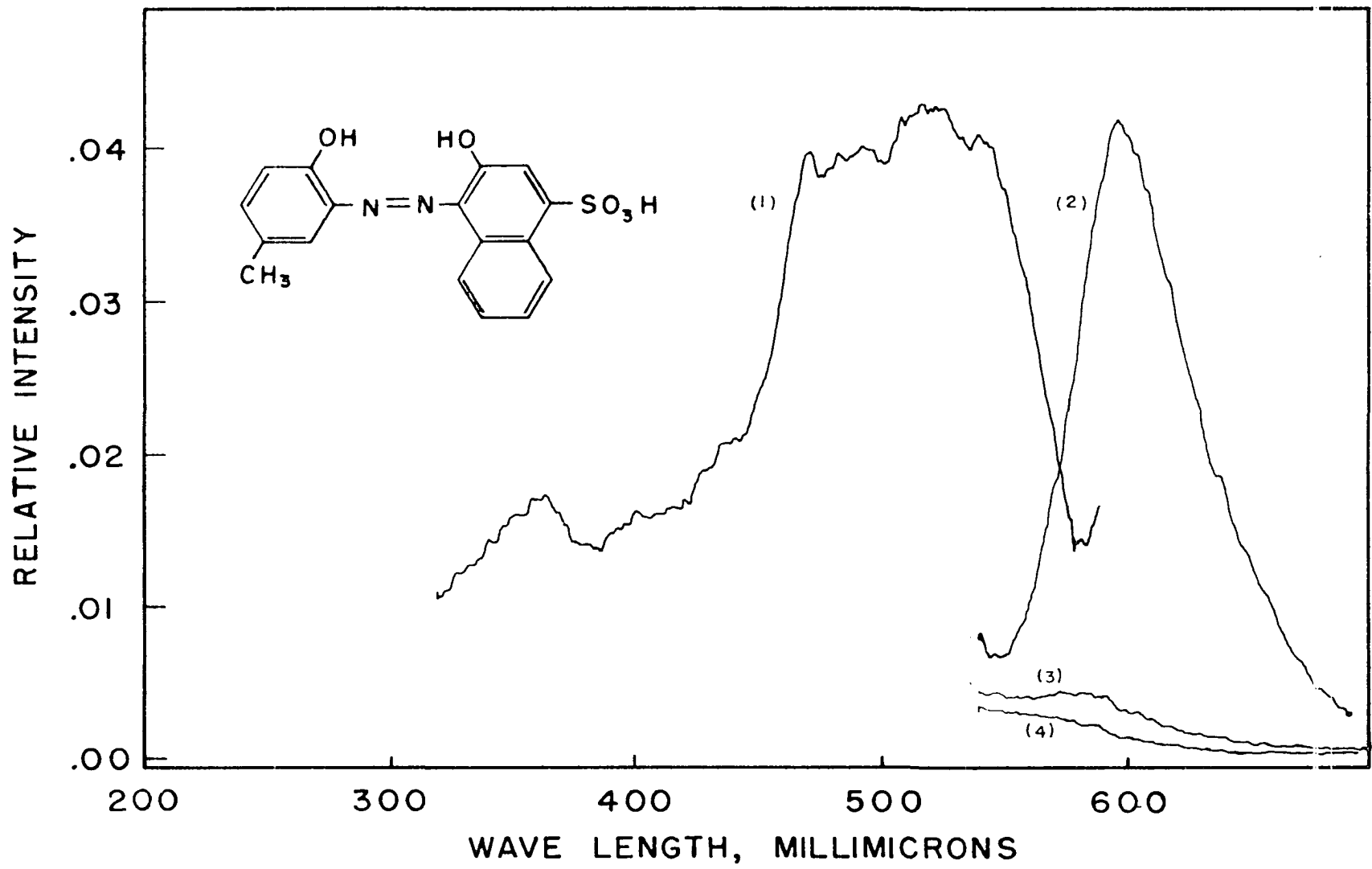


Table 1. Fluorescence of azo compounds and their magnesium derivatives^a

Compound	Wave length, m .		Relative intensity
	Maximum excitation	Maximum fluorescence	
B-1	328	412	0.5
B-1 (Mg)	480	578	0.04
B-2 (Mg)	468	580	0.22
B-3 (Mg)	466	558	0.13
B-6	288	404	0.2
B-6 (Mg) ^b	428	548	0.12
B-6 (Mg) ^c	472	580	0.04
B-7 ^d	290	412	2.8
Calmagite	360 (246,292)	424	1.0
Calmagite (Mg)	520	596	0.04

^aData taken at pH 11.4.

^bSolutions freshly prepared.

^cData taken at pH 10.2.

^dWhen freshly prepared solutions of this compound have no fluorescence characteristic of the presence of magnesium.

Table 2. Fluorescence of azo compounds in the presence of calcium^a

Compound	Wave length, m .		Relative intensity	Intensity ratio Mg deriv. Ca deriv.
	Excitation	Fluorescence		
B-1	480	578	0.006	6.7
B-2	468	580	0.013	17.0
B-3	466	558	0.002	60.
B-6	428	548	0.06	2.
B-6 ^b	472	580	0.002	20.
Calmagite	520	596	0.003	13.5

^aData taken at pH 11.4.

^bData taken at pH 10.2.

Discussion

On the basis of the compounds studied, it is concluded that of the azo compounds which form calcium or magnesium derivatives two hydroxyl groups, *o*- and *o'*-, are necessary for there to be fluorescence characteristic of the magnesium derivative. Generally there is no fluorescence characteristic of the presence of calcium. At high pH, however, such fluorescence may develop over a period of time as was observed with compounds B-6 and B-7. It appears that the fluorescence intensity of the magnesium derivatives decreases with the complexity of the parent azo compound.

The fluorescence reported for the uncomplexed azo compounds is observed to be essentially the same when calcium or magnesium is present. That fluorescence characteristic of the presence of magnesium is in addition to that shown by the azo compound itself.

Most of the data reported is for solutions at pH 11.4. Behavior of the compounds at pH 10.2 was very similar except when indicated otherwise. It was found in the investigation of B-2 as a fluorometric reagent for magnesium that if the magnesium was not in excess (it was present in fivefold excess in this part of the study) then the intensity was greater at the higher pH.

The effects of beryllium, strontium and barium were also investigated. Beryllium and barium gave no indication of reaction with compounds B-1, 2, or 3 at either pH 10.2 or 11.4. The reaction of strontium was very slight and, if observed at all, was similar to that of calcium.

The wave length and intensity data presented are as observed with the instrument described. The data reflect, in addition to the properties of the compounds studied, instrumental and other minor variables. The intensity of the source and the sensitivity of the detector both vary with wave length. These variations are not easily compensated as they are in spectrophotometry. Ideally the solutions studied should have negligible absorbance at all wave lengths at which measurements are made. This was not possible. Trace impurities in the reagents and especially the deionized water may have contributed slightly to the observed fluorescence.

III. O,O'-DIHYDROXYAZOBENZENE AS A FLUOROMETRIC REAGENT FOR MAGNESIUM

The simplest member of the series of azo compounds studied in the previous section appears ideally suited for the fluorometric determination of magnesium in the presence of calcium. This application of compound B-2, o,o'-dihydroxyazobenzene or DHAB, was investigated, and it was indeed found well suited.

Preparation of o,o'-Dihydroxyazobenzene

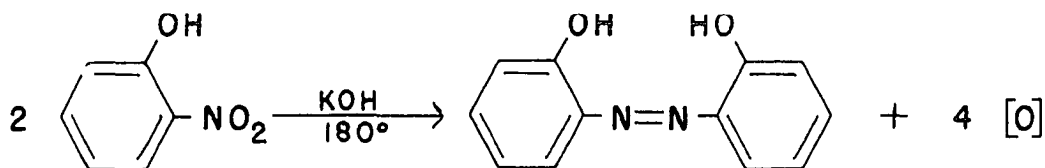
o,o'-Dihydroxyazobenzene can be prepared by the method of Willstätter (17) by the interaction of o-nitrophenol and sodium hydroxide. The reaction has its spectacular aspects and the operator is advised to keep his head well back to avoid being singed by the fireworks. By controlling the reaction as described below, the reaction can be moderated and the yield increased. A second method of synthesis is that of Freeman and White (8) in which diazotized o-aminophenol is treated with ammoniacal cuprous chloride and the precipitated, brown copper compound decomposed with strong hydrochloric acid. The yield is poor after the necessary recrystallization. It has been especially difficult to obtain satisfactory yields on the preparation of large batches. The material is available in satisfactory quality from the G. Fredrick Smith Chemical Company, Columbus, Ohio.

Recommended procedure

Mix 190 g. of potassium hydroxide with 65 ml. of water in a 1 l. beaker. Stir the mixture until it has cooled considerably. With

vigorous stirring add 35 g. of o-nitrophenol which has been ground sufficiently to break up the lumps. Heat the mixture, stirring it continuously with a stirring rod and a 250^o thermometer. When the temperature reaches 170^o decrease the rate of heating. The rate of temperature increase can be partially controlled by the rate of stirring. The reaction, which begins to take place at about 180^o, is strongly exothermic. Above 180^o allow the temperature to increase only as long as the foam can be kept in the beaker and no puffs of smoke are evolved. When the mixture can be heated to 190^o without foaming, the reaction is complete. It is important that certain safety precautions be observed in connection with the above procedure. Ascertain that there are no small cracks in the beaker used. Carry out the reaction behind a safety shield. Wear a cloth glove while stirring the mixture. If the mixture is not stirred adequately combustion will begin, sparks and smoke will be evolved and a beaker of soot will result. It might be advisable to practice the operation on a smaller scale.

As soon as the semi-liquid reaction mixture has cooled to around 150^o transfer it completely to 2.5 l. of water, stirring rapidly with a magnetic stirrer. Bring the volume of the solution to about 3 l. and, stirring rapidly, add 1:1 hydrochloric acid until precipitation of the product is complete (pH = 2). Allow the precipitate to settle and filter it using suction. Allow the filter cake to air dry for several hours and then overnight in a vacuum desiccator over anhydrous magnesium perchlorate. Assuming the reaction to be:



the yield of the crude product is about 50%.

Using a Soxhlet extractor extract the crude, dry product with 350 ml. of benzene for about 3 hours. After the first four or five extractions (not before), the extraction chamber should be heated in some manner, with an electrical heating tape, for example.

Transfer the benzene solution to a beaker, add 2-3 g. of decolorizing charcoal, and heat to boiling. Filter as hot as possible using a fluted filter. Allow the filtrate to cool slowly to room temperature, stirring occasionally. Continue cooling the mixture in a refrigerator. Do not allow the temperature to drop below 8° as a solid solution containing mostly benzene begins to form at about that temperature. Using suction, filter the crystals of product and wash it with a few milliliters of cold benzene. An additional quantity of product can be obtained by evaporating the benzene from the filtrate and recrystallizing the residue from a minimum of boiling benzene and a little decolorizing charcoal. The overall yield of *o,o'*-dihydroxyazobenzene is about 25 per cent. The product is in the form of bright yellow needles melting sharply at 174°C .

Apparatus and Reagents

The apparatus used was essentially the same as that described in the previous section. In addition, a Coleman Model 12 Electronic

Photofluorometer was used for some fluorescence measurements. In the excitation beam a Corning filter, CS-5-60 was used and in the fluorescence beam a Corning filter, CS-3-67.

Prepare a 2.50×10^{-3} M stock solution of DHAB by dissolving 0.5355 g. of the crystals in 10 ml. of ethanol and 10 ml. of 2N potassium hydroxide, adding water as necessary to effect complete solution. Avoid the use of more ethanol or potassium hydroxide. Transfer the solution to a 1 liter volumetric flask, dilute to the mark and mix. Store the solution in a polyethylene container. A standard magnesium solution was prepared by dissolving 0.2432 g. of Grignard-grade magnesium metal in a slight excess of hydrochloric acid and diluting to exactly 1 liter. Working solutions of magnesium chloride were then prepared from this stock solution by appropriate dilutions. The ethylenediamine used was anhydrous and redistilled. The isoamyl alcohol used for extractions was reagent grade or redistilled. Reagents used in interference studies were of the best grade commercially available. The water used for preparing all solutions was distilled and deionized (passage through Amberlite MB-1 ion exchange resin).

Experimental Part

The excitation and fluorescence spectra of magnesium-o,o'-dihydroxyazobenzene are shown in Figure 3. Also illustrated is the very slight fluorescence exhibited by the free compound itself and in the presence of calcium.

Effect of pH on the fluorescence of magnesium-o,o'-dihydroxyazobenzene

The intensity of the fluorescence of magnesium-o,o'-dihydroxyazobenzene is essentially linear over the pH range 11.0 to 12.2, falling off at higher and lower values of pH, Figure 13. o,o'-Dihydroxyazobenzene is a weak dibasic acid, pK_1 7.8 and pK_2 11.5 (3), and the decrease in intensity of the fluorescence at low pH is probably caused by incomplete formation of the magnesium compound. The decrease at high pH is probably a result of the competition of the hydroxy ion for magnesium; the solubility product constant for magnesium hydroxide is about 10^{-11} and the decrease in intensity of the fluorescence is not eliminated by extraction into isoamyl alcohol.

Effect of excess o,o'-dihydroxyazobenzene on the fluorescence

To insure complete formation of the magnesium compound it is necessary to have a moderate excess of the reagent present. The uncombined o,o'-dihydroxyazobenzene has a strong absorption peak at 475 $m\mu$, which is approximately the wave length for the maximum excitation of the magnesium derivative. This precludes use of a large excess of reagent and also necessitates working in a low concentration range. The effect of reagent concentration is illustrated in Figure 14.

Effects of time and temperature

Magnesium-o,o'-dihydroxyazobenzene appears to form instantly and to be stable indefinitely. If the fluorometer lamp has been turned on for some time the sample cell compartment is warmed. Under this condition the intensity of the fluorescence of the compound decreases slowly as the sample stands in the cell compartment. If the cell compartment is

Figure 13. Effect of pH on fluorescence intensity of magnesium-
o,o'-dihydroxyazobenzene

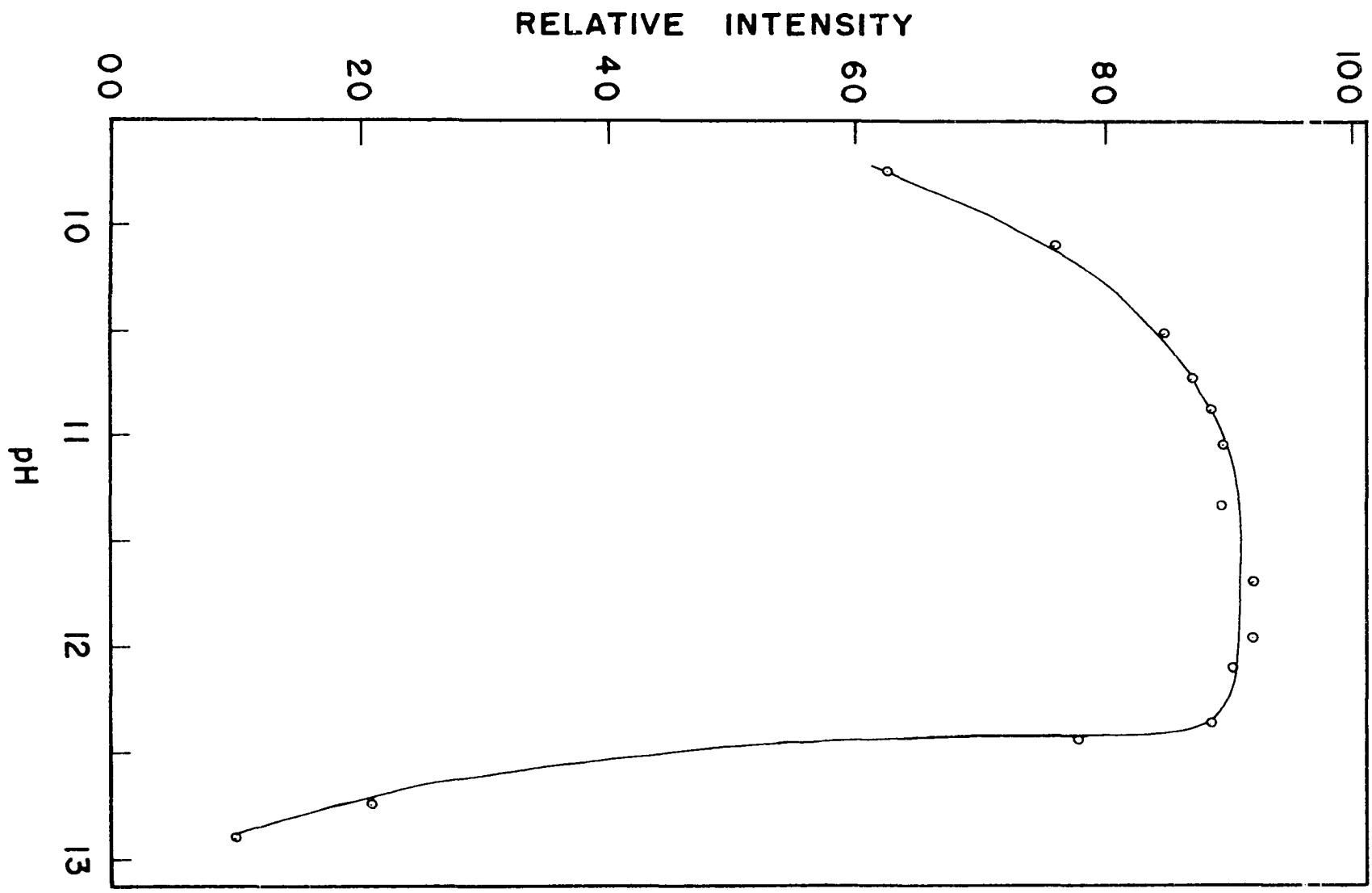
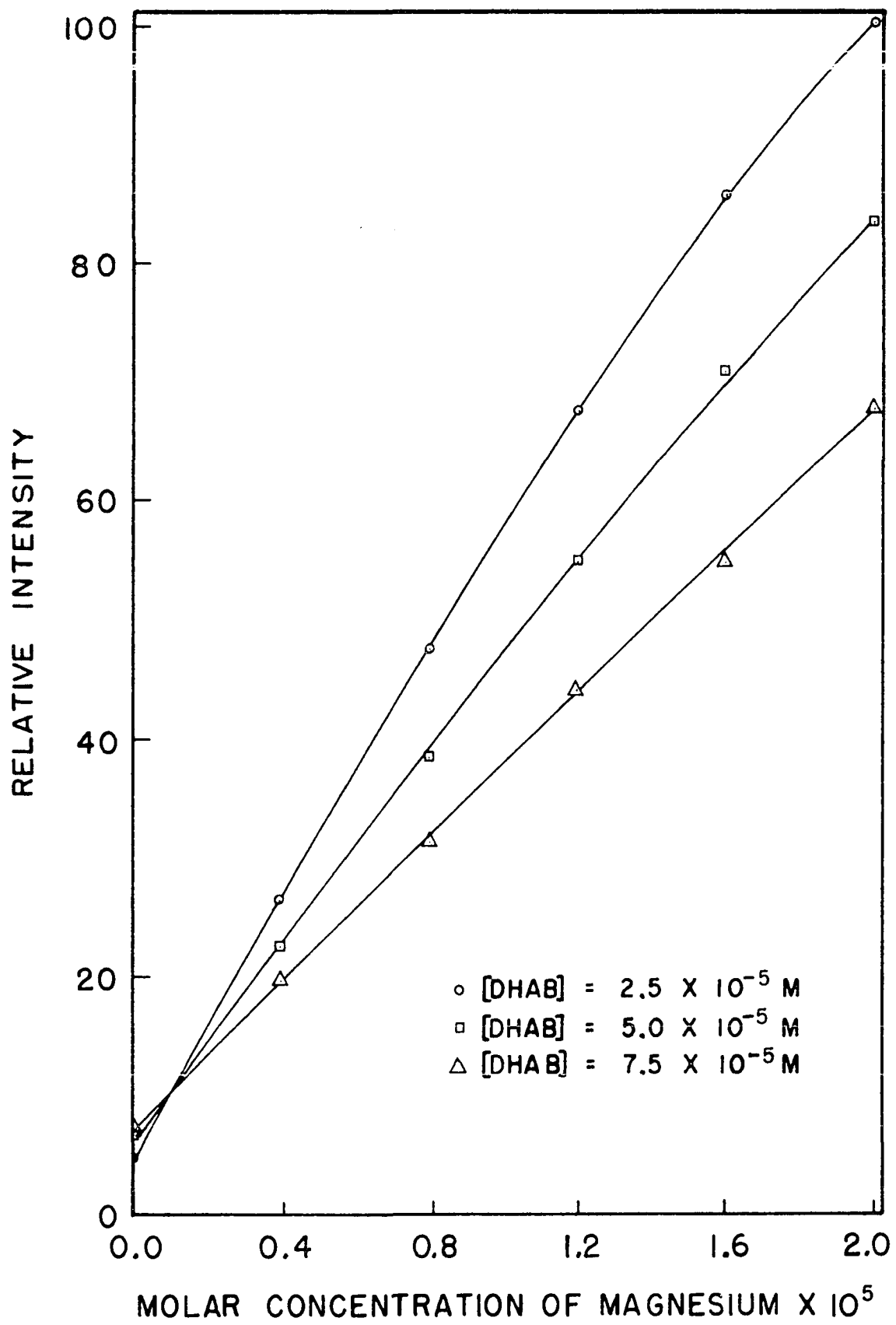


Figure 14. Calibration curves for the fluorometric determination of magnesium using various quantities of DHAB working solution



cool the effect is not observed. Refer to Figure 15. The fluorescence intensity readings of identical solutions were found to vary from day to day. It appears that certain characteristics of the fluorometer are influenced by large variations in A.C. line voltage and room temperature.

Effect of various foreign ions

The presence of large amounts of alkali and barium chlorides appears to have no detrimental effect on the fluorometric determination of magnesium. The presence of large amounts of calcium, strontium or beryllium tends to suppress the fluorescence; moderate amounts can be tolerated as can be seen from the data presented in Table 3. It is possible to compensate for larger amounts of these alkaline earth metal ions by having the composition of the standards approximate that of the unknown with respect to the interfering ion. The effect of calcium is illustrated in detail by Figure 16.

Copper, iron manganese, aluminum and zinc form highly colored compounds with o,o'-dihydroxyazobenzene and therefore represent a serious interference in the determination of magnesium. The copper and zinc interference can be overcome by the addition of cyanide. The interference of iron can be obviated by reduction to the ferrous state with sodium hydrosulfite (small quantities with hydroxylammonium chloride) and complexing it with cyanide. Moderate amounts of aluminum can be masked with triethanolamine.

Extraction of magnesium-o,o'-dihydroxyazobenzene

Several extractants were tried. Benzene, nitrobenzene, ether, petroleum ether, and chlorinated hydrocarbons would not extract any

Figure 15. Effect of time and temperature on fluorescence intensity of magnesium-o,o'-dihydroxyazobenzene

- (1) Effect on sample solution standing in warm cell compartment
- (2) Effect on sample solution standing in cool cell compartment
- (3) Temperature of sample solution standing in warm cell compartment

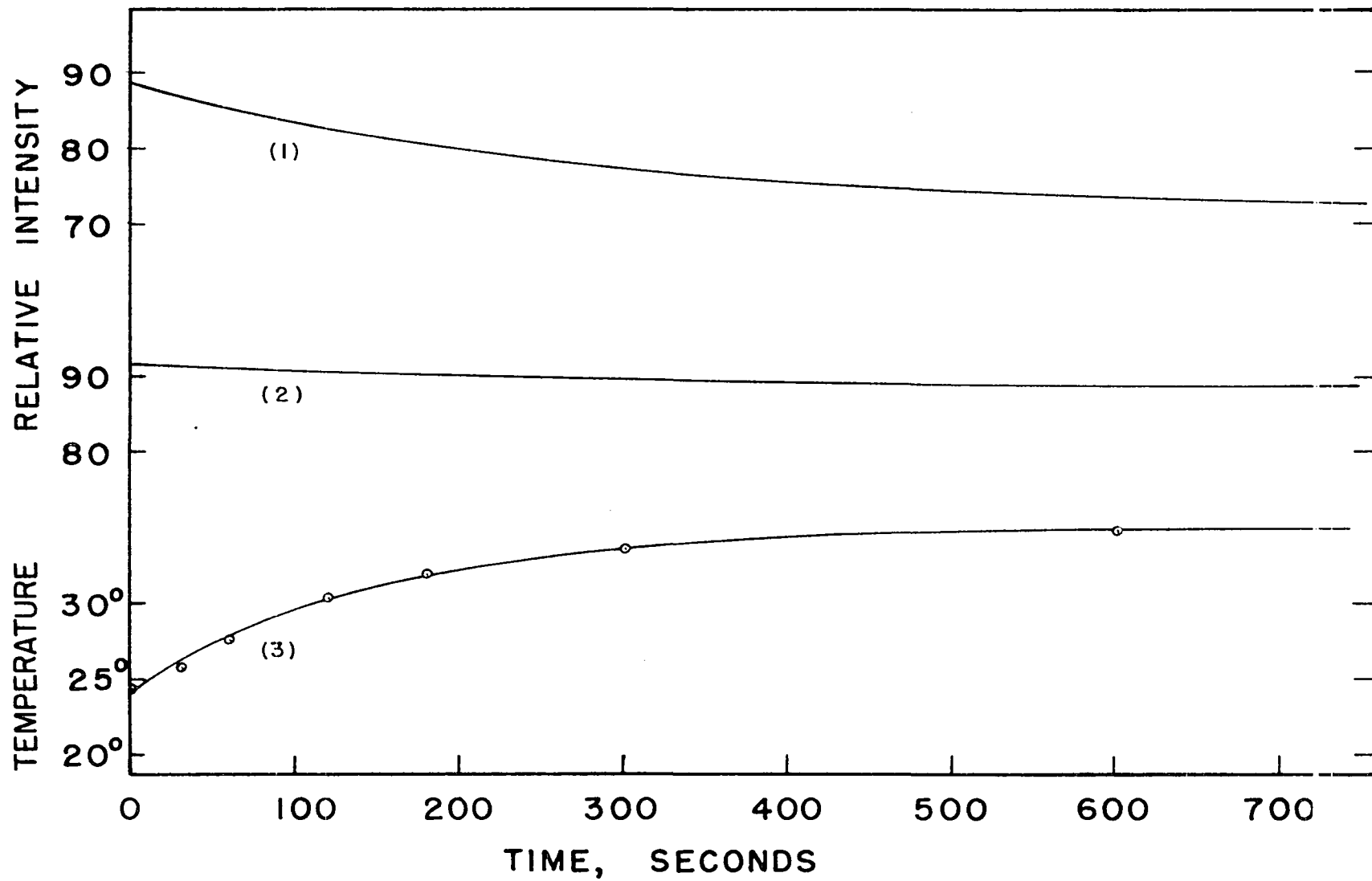
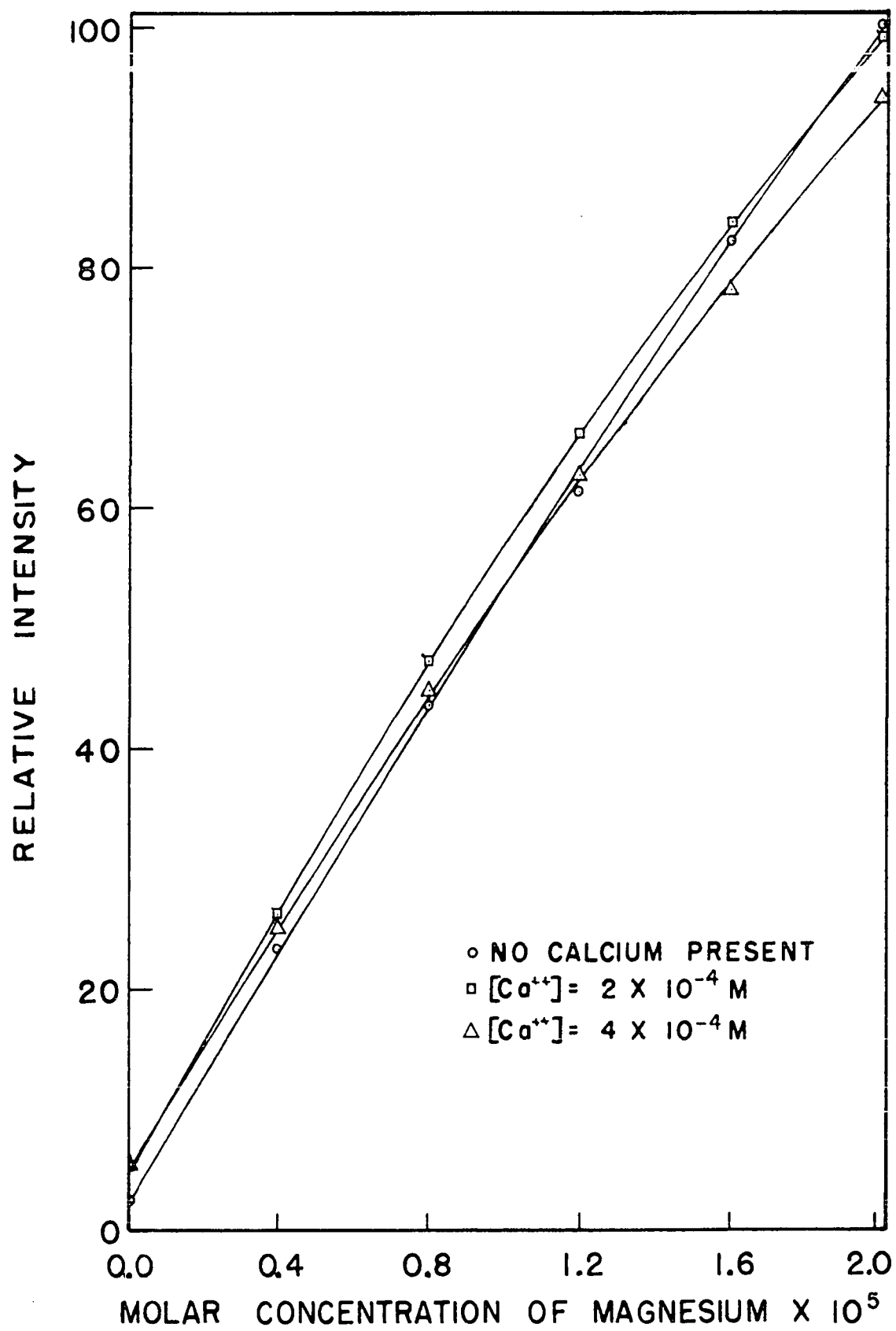


Figure 16. Calibration curves for the fluorometric determination of magnesium at various levels of calcium concentration



appreciable amount of either the free DHAB or its magnesium derivative from a solution of pH 11.4. Esters were found to be hydrolyzed, and thus lowered the pH of the aqueous phase so that the free DHAB would be extracted. Isoamyl and isobutyl alcohols were found to be reasonably suitable extractants. Isoamyl alcohol was chosen for study since it is less miscible with water. The distribution coefficient of the magnesium derivative was found to be about 9 at pH 11.4. At the same pH, that of the free DHAB was found to be about 6. If one wished, as part of an analytical procedure, for example, to extract at least 99% of the Mg-DHAB from a 25 ml. of solution, three 10 ml. portions of isoamyl alcohol would be required. The application of an isoamyl alcohol extraction is desirable, not to obtain a concentration effect, but because the intensity of the fluorescence is greatly enhanced by the organic solvent. A series of calibration curves representing different levels of calcium concentration was prepared using an extraction procedure. This procedure did not result in complete extraction of the Mg-DHAB but the volume of each of these phases was controlled so that the degree of extraction would be reproducible. Solutions 50 ml. in volume, up to 2×10^{-5} M in magnesium, and at pH 11.4 were extracted with one 10 ml. portion and one 5 ml. portion of isoamyl alcohol. The extracts were combined and diluted to 50 ml. with 95% ethanol. The intensity of each was measured with the Aminco-Kiers fluorometer setting the excitation and fluorescence monochromators at 480 m μ and 570 m μ , respectively. The fluorescence of the extracts when so diluted is about ten times as intense as that of the aqueous solution from which the extractions were

made. These calibration curves are shown in Figure 17. Utilizing this extraction procedure, a Coleman filter fluorometer could be used.

Table 3. Fluorometric determination of magnesium in the presence of various interfering substances

Magnesium taken, g.	Interference	Magnesium found, g.	Difference g.
20	1 mg. Be	19.2	-0.8
20	10 mg. Sr	20.4	+0.4
20	0.1 mg. Mn	14.0	-6.0
20	1 mg. Al ^a	21.6	+1.6
20	0.1 mg. Al ^a	20.6	+0.6
20	1 mg. Cu ^a	19.1	-0.9
20	1 mg. Zn ^a	19.7	-0.3
20	1 mg. Fe ^a	19.9	-0.1
20	Na ₂ B ₄ O ₇ , NaCl ^b	20.0	0.0

^aTreatment appropriate for the interference applied in accordance with the recommended procedure.

^bQuantity equivalent to 8 mg. of the fusion mixture used in analysis of the N.B.S. samples.

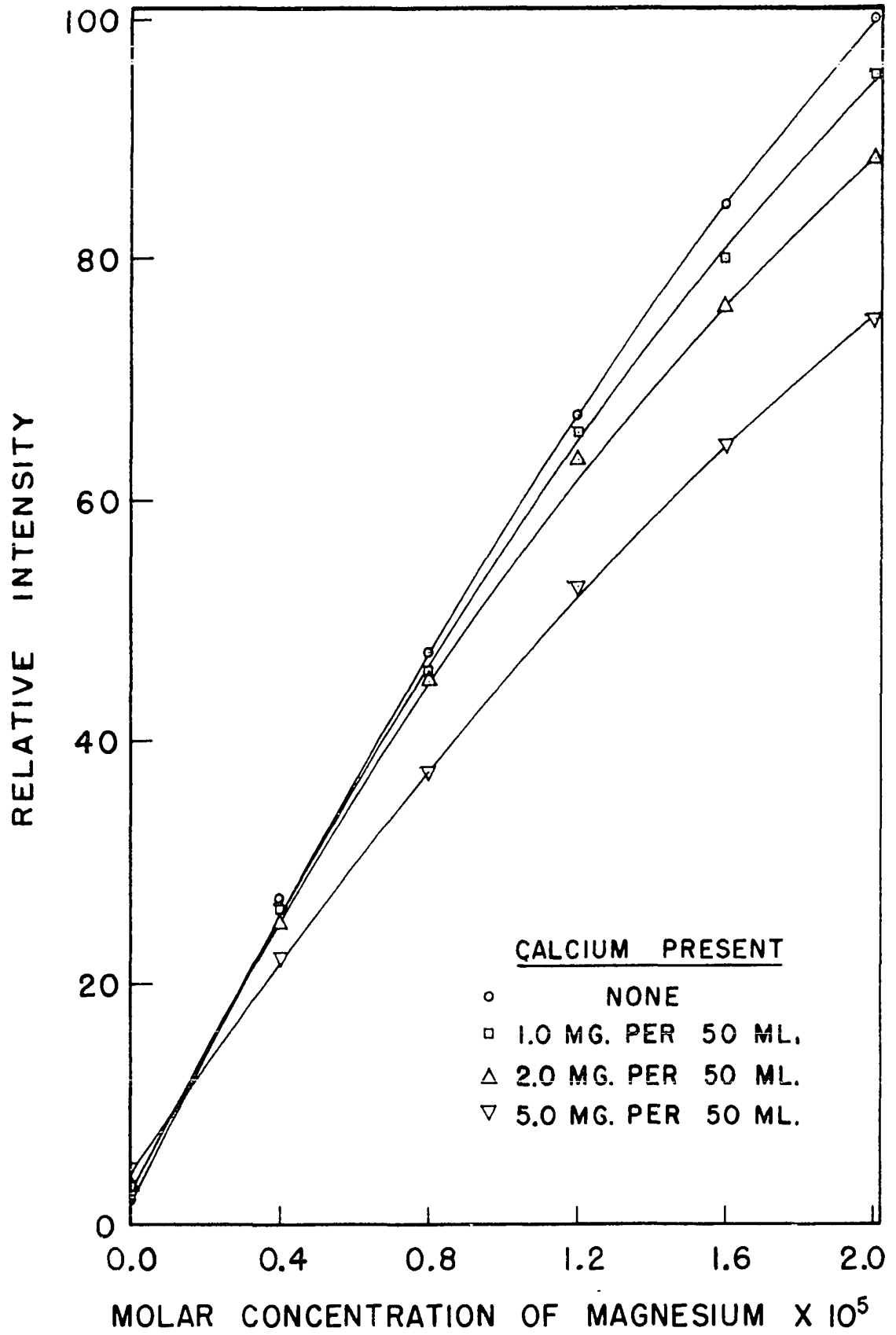
Analytical Procedure

Prepare a working solution of DHAB as follows: To 100 ml. of water and 100 ml. of 2.5 M potassium chloride in a 1 liter volumetric flask add 67 ml. of ethylenediamine. Mix and allow the solution to cool. Add exactly 100 ml. of the DHAB stock solution and dilute to exactly 1 liter.

Prepare the sample in such a manner as to bring all the magnesium into solution. The solution should be relatively unbuffered and free of organic substances.

For analysis take aliquots containing from 5 to 25 g. of magnesium. If the sample contains iron, add to the aliquot in rapid succession

Figure 17. Calibration curves for fluorometric determination of magnesium utilizing isoamyl alcohol extraction



10-20 mg. of sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$), 1 ml. of concentrated ammonium hydroxide and 2 ml. of 5% potassium cyanide. Heat the solution and allow it to boil gently for one or two minutes. Avoid using more sodium hydrosulfite than necessary. If aluminum is present add 0.5 ml. of pure triethanolamine. If copper or zinc may be present add 2 ml. of 5% potassium cyanide unless the above treatment for iron was applied. Add 10 ml. of the DHAB working solution and dilute to exactly 50 ml. A convenient device for dispensing a reproducible volume of the working solution is shown in Figure 18. In a similar manner, prepare a series of standards covering the range 2 to $25\mu\text{g}$. of magnesium (a 1.00×10^{-4} solution of magnesium chloride contains $2.432\mu\text{g}$. of magnesium per ml.). If the unknown contains sufficient calcium, strontium or beryllium to influence the fluorescence add the necessary quantity of each of these to the standards so their composition approximates that of the unknown.

Set the excitation monochrometer at $470\text{ m}\mu$. and the emission monochrometer at $580\text{ m}\mu$. Read the fluorescence intensity of each of the standards and unknown as quickly as possible. From the data obtained on the standards, prepare a plot of concentration versus fluorescence intensity; the curve will probably be slightly concave toward the concentration axis.

If a filter fluorometer is used rather than the spectrophotofluorometer, use Corning filters CS-5-60 and CS-3-67 or equivalent.

If samples containing widely differing amounts of calcium are to be analyzed from time to time, prepare once a family of calibration curves representing different levels of calcium concentration. Refer to

- Figure 18. A device for dispensing a reproducible volume of solution
- (A) 300 ml. Bulb
 - (B) Ball and socket joint, No. 12
 - (C) Capillary tube, 1-2 mm. i. d.
 - (D) Flexible plastic tube
 - (E) Volumetric pipette, 5-20 ml.
 - (F) Standard taper joint, 7/25

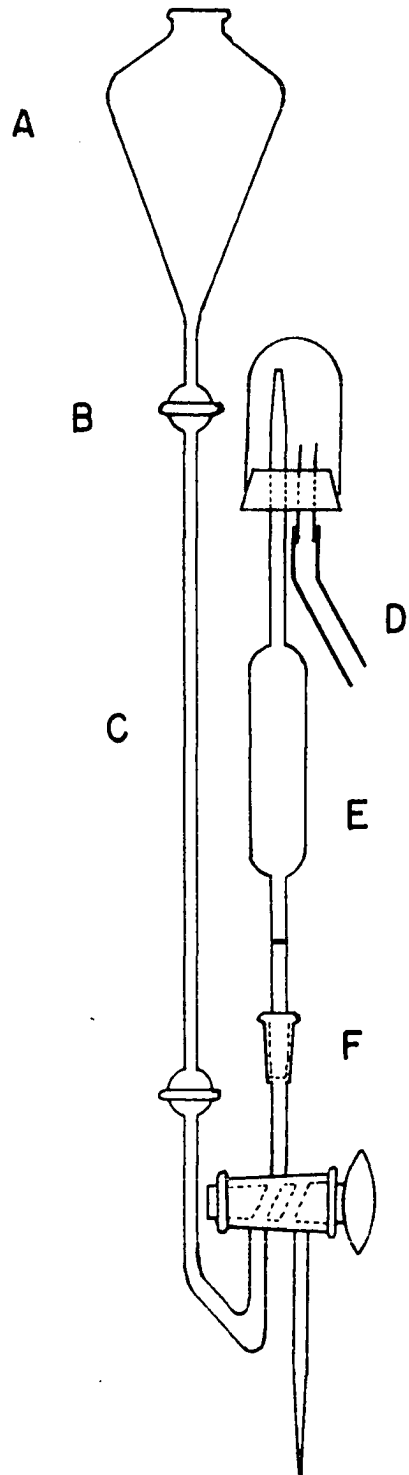


Figure 16. From then on prepare only one calibration curve. Refer to the previously prepared family of curves for an estimate of the correction which must be applied to observed magnesium concentration.

Analysis of Standard Samples

Four National Bureau of Standards samples were subjected to the analytical procedure described above. Samples of No. 1a (Argillaceous Limestone), No. 26 (Iron Ore), No. 177 (Portland Cement), and No. 88 (Dolomite) weighing on the order of 100 mg. were taken for analysis. Each sample was fused in a platinum crucible for 10-15 minutes with 1.0 g. of a 1:1 mixture of sodium carbonate and sodium tetraborate decahydrate. The cooled fusion mixtures were taken up with 10 ml. of water and 5 ml. of concentrated hydrochloric acid. In no case did any dark colored residue remain. The resulting solutions were diluted to exactly 250 ml. without separating the silica. Standard dilutions were made, avoiding transference of the small amount of silica present. Appropriate aliquots were treated as necessary for the interfering ions present. The solutions of the Dolomite required no treatment. The others were treated with sodium hydrosulfite, ammonia and cyanide to prevent interference of the iron present and triethanalamine to mask the aluminum. The results are summarized in Table 4.

Table 4. Fluorometric determination of magnesium in N.B.S. samples

Sample	Magnesium found, % MgO	Magnesium reported, % MgO	Relative error, %
No. 1a, Argillaceous Limestone	2.12, 2.20, 2.42 (Ave. 2.25)	2.19	+2.7
No. 26, Iron Ore	3.43, 3.43, 3.40 3.39 (Ave. 3.41)	3.27 ^a	+3.7
No. 88, Dolomite	21.4, 21.7, 21.5, 21.5 (Ave. 21.5)	21.48	+0.1
No. 177, Portland Cement	2.53, 2.53, 2.47, 2.50 (Ave. 2.51)	2.45	+2.4

^aN.B.S. lists 3.44% as the average of the analyses reported but recommends the value 3.27% as more nearly correct.

IV. O,O'-DIHYDROXYAZOBENZENE AS A SPECTROPHOTOMETRIC REAGENT FOR MAGNESIUM

According to Diehl and Ellingboe, (7) the compound o,o'-dihydroxyazobenzene (DHAB) forms a chelate with magnesium ($K_f = 10^{4.85}$ at pH 10) but not with calcium. They also stated that the color change of the compound on chelating with magnesium was not easily detected by eye. The molar extinction coefficient of the magnesium derivative was reported to be about fifty per cent greater than that of the compound itself. This and the fact that the compound was relatively nonreactive toward calcium suggested its use as a reagent for magnesium. Since the compound does absorb at the same wave length as the magnesium derivative a differential spectrophotometric technique would be necessary, but the method should be relatively free from interference of calcium. The possibility has been investigated and a workable procedure devised. Although the method is beset with some rather severe limitations, it is quite suitable for analysis of materials in which magnesium is more than a trace constituent. It would be especially useful in water analysis since magnesium in this is usually determined by difference from the total hardness and the calcium only. Considerably less sample is required than for the conventional volumetric procedure just mentioned (4).

Experimental Part

Apparatus and reagents

Absorption spectra were obtained using a Cary Model 14 recording spectrophotometer. Absorbance measurements were made with a Beckman

Model DU spectrophotometer. pH measurements were made with a Beckman Model G pH meter and a Beckman No. 40495 glass electrode.

Reagents used were essentially the same as those used for the fluorometric study. The working solution, however, was prepared in such a way that the final sample solutions would have a pH of about 10. To a 1 liter volumetric flask were transferred in sequence about 100 ml. of water, 100 ml. of 2.5 M potassium chloride and 67 ml. of ethylenediamine. The mixture was cooled. A volume of 82 ml. of 1:1 hydrochloric acid was added, the solution mixed and cooled to room temperature. Exactly 100 ml. of the stock DHAB solution was added, the mixture diluted to volume and mixed thoroughly. Polyethylene was found unsatisfactory for storage of this solution.

Absorption spectra of o,o'-dihydroxyazobenzene and its magnesium derivative

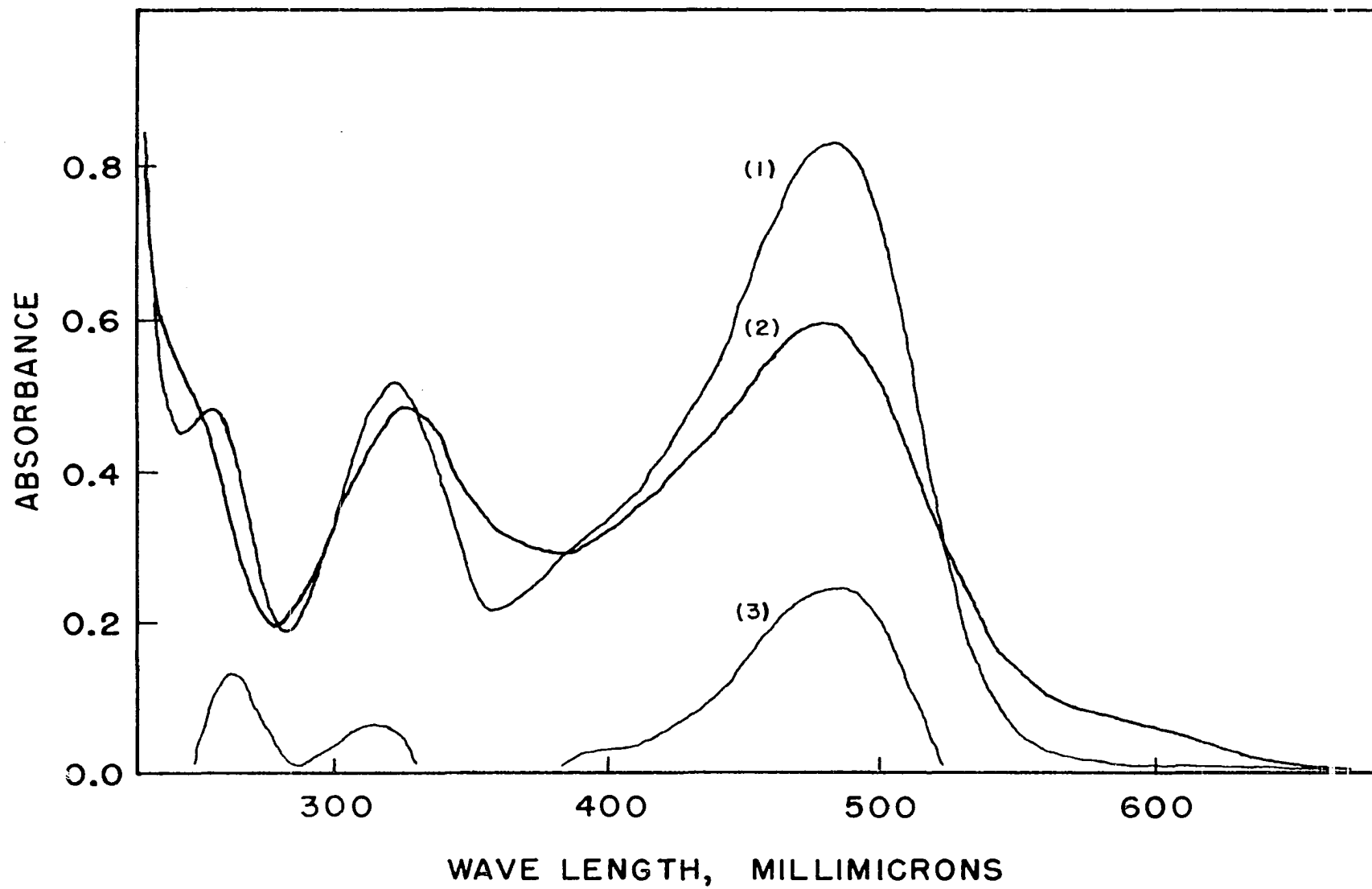
Absorption spectra of 5×10^{-5} M solutions of each at pH 10 and 0.05 M in potassium chloride are shown in Figure 19. In addition the spectrum of the Mg-DHAB, using the solution of the free DHAB in the reference cell, is shown. Quartz 1 cm. cells were used.

Necessity for presence of inorganic salt

In preliminary studies satisfactory calibration curves could be obtained only at the higher levels of calcium concentration. Without the calcium the absorbance was not reproducible. A series of standards was then prepared, each one being made 0.1 M in potassium chloride. With the uniform inorganic salt concentration, smooth calibration curves were obtained.

Figure 19. Absorption spectra of *o,o'*-dihydroxyazobenzene and its magnesium derivative at pH 10.2

- (1) Spectrum of 5×10^{-5} M Mg-DHAB
- (2) Spectrum of 5×10^{-5} M DHAB
- (3) Spectrum of Mg-DHAB, DHAB as reference



Effect of pH on the absorbance of magnesium-o,o'-dihydroxyazobenzene

The absorbance is very much a function of the pH as can be seen in Figure 20. It would appear desirable to operate at a pH higher than the recommended value of 10. At the higher pH, however, the influence of calcium on the absorbance is much more pronounced. Thus pH control is quite critical but, with the effective buffer specified, is not difficult in the analysis of typical water samples.

Effects of time and temperature

The absorbance of the Mg-DHAB is stable with time but varies with temperature. The absorbance decreases with increase in temperature, the variation being about 0.5% per degree Centigrade at room temperature. This is not a serious problem if the absorbance of each of the standards and unknowns is read at the same time.

Effect of foreign ions

The effect of foreign ions is more serious here than in the fluorometric procedure. Spectrophotometric calibration curves representing various levels of calcium concentration are shown in Figure 21. Effects of several ions on the determination of magnesium are summarized in Table 5. The hydrosulfite reduction of iron was not satisfactory here as it introduced considerable uncertainty in the pH.

Procedure for the Analysis of Water

The water to be analyzed should be free from turbidity or suspended matter. To each liter add about 0.5 ml. of concentrated hydrochloric acid and, if the water may contain partially oxidized iron, 1 g. of solid

hydroxylammonium chloride. The sample should be stored in a polyethylene bottle, leaving no air space.

Prepare a series of standards by pipeting into 50 ml. volumetric flasks 0.0, 2.0, 4.0, 6.0, 8.0 and 10.0 ml. of a 5×10^{-4} M magnesium solution and an appropriate quantity of 0.01 M calcium chloride (if the samples to be analyzed contain calcium equal to or more than the magnesium present). Add exactly 20 ml. of the DHAB working solution. A convenient device for dispensing this solution is shown in Figure 18. Dilute the standards to volume and mix well. Treat appropriate aliquots of the water sample to be analyzed in the same manner. If the sample contains iron or copper transfer 2 ml. of 5% potassium cyanide to the 50 ml. flask before the sample is added. If aluminum is present, add 0.5 ml. of pure triethanolamine to the 50 ml. flask after adding the sample of water. Read the absorbance of the standards and sample at 485 $m\mu$. using 1 cm. cells. Set the transmittance at 100% using the solution containing no magnesium. From the standards prepare a calibration curve. This will not be a straight line but rather slightly concave toward the concentration axis. If several samples containing widely differing amounts of calcium are to be run from time to time, prepare once a family of calibration curves at different levels of calcium concentration. From then on when analyses are to be made prepare only a calibration curve from standards containing no calcium. Refer to the family of calibration curves for an estimate of the correction which must be applied to the observed magnesium concentration.

Figure 20. Effect of pH on the absorbance of magnesium-
o,o'-dihydroxyazobenzene

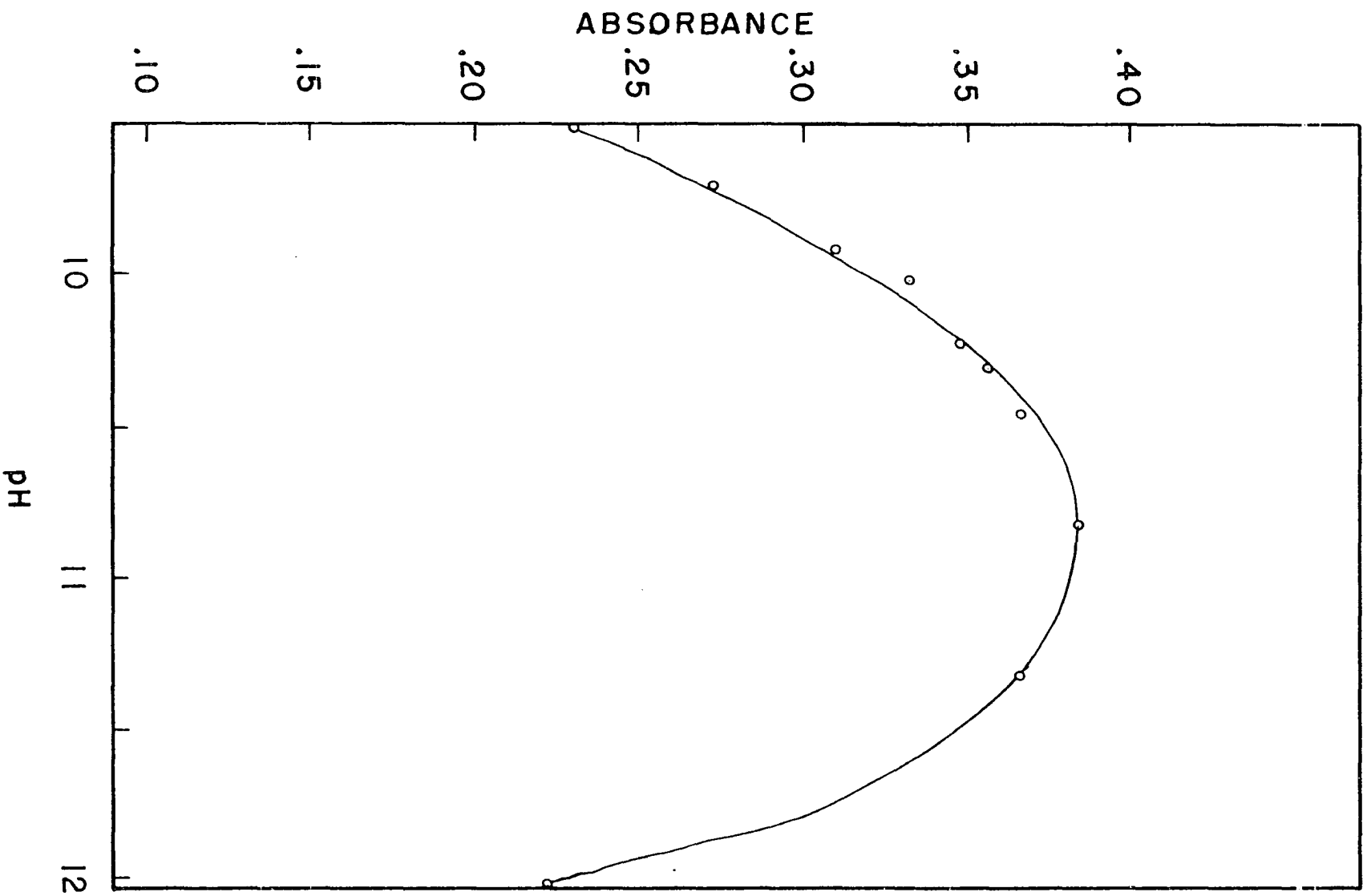


Figure 21. Calibration curves for the spectrophotometric determination of magnesium

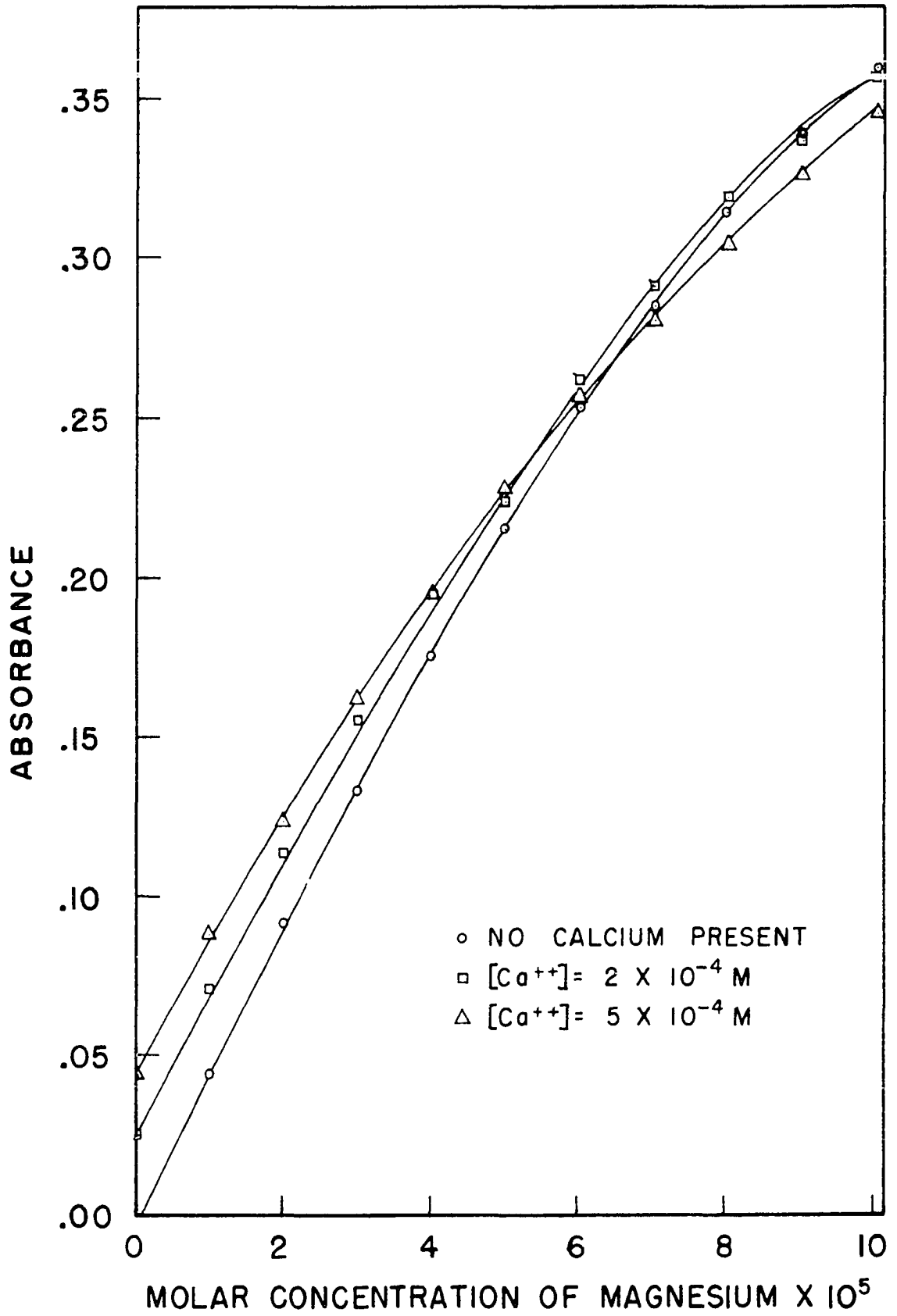


Table 5. Spectrophotometric determination of magnesium in the presence of various interfering substances

Magnesium taken, μg .	Interference added	Magnesium found, μg .	Difference, μg .
100	none	99.0	-1.0
100	none	98.2	-1.8
100	0.1 mg. Zn ^a	97.7	-2.3
100	0.1 mg. Cu ^a	98.2	-1.8
100	1.0 mg. Sr	99.0	-1.0
100	1.0 mg. Be	97.5	-2.5
100	10 mg. Ba	97.7	-2.3
100	15 mg. La	97.5	-2.5
100	10 g. Mn	81.5	-18.5
100	10 mg. KH ₂ PO ₄	94.2	-5.8
100	2 g. Mn	94.5	-5.5
80	1 mg. Fe ^a	86.0	+6.0
20	0.25 mg. Fe ^a	21.0	+1.0

^aTreatment appropriate for the interference applied in accordance to the procedure for analysis of water.

Results

A variety of water samples were analyzed according to the procedure above and by the conventional EDTA titration for comparison (4). The results are summarized in Table 6. A set of typical calibration curves is shown in Figure 21.

Table 6. Determination of magnesium in various water samples

Source	Magnesium found, p.p.m.		Difference, %
	Volumetric	Spectrophotometric	
Iowa State University (Tap)	37.8	38.4, 38.0, 36.6, 37.9 (Ave. 37.7)	-0.3
Ames, Iowa, Municipal (Tap)	4.62	4.52, 4.53, 4.45, 4.48 (Ave. 4.48)	-3.1
Ames, Iowa, Municipal (Untreated) ^b	37.1	37.0, 37.3, 37.0 (Ave. 37.1)	0.0
Duluth, Minn., Municipal (Tap)	2.86	2.73, 2.70, 2.85, (Ave. 2.79)	-2.4

^a Calculated as the difference between calcium plus magnesium and calcium only (4).

^b Analyzed for iron by the method of Collins *et al.* (1) and found to contain 6.7 p.p.m. The other samples contained virtually no iron.

V. PREPARATION OF CRYSTALLINE MAGNESIUM-
O,O'-DIHYDROXYAZOBENZENE

Because o,o'-dihydroxyazobenzene, DHAB, is a very weak acid ($pK_2 = 11.5$) and magnesium hydroxide is relatively insoluble ($K_{sp} = 10^{-11}$), there is no one pH value at which a high concentration of both magnesium ions and DHAB anions can be obtained. Since the magnesium-DHAB is only slightly dissociated ($K_f = 10^{4.85}$ at pH 10) a high concentration of both ions is not absolutely essential.

Several grams of magnesium oxide, 1 g. of DHAB and a few drops of 2-ethylhexanol (wetting agent) were stirred vigorously with about 500 ml. of water for about an hour. The slurry was filtered and the filtrate evaporated slowly over a period of 12-16 hours. Fine red brown needles were obtained. A sample of the material was analyzed by Huffman Microanalytical Laboratory. Found: C 52.61%, H 4.37%, N 9.92%, Mg 9.62% (calculated from weight of residue, assumed to be MgO after ignition at 900°C). Composition calculated for monohydrate of Mg-DHAB: C 56.63%, H 3.96%, N 11.01%, Mg 9.56%; dihydrate of Mg DHAB: C 52.89%, H 4.44%, N 10.28%, Mg 8.92%. Except for the discrepancy in the magnesium content the observed composition corresponds closely to the formula of the dihydrate. Thermogravimetric analysis also indicates that the compound is the dihydrate. The observed composition would be accounted for almost perfectly if the material was the dihydrate contaminated with about 8% excess magnesium.

Later, another batch of crystals was prepared as follows: A mixture of 0.4 g. of magnesium oxide and 400 ml. of boiling water was

stirred until it cooled to about 50°C. A quantity of 1 g. of DHAB and 5 drops of 2-ethylhexanol was added and the stirring was continued for several hours. The mixture was filtered. The filtrate was evaporated over very low heat in a partially covered crystallizing dish. Before the evaporation was complete, the mixture was suction filtered and the yellow-orange crystals were washed lightly with water. The material was air dried. Results of thermogravimetric analysis were nearly identical to those on material from the first batch. The magnesium analysis was much more nearly that calculated for the dihydrate, 9.18%. The compound does not melt but decomposes leaving magnesium oxide in exactly the same shape as the original crystals.

VI. CONCLUSION

It has been found that the magnesium derivatives of several *o,o'*-dihydroxyazo compounds are fluorescent. The optimum excitation wave length for compounds studied is around 470 $m\mu$. while the maximum fluorescence occurs at around 580 $m\mu$. Compounds of other alkaline earths with the dihydroxyazo compounds, if they form at all, are not generally fluorescent.

The data assembled suggested that many of the azo compounds studied might be used as fluorometric reagents for magnesium. One in particular, *o,o'*-dihydroxyazobenzene, was investigated for such use and was found to be excellent. By use of a suitable procedure, natural and manufactured materials containing large amounts of interfering substances can be analyzed successfully.

o,o'-Dihydroxyazobenzene was also studied as a spectrophotometric reagent for magnesium. Only moderate success was met in this endeavor. Since the compound itself absorbs at the same wave length as its magnesium derivative, only to a lesser degree, the procedure must be that of a differential spectrophotometric method. Rigid control of pH was found to be necessary. The method is satisfactory for materials containing no more than moderate amounts of calcium or small amounts of other interferences such as iron, aluminum or manganese.

Crystalline magnesium-*o,o'*-dihydroxyazobenzene has been isolated. Its composition appears to be that of a dihydrate although on various preparations the magnesium analysis is a little higher than that calculated.

VII. SUMMARY

The fluorescence and excitation spectra of several *o,o'*-dihydroxyazo compounds and their magnesium derivatives have been recorded.

The effect of the other alkaline earths, especially calcium, on the fluorescence of these azo compounds has been studied and generally found to be slight.

An efficient preparation of *o,o'*-dihydroxyazobenzene has been worked out and described in detail.

The simplest member of the series, *o,o'*-dihydroxyazobenzene, has been investigated as a fluorometric reagent for magnesium and found suitable for determination of 2 to 25 μ g. of magnesium. Interference of calcium and several other common materials is easily handled.

A spectrophotometric method for magnesium utilizing *o,o'*-dihydroxyazobenzene has been developed. It is suitable for the determination of 10 to 100 μ g. of magnesium but is beset with several problems such as pH control and the interference of large amounts of calcium and even moderate amounts of several transition metals.

Crystalline magnesium-*o,o'*-dihydroxyazobenzene, apparently the dihydrate, has been prepared.

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