1973

The Isolation and Purification of a Fluorescent Compound from Pseudomonas

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The Isolation and Purification of a Fluorescent Compound from Pseudomonas

(TITLE)

BY

Thomas M. Davey
B.S. Rose Polytechnic Institute
Terre Haute Indiana June 1969

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1973
YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
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The Isolation and Purification of a Fluorescent Compound from Pseudomonas

Thesis Approved

Dr. C. D. Foote, Thesis Advisor

Dr. D. H. Buchanan

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Abstract

Title of Thesis: The Isolation and Purification of a Fluorescent Compound from Pseudomonas

Thomas M. Davey Master of Science 1973

Thesis Directed by Dr. C. Dan Foote

Fluorescence in certain land snails has been found to arise from a bacteria carried by the snails. The bacteria, a genus of Pseudomonas, produce a pigment complex in iron-deficient media. The pigment complex consists of both fluorescent and non-fluorescent pigments. One of the fluorescent compounds was isolated and purified by paper chromatography using several different solvent systems.

Absorption and fluorescent spectra of the compound at a number of pH values were measured and the changes with pH of both the intensity and the color of the fluorescence were observed. The purified compound was ninhydrin negative but after acid hydrolysis several amino acids were identified and were thought to occur in peptide bonds. The amino acids identified were: serine, glutamic acids, aspartic acid, cystine, threonine, lysine, and alanine.

A comparison with other fluorescent compounds isolated from the Pseudomonas pigment complex showed the compound to be one which had not been previously isolated.

Suggestions on a more efficient method of purification and precautions necessary during isolation are given.

Introduction

Recently Rawls (1) observed an intense blue-green fluorescence in certain endodontid snails. The fluorescence was found to be caused by a pigment complex produced by bacteria within the snail. The bacteria were identified as of the Pseudomonas genus. Production of the pigment complex by Pseudomonads has been known since 1899 as reported by Jordan (2). The pigment complex is not a single pigment but is a mixture of both fluorescent and non-fluorescent pigments (3). The pigment complex is most commonly called pyoverdine, although other names, such as fluorescin (4), have been used. Pigments isolated from the complex have been called Fluorescence I and II, C substance (5) and Compound I (6).

Several studies have been done on the nutritional requirements for the production of the pigment complex. The presence of magnesium ion, sulfate ion, and phosphate ion was found necessary by King (7) and Burton (8). The presence of iron in the growth medium was found by a number of workers to decrease the production of the pigment complex (7, 8, 9). The pigment complex has been thought to combine with iron to form a respiratory cytochrome (10) or to be a substitute for an iron-containing cyctochrome (11).

Color changes in the pigment complex with changes in concentration and pH have been examined by Osawa et. al. (12),

Newkirk and Hulcher (6), and Elliot (3).

Isolation of a single fluorescent compound from the complex by using a gel filtration method has been reported by Osawa et. al. (13) and by Newkirk and Hulcher (6). Both used Sephadex G-25, which is a cross-linked dextran gel. The Sephadex G-25 excludes compounds of molecular weight greater than 2500. Osawa reported an impure preparation with the separation of a fraction, that he called Fluorescence I, into two bands during electrophoresis. Osawa examined the absorption and emission spectra and the physical properties of Fluorescence I, but gave no information on the structure of the compound. Hulcher reported the isolation of a fraction that he called Compound I. He reported that the compound was composed partly of a peptide containing the amino acids threonine, serine, glutamic acid, and lysine. The peptide comprised 26% by weight of the compound. The compound appeared pure by thin layer and paper chromatography, but showed some impurities during polyacrylamide-gel electrophoresis.

The purpose of this work is to attempt to cleanly isolate a fluorescent compound from the Pseudomonas pigment complex, to study its properties, and to investigate its composition.

Materials and Methods

Bacteria

Snail mucus containing the Pseudomonas bacteria was obtained from Dr. Hugh Rawls of the Eastern Illinois University Zoology Department. Cell suspensions were made by mixing the mucus with distilled water.

Culture Media

The culture medium, called Pseudomonas Medium B, consisted of 20 gr of Proteose Peptone #3, 10 ml of glycerol, 1.5 gr of K_2HPO_4, 1.5 gr of $MgSO_4$, and 15.0 gr of agar dissolved in one liter of distilled water. The solution was autoclaved for 20 min at 120° C. The medium was kept in sterile containers at 4.0° C until needed. The medium was resterilized prior to use.

Bacteria Purification

Cell suspensions from the mucus were streaked on agar plates to isolate the fluorescent pigment-producing bacteria. After 24 hours at room temperature the plates were examined for isolated fluorescing bacterial colonies. Several of these isolated colonies were restreaked onto fresh agar plates. Isolated colonies from this second set of plates were used to produce the pigment and were streaked onto agar

slants for the storage of the live bacteria. The agar slants were kept at 4.0 C. Bacteria from the slants were streaked on agar plates every three months and new slants were made.

Pigment Production

Cell suspensions from the agar slants were poured over agar plates to obtain maximum growth. The plates were kept in the dark at room temperature for three days. After the incubation period the agar plates were soaked with a few milliliters of 95% ethanol to kill the bacteria. The plates were washed with water several times and allowed to stand for several hours. The washings were combined and flash evaporated to a volume of about 200 ml. Bacterial cells were removed by centrifugation at 5000 RPM for five minutes. The washings were then flash evaporated to a point where the liquid became slightly viscous. A second method of preparing the pigment from the agar plates using a solid-liquid extractor was tried. The agar plates were extracted with methanol for four hours at 65 C. The methanol extract was concentrated by evaporation and purification continued in the same manner as the water washings of the plates. The solid residue was washed with cold (4 C) water to remove the remaining pigment without dissolving the agar. The pigment prepared this way had fewer of the fluorescing impurities contained in the medium.

Pigment Purification

About 0.3 to 0.5 ml of the concentrated pigment solution was applied to a 19 x 45 cm sheet of Whatman #1 paper. The sheets were placed in a chromatographic tank saturated with solvent vapor and developed by descending chromatography for 12 to 15 hours at room temperature. The initial solvent was a 12:5:3 v/v ratio of n-propanol, water, and formic acid. The sheets were air dried in a hood overnight. The fluorescent bands were viewed using a Mineralight Ultra Violet lamp, model S1-2537, and were marked with pencil. The bands were cut out and eluted with water or 0.01N HCl. The eluate were pooled from a number of sheets and evaporated to dryness. The residue was redissolved in a minimum of distilled water. This solution was then applied to another sheet and developed with n-propanol, water, and aqueous ammonia solvent with a ratio of 12:5:3 v/v. The procedure for the elution of the fluorescent bands was repeated and the eluate was chromatographed in a n-propanol, water, and formic acid solvent with a ratio of 4:2:1 v/v. The fluorescent band was collected as before.

Hydrolysis of the Fluorescent Compound

The purified compound was placed in a glass tube with an equal amount of 12M HCl to give a solution that was six

molar. The tube was purged with nitrogen and sealed. The tube was placed in a 110° C oven for 36 hours. The tube was opened and the solution and water washings of the tube were collected. The combined solution was filtered and flash evaporated to dryness. The residue was redissolved in an amount of water equal to the initial volume, about two milliliters.

Paper Chromatography

The periodate-benzidine reagents for sugars were prepared by making a 0.5% solution of $NaIO_4$ in water and a 0.5% benzidine solution in 95% ethanol and glacial acetic acid (4:1 v/v). The chromatograms were sprayed with the periodate solution until damp. After five minutes, the chromatograms were sprayed with the benzidine solution. Sugars and polysaccharides appear as white spots on a blue background. Chromatograms for amino acid analysis were sprayed with a 0.2% solution of ninhydrin in 95% ethanol. Color was developed by placing the sheets in a 110° C oven for five minutes. The amino acids appear as blue or purple spots on a white background. The solvent systems used were the n-propanol, water, formic acid (12:5:3 v/v) called solvent system A and the n-propanol, water, aqueous NH_3 (12:5:3 v/v) system B. Thin layer plates of Eastman silica gel sheets

K301R2 were also run using the same systems. Two dimensional chromatograms were run in an Eastman chromatogram chamber.

Electrophoresis

Electrophoresis runs were carried out in 0.1M buffer solutions on sheets of Whatman #1. The runs were made at 100V and five mA for six hours at 4.0 C. A Gelman electrophoretic apparatus with a Heathkit Regulated Power supply Model 1P-32 was used. Buffer composition is specified in section on absorption spectra.

Absorption Spectra

Absorption spectra were taken in quartz cells (one cm) using a Beckman DB-G UV Spectrophotometer. The tungsten lamp was used for the 340nm to 600nm region of the spectra. The hydrogen lamp was used in the 200nm to 340 nm region. Fluorescence and Emission spectra were taken with an Aminco-Bowman spectrophotofluorometer using one cm quartz cells. The buffers used in the spectra and the electrophoresis were 0.1M solutions of: sulfate buffer for pH 2, formate buffer for pH 3 and 4, acetate buffer for pH 5, phosphate buffer for pH 6, 7, and 8 ammonia buffer for pH 9, and carbonate buffer for pH 10 and 11.

Dialysis

Dialysis of the water washings from the agar plates, of

the methanol-extracted agar plates and of the purified compound were carried out using dialysis tubing, against distilled water for 24 hours.

Iron Complexing of the Compound

A one millimolar solution of iron (III) nitrate was prepared and added to the purified compound. Absorption and fluorescence measurements were taken and compared to the original compound measurements.

Results

Several fluorescent spots were detected using solvent system A. The most intense band fluoresced blue-white at a distance 11.25 cm from the origin compared to the solvent front which moved 45 cm. The ratio of the distances is called the R_f and for this band had a value of 0.25. Other strong bands were a greenish-blue band at an R_f of 0.50 and a yellow band at 0.70. The main band was cut out, eluted, and rechromatographed using the "B" solvent system. The main band in the second chromatogram was found at an R_f of 0.15 with only very minor bands well separated from the main band. The main was cut out, eluted and rechromatographed using propanol, water, formic acid (4:2:1 v/v). Two bands of approximately equal intensity were found, one at an R_f of 0.40 and one at an R_f of 0.55. Only the band at 0.55 was

eluted and examined further due to the length of time involved in preparing the compound.

The purified compound traveled as a single spot (R_f 0.65) on thin layer chromatography plates using the acid solvent. In the basic solvent the spot was not moved from its origin. Paper electrophoresis of the pigment complex at pH 3, 7, and 10 showed the material to be a weak cation at pH 10 but immobile at pH 3 and 7. Evaporation of the solution to dryness left a brownish-yellow solid that decomposed at 145 to 150 ° C. Evaporation also caused some decomposition of the compound. The dialysis of the compound and the pigment complex showed the pigment was small enough to pass through the dialysis tubing. A chromatogram of the dialysate of the pigment complex showed that there were several fluorescent compounds which were able to pass through the membrane. Chromatographs of the methanol-extracted agar plates also showed several bands. The absorption of a 1% by weight solution in a 1 cm cell or the $E_1^1\%$ of the purified compound at 400nm had a value of 13.3 and the absorption and emission spectra were linear in the range of 0.15 mg/ml to 1.0 mg/ml (figures 1 and 2).

The absorption spectra of the purified compound in the various buffer solutions from pH 1.5 to 11 show a gradual shift of the visible absorption peak from 375nm to 420nm. This shift can be seen in table 1 along with the smaller

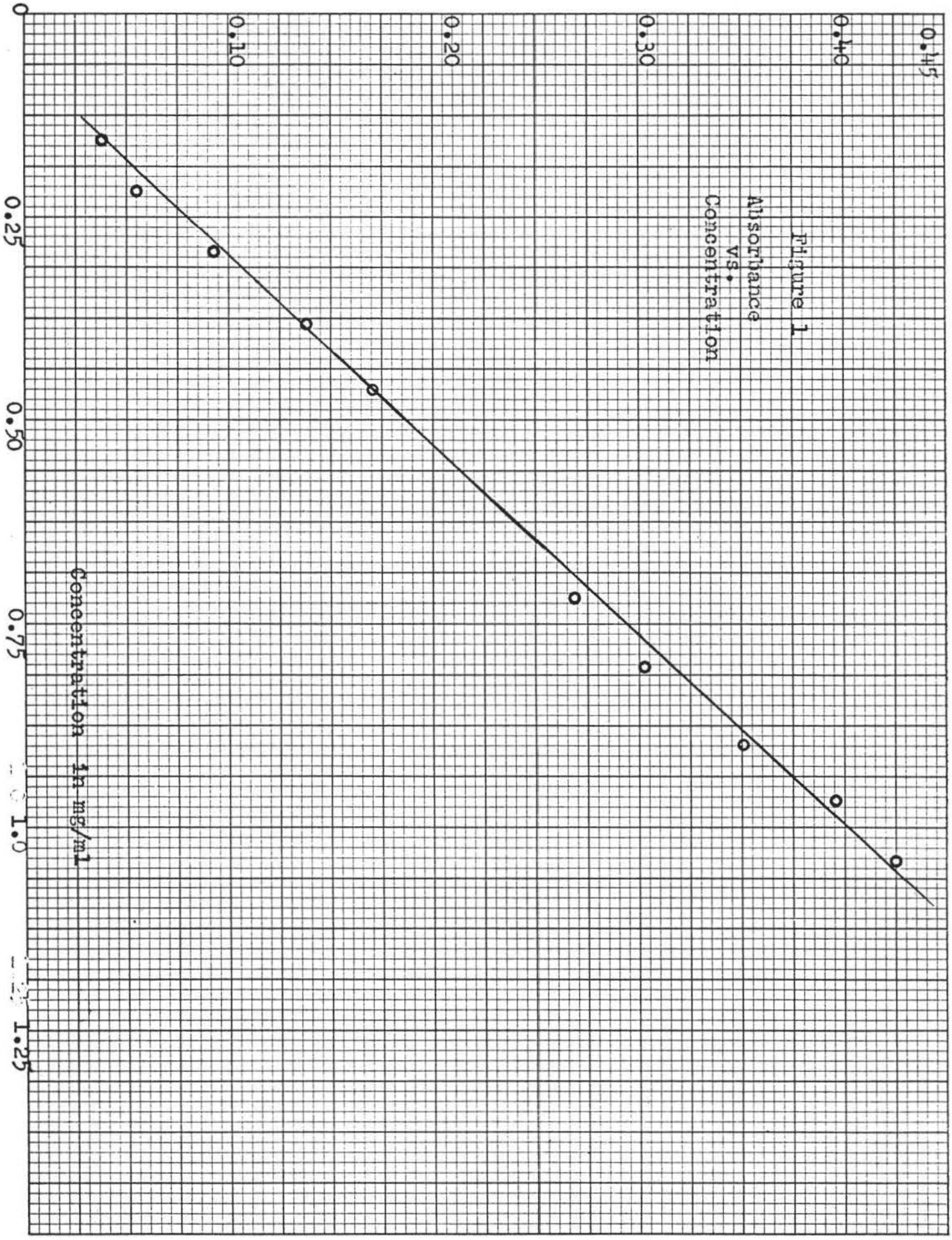


Figure 1
Absorbance
vs.
Concentration

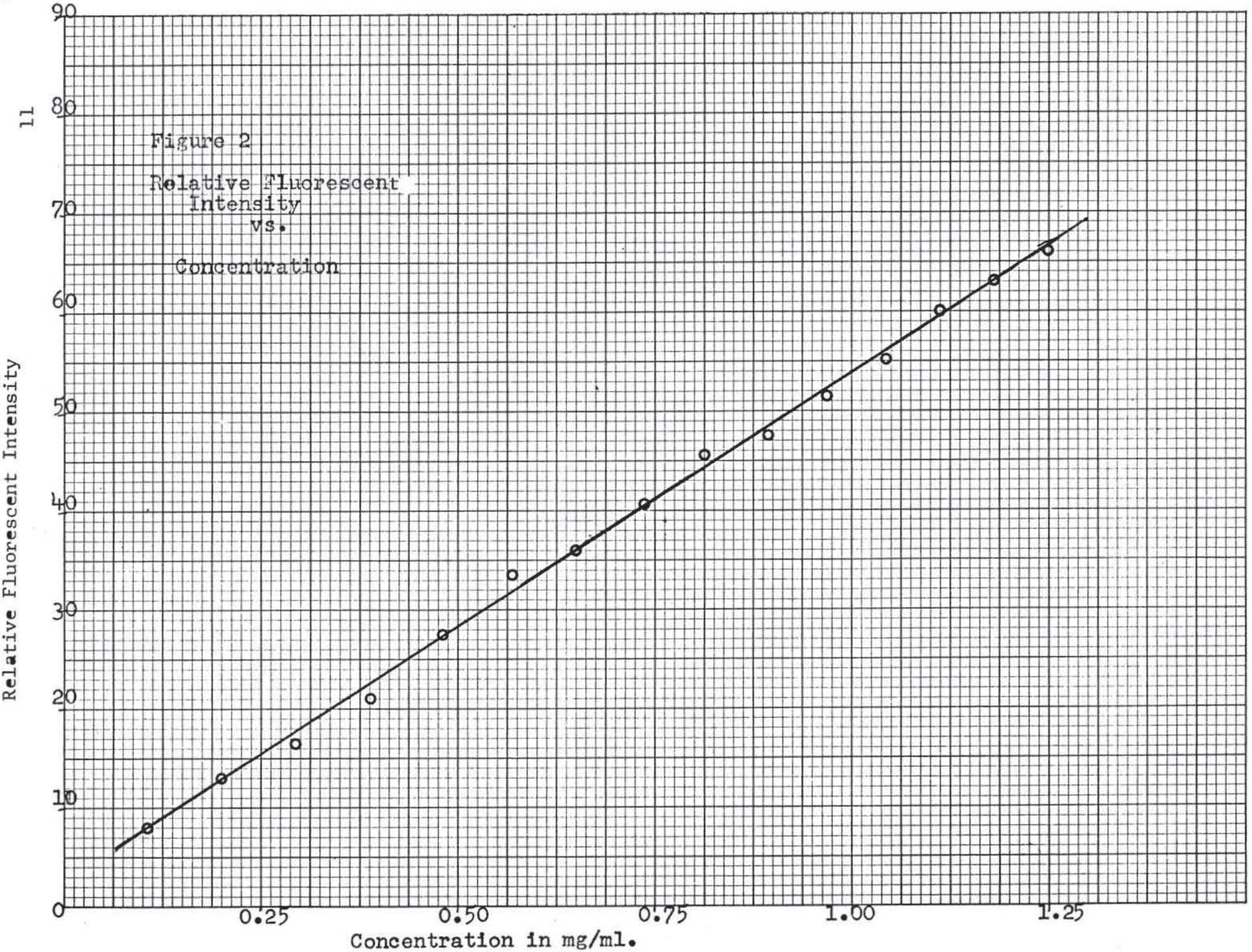


Figure 2
Relative Fluorescent Intensity
vs.
Concentration
Relative Fluorescent Intensity
90
80
70
60
50
40
30
20
10
0
0.25
0.50
0.75
1.00
1.25
Concentration in mg/ml.

Table I

Absorbance Spectra of the compound at different pH's in 0.1M buffer

pH value	Max (nm)	Percent Transmission	Concentration (mg/ml)
1.5	375	69	0.35
	250	36	
	210	0	
2.0	375	72	0.35
	250	42	
	210	0	
3.0	375	71	0.35
	250	40	
	225	15	
3.5	375	75	0.35
	250	43	
	225	15	
4.0	375	72	0.35
	270	45	
	225	5	
5.0	400	74	0.35
	260	45	
	220	5	
6.0	406	72	0.35
	265	39	
	210	0	
7.0	412	74	0.35
	365	46	
	210	0	
8.0	412	70	0.35
	265	40	
	210	0	
9.0	410	72	0.35
	262	43	
	210	0	
10.0	265	41	0.70
	225	0	
11.0	275	45	0.70
	225	0	
12.0	260	47	0.70
	220	0	

variations of the peaks in the ultra violet region at 210nm and 265nm. Figures 3 to 6 show the absorption spectra of the purified compound at pH's of 3, 4, 7, and 9.

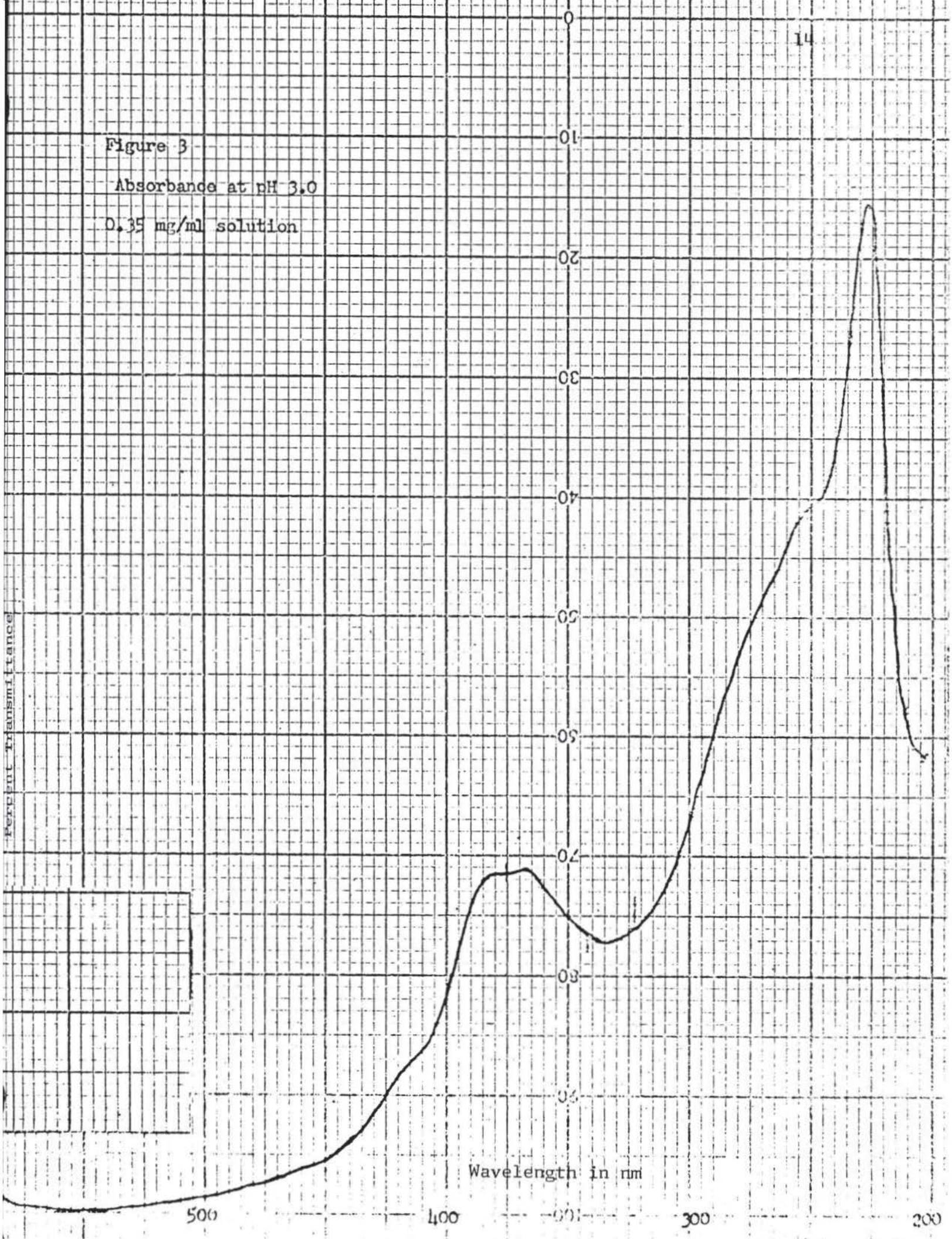
The fluorescence spectra of the compound in the different buffer solutions show both a change in the color of the fluorescence and the intensity of the emitted light. The most notable of these is the nearly tenfold increase in intensity of the compound at pH 5 as compared to pH 4 and 6. These changes can be seen in table 2. Figures 7 to 10 show the fluorescent spectra at pH's 3, 5, 7, and 9. The two curves shown in the figures are the excitation and emission spectra from left to right respectively. In figure 7, a radical change that takes place at pH values of three or below is seen in the spectrum. The emission maximum changes with pH are completely reversible between pH3 and pH9. Outside of that range a decomposition of the compound begins. Above pH9 decomposition products can be seen to precipitate. Figure 11 shows a graph of the relative fluorescent intensity against pH.

A chromatogram of the hydrolyzed compound showed eight ninhydrin positive spots and a single long fluorescent spot. One of the ninhydrin positive spots had the same R_f as the purified compound and was fluorescent. This spot was thought to be the partially hydrolyzed compound. The

Figure 3

Absorbance at pH 3.0

0.35 mg/ml solution



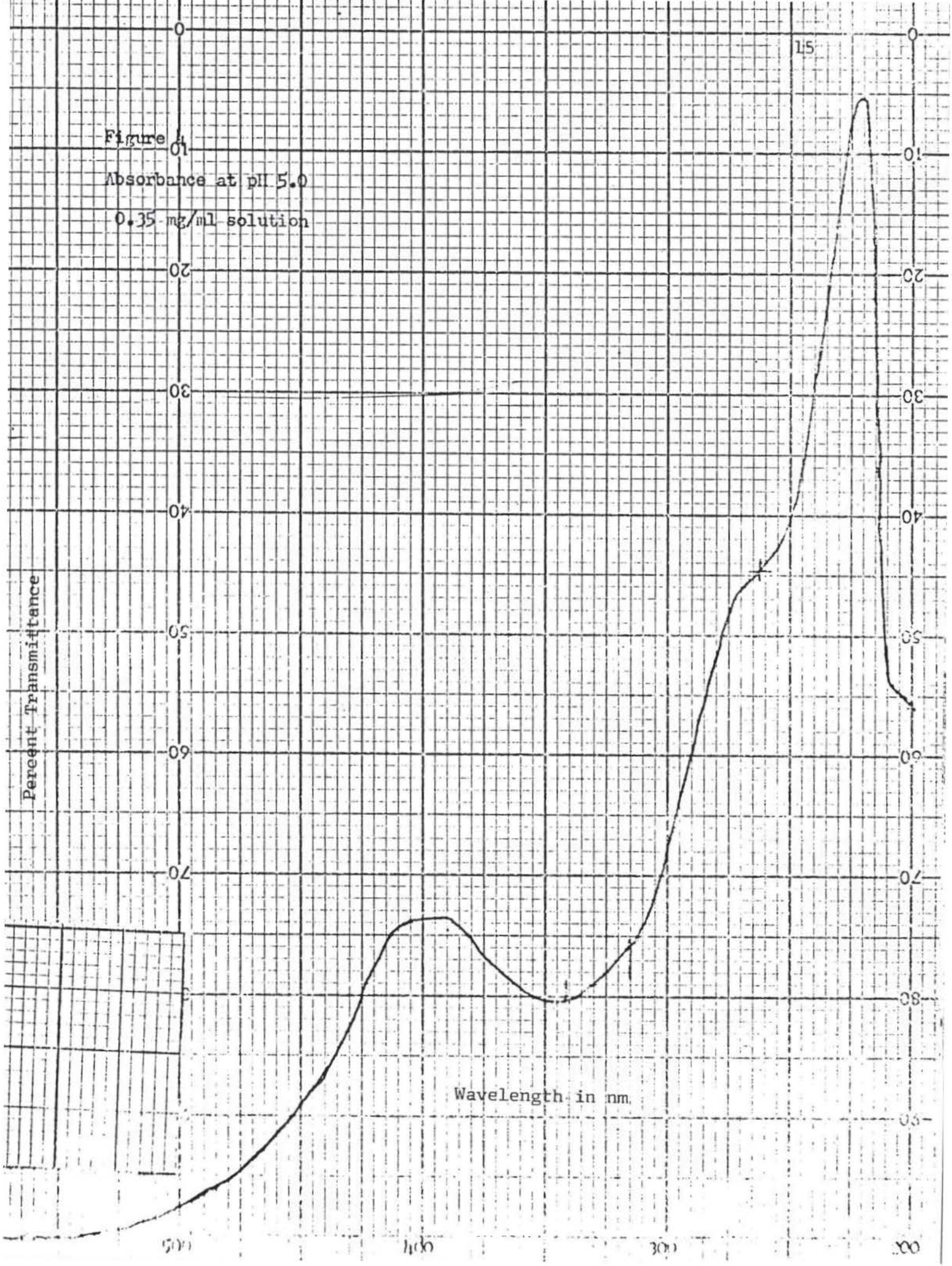


Figure 4
Absorbance at pH 5.0
0.35 mg/ml solution
Percent Transmittance
Wavelength in nm.

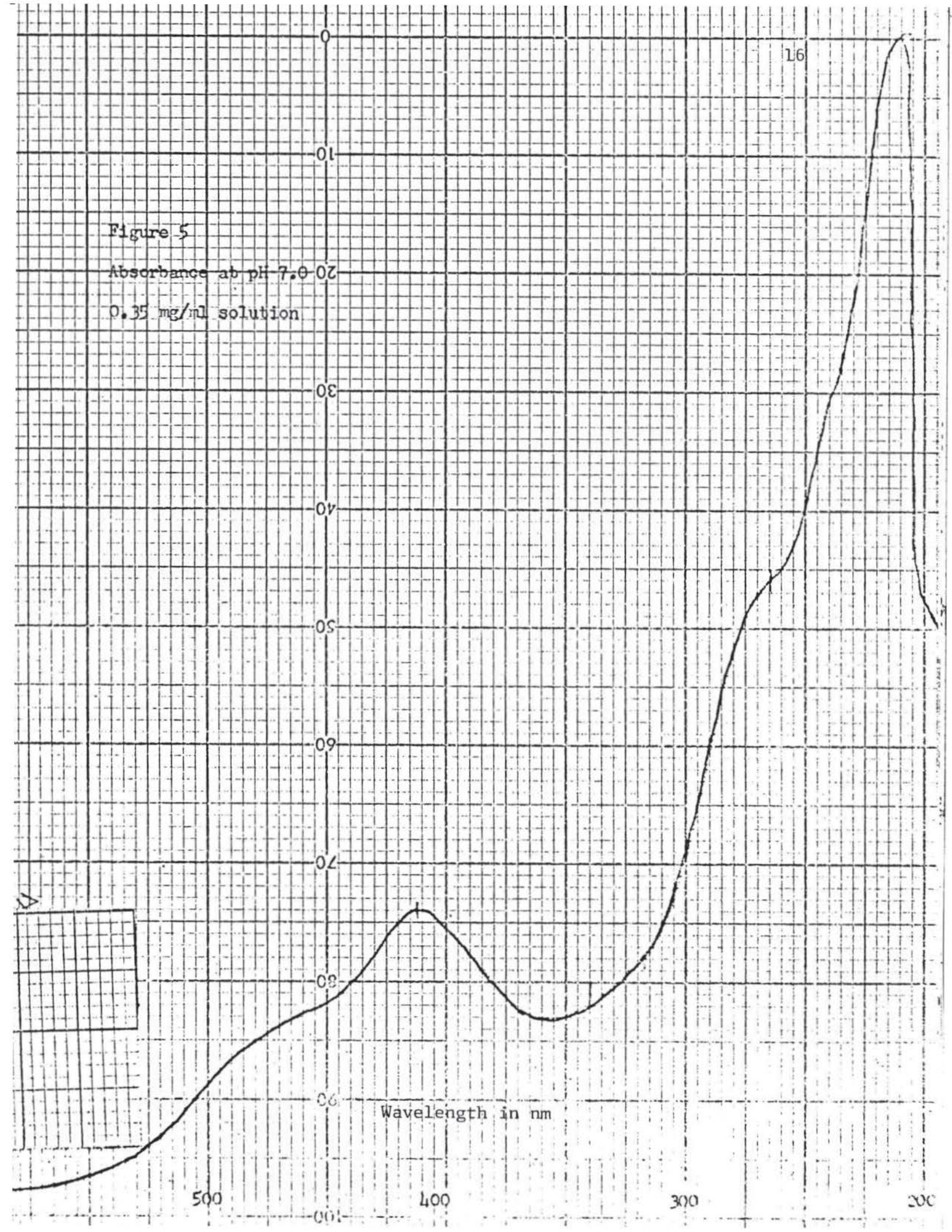


Figure 5
Absorbance at pH 7.0
0.35 mg/ml solution
Wavelength in nm
500
400
300
200

Figure 6

Absorbance at pH 9.0

0.35 mg/ml solution

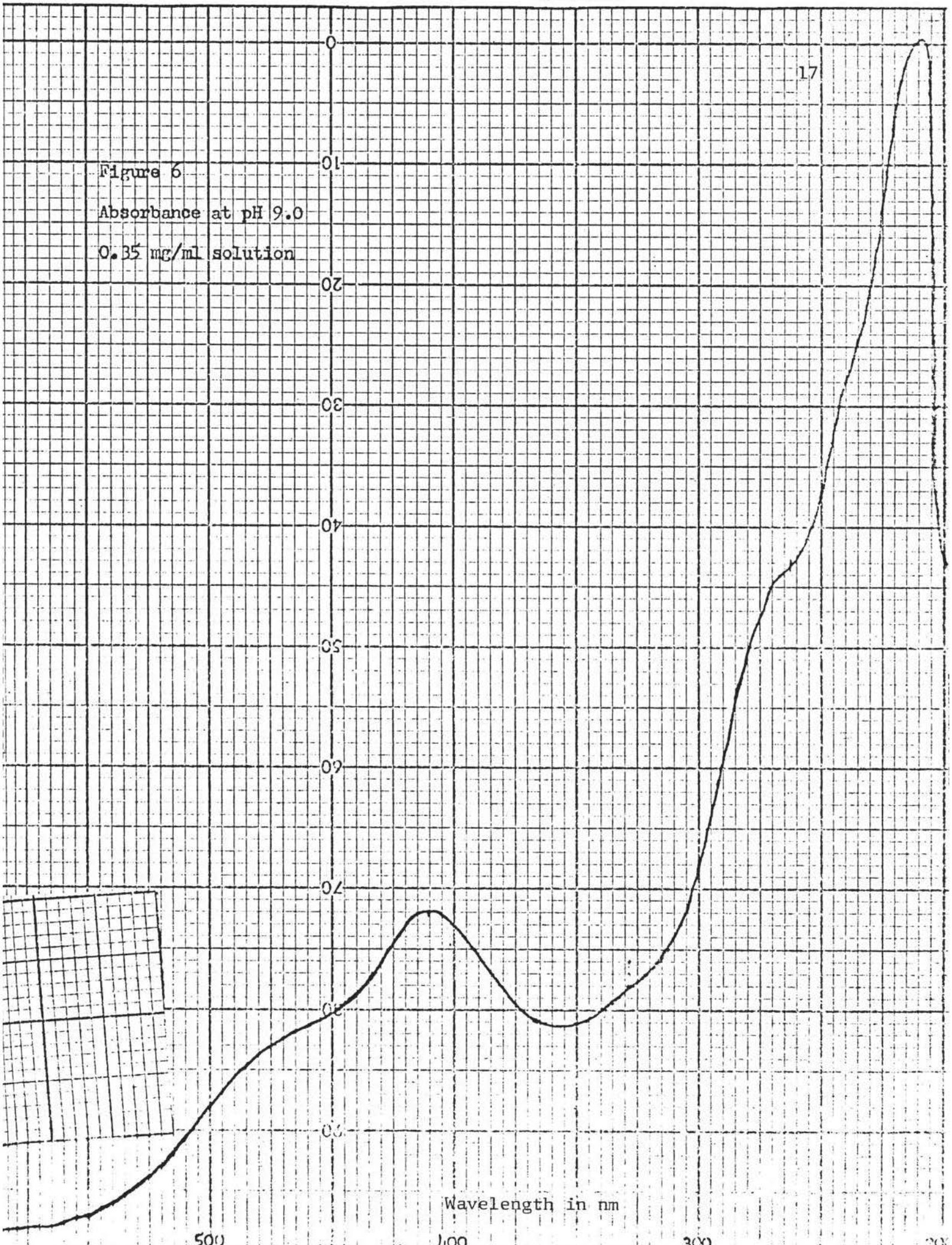


Table II

Fluorescence Spectra of the compound at different pH's in 0.1M buffer

pH value	Emission max. (nm)	Excitation max. (nm)	Relative Intensity
1.5	420,502	372	3.0
2.0	425,505	370	3.0
3.0	430,500	380	3.0
4.0	482	388	4.5
5.0	465	400	30.0
6.0	464	404	9.0
7.0	456	404	5.5
8.0	462	404	7.5
9.0	455	392	2.0
10.0	500	414	1.8
11.0	502	430	4.5
12.0	500	430	7.5

Figure 7

Fluorescence spectra in pH 3 buffer (Uncorrected)

Emission Maximum 430, 500 nm

Excitation Maximum 364 nm

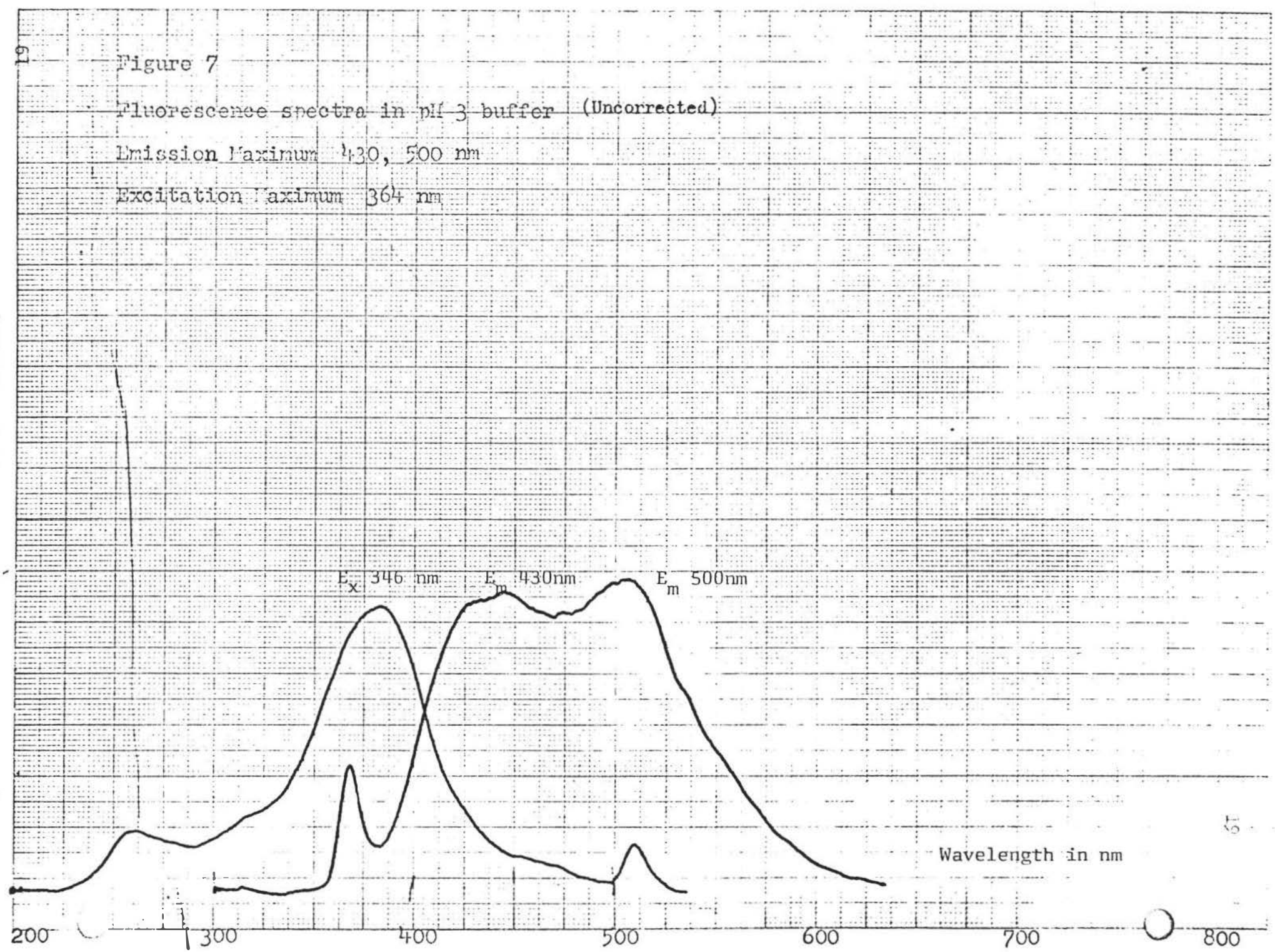


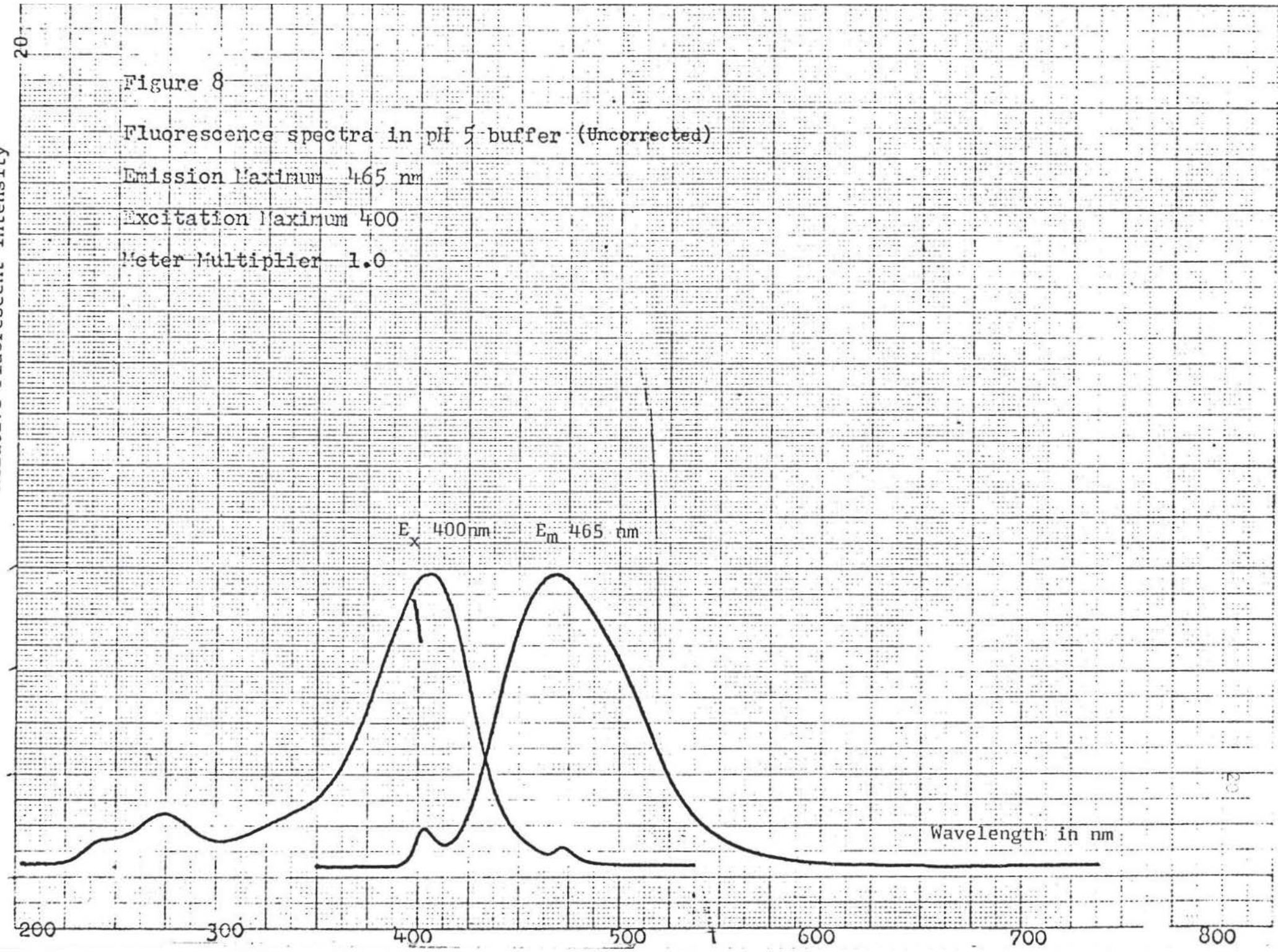
Figure 8

Fluorescence spectra in pH 5 buffer (Uncorrected)

Emission Maximum 465 nm

Excitation Maximum 400

Meter Multiplier 1.0



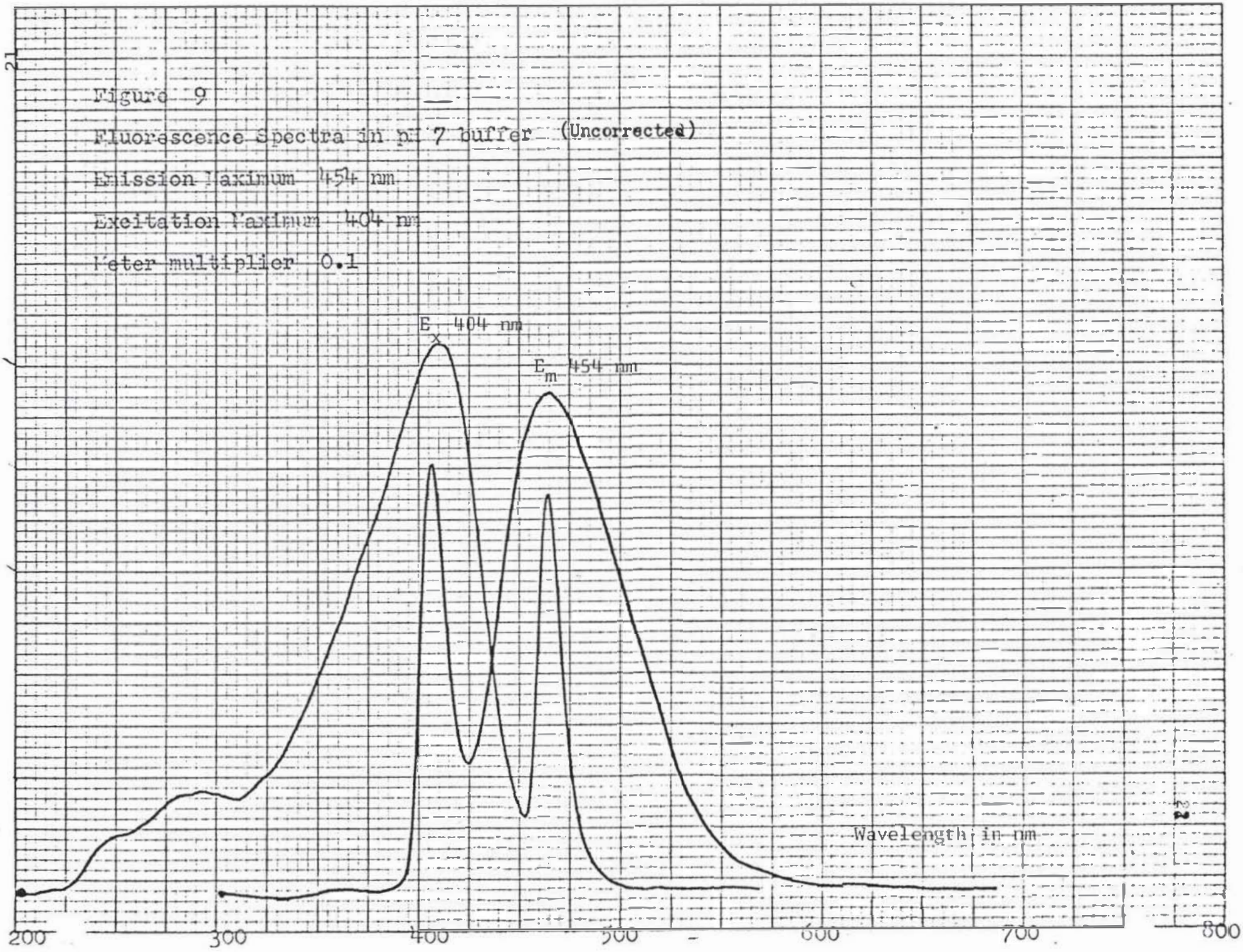


Figure 9
Fluorescence Spectra in pH 7 buffer (Uncorrected)
Emission Maximum 454 nm
Excitation Maximum 404 nm
Meter multiplier 0.1
E 404 nm
E_m 454 nm
Relative Fluorescent Intensity
Wavelength in nm
200
300
400
500
600
700
800

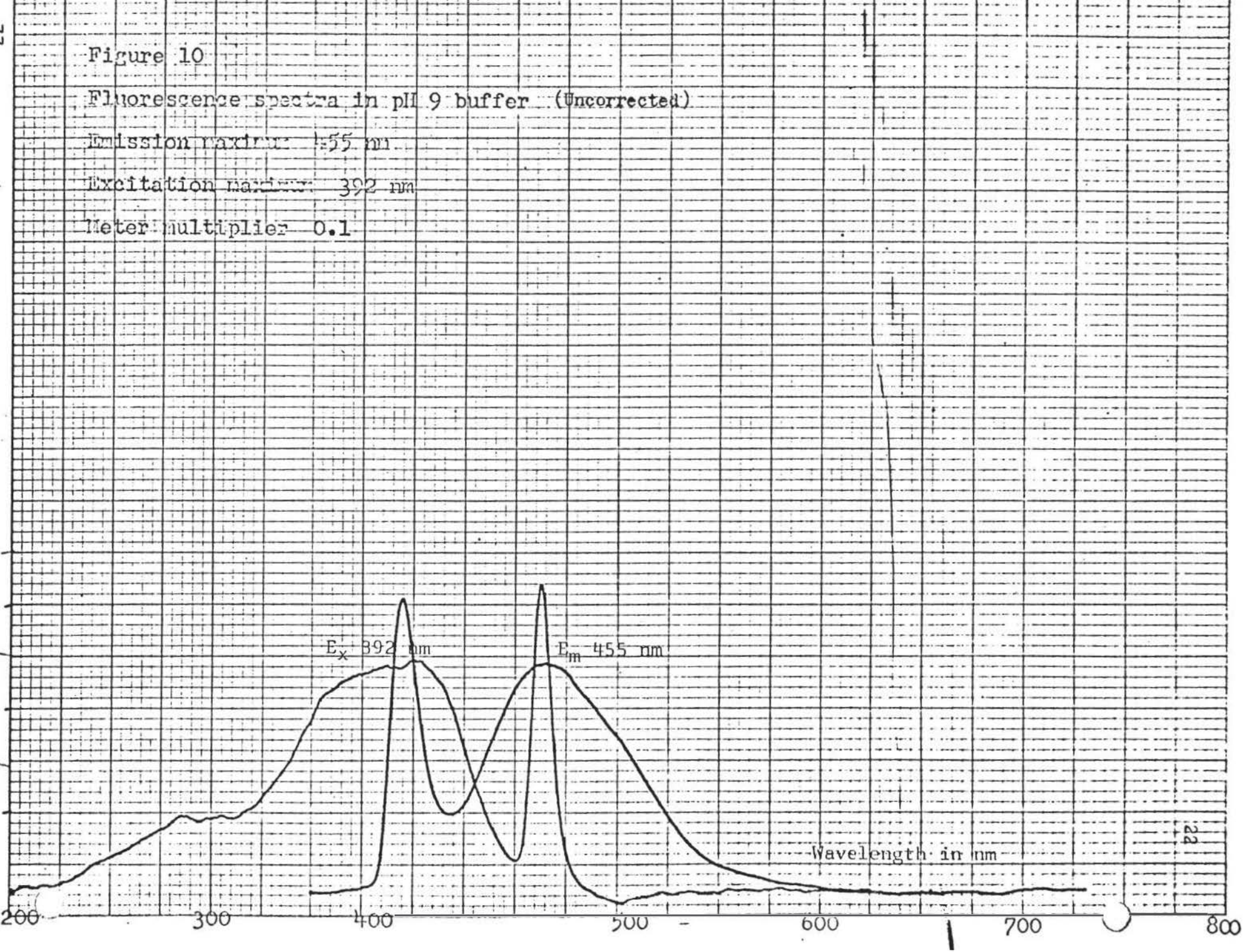
Figure 10

Fluorescence spectra in pH 9 buffer (Uncorrected)

Emission maximum 455 nm

Excitation maximum 392 nm

Meter multiplier 0.1



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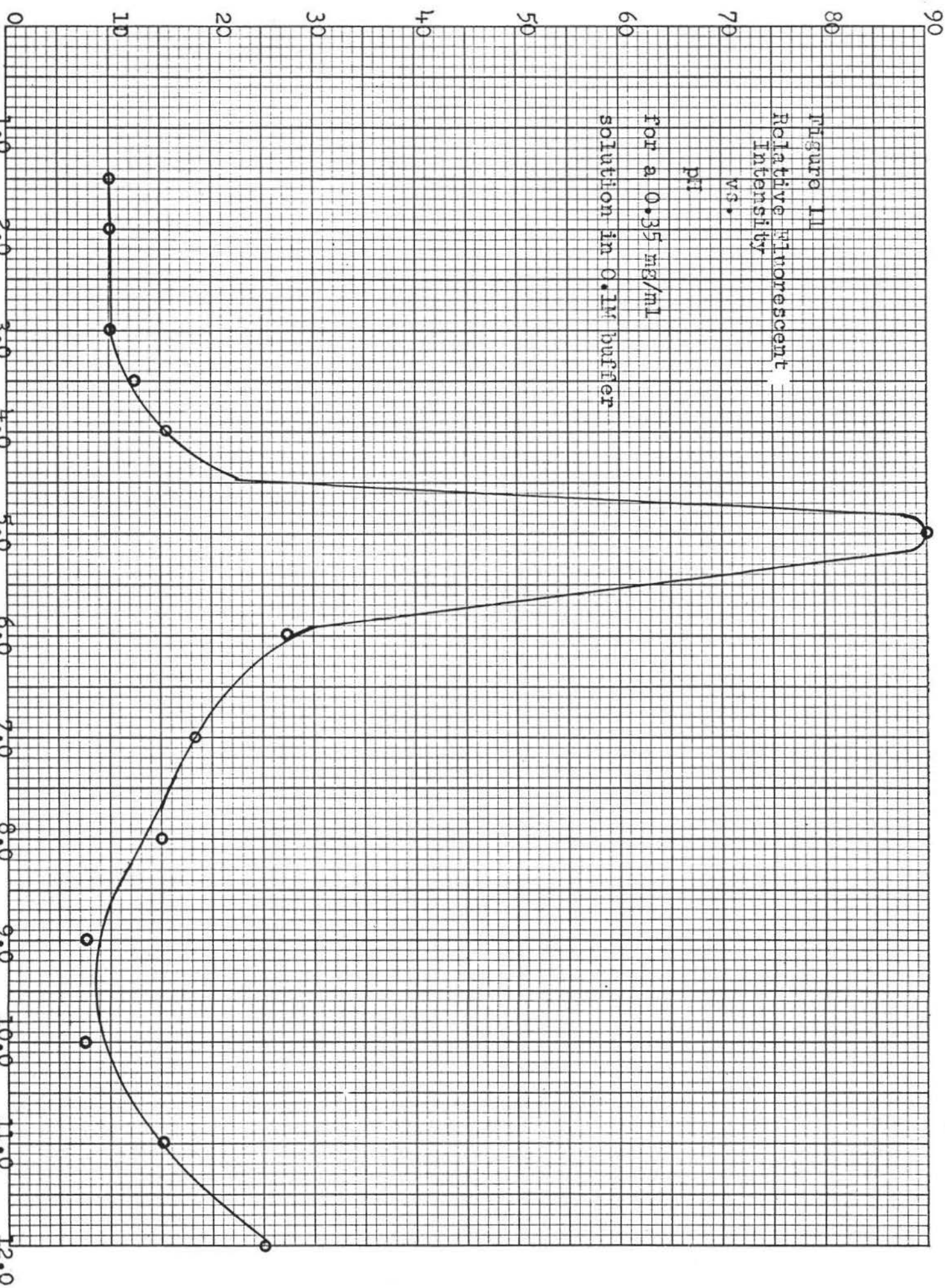


Figure 11
Relative Fluorescent Intensity vs. pH
for a 0.35 mg/ml solution in 0.1M buffer

chromatogram was also periodate negative as was the purified compound. Amino acids identified from the hydrolyzed compound were aspartic acid, glutamic acid, serine, threonine, lysine, alanine, and cystine. Table 3 gives the R_f's of the amino acids in the solvent systems. Electrophoresis was also used to confirm the presence of several of the amino acids. In pH 3 buffer solution aspartic acid and glutamic acid were easily determined as they moved toward the anode. Several of the amino acid spots were very ninhydrin positive and may have obscured other amino acids.

The addition of 10 µl of the $Fe(NO_3)_3$ solution to 3 ml of a 5.8 mg/100 ml quenched 97% of the fluorescence at 460 nm almost immediately. The addition of 30 µl of the iron solution to 3 ml of a 1 mg/ml solution of the compound caused the changes in the absorption spectra seen in figure 12.

Discussion

In 1963, Osawa et. al. isolated a fluorescent compound by Sephadex gel filtration, called Fluorescence I. He conducted spectral and pH studies on it and on another compound later isolated and called "C" substance. He did not attempt to determine the chemical nature of the compounds and felt that Fluorescence I had been isolated impurely. In 1969, Newkirk and Hulcher reported the isolation of a

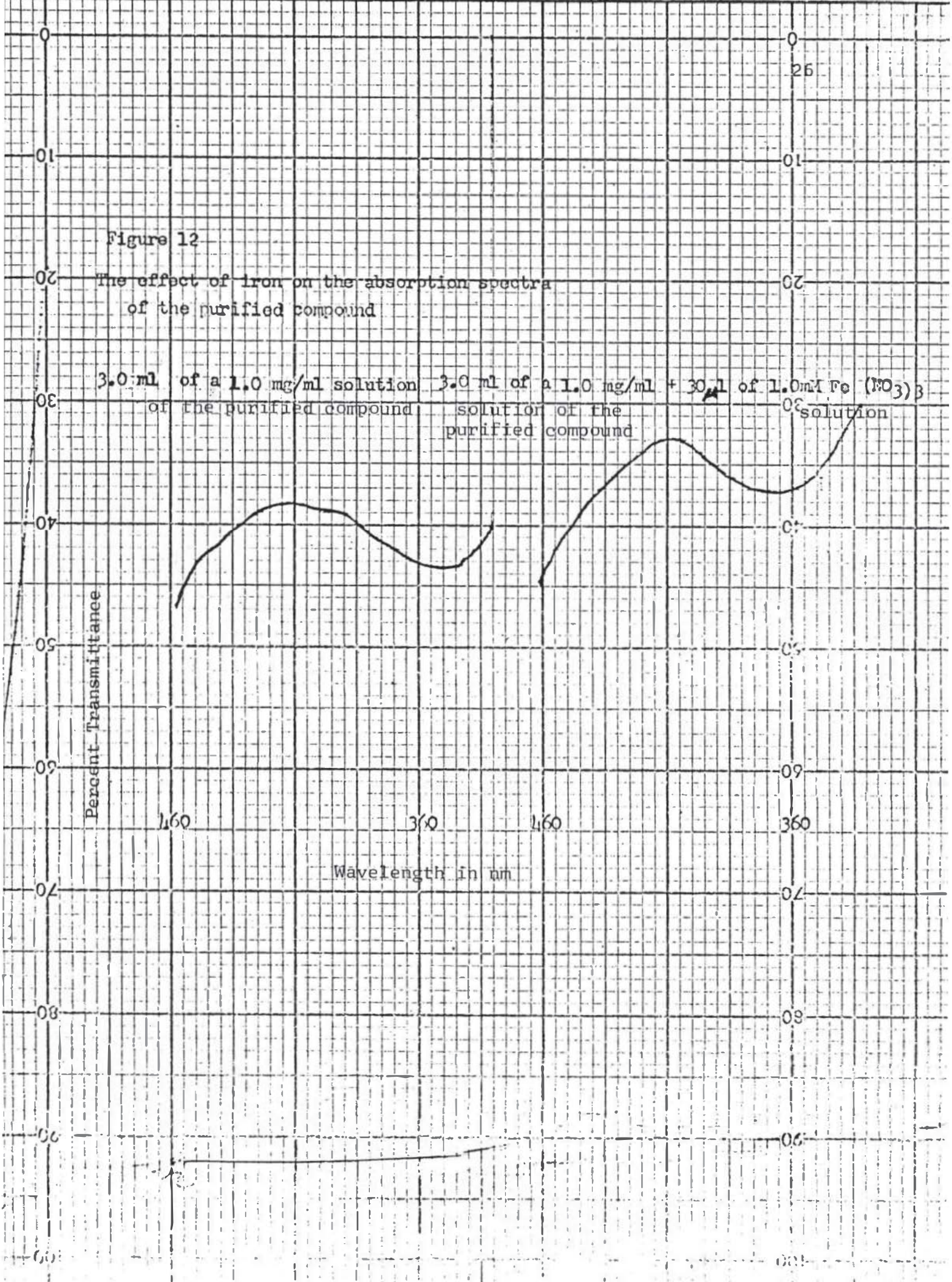
Table III

R_f values of the amino acids in chromatographic systems

Amino acid	R_f in n-propanol, water, formic acid (12:5:3)	R_f in n-propanol, water, NH_3 (12:5:3)
Alanine	0.63	0.44
Leucine	0.95	0.63
Valine	0.90	0.55
Tyrosine	0.63-0.90	0.47
Tryptophan	0.74-0.90	0.53
Threonine	0.58	0.55
Proline	0.65	0.47
Glycine	0.54	0.38
Lysine	0.37-0.47	0.39
Histidine	0.37	0.40
Glutamic acid	0.60	0.21
Cystine	0.29	0.00
Aspartic acid	0.55	0.19
Serine	0.53	0.38

Figure 12

The effect of iron on the absorption spectra of the purified compound



similar compound called Compound I from Pseudomonas by repeated gel filtration. They also referred to a compound they called Pyoverdine. Newkirk and Hulcher found that the compounds were partly composed of amino acids. They identified four amino acids: serine, glutamic acid, lysine, and threonine. They also determined the molar ratio of the amino acids in the compounds as 5.0:2.6:1.8:1.0 and 4:2:1:1 respectively and calculated that on the basis of the weight of the starting material that the amino acids comprised 26% of the mass of Compound I and 43% of the mass of Pyoverdine. This led them to suppose that the compound contained more than one chromophore or one of very high molecular weight. A molecular weight of 2380 was given to Pyoverdine and a significantly higher weight was suggested for Compound I.

The compound reported here is of the same general type as those reported by Osawa and Hulcher, but does not appear to be exactly the same as any of them. The difference in the absorption and fluorescence spectra are shown in table 4. The table shows that this compound more closely resembles Osawa's Fluorescence I and Hulcher's Pyoverdine. There are, however, differences particularly in the peak at about 205nm, which is not present in Fluorescence I, and the absence of several peaks seen in Pyoverdine. Table 4 also shows the differences in the fluorescence spectra of the compounds.

Table IV

Absorbance maxima in nm

pH	Compound I[a]	Fluor-escence I*[b]	Fluor-escence II[c]	Pyoverdine[d]	C-substance[e]	Fluor-escent Compound
2	370-376 277	265		373, 360 308, 245 217		375, 250 210
7	401 278	400, 300 260, 220	360 250	405, 262 232, 203		412, 265 210
10		260 220 210			346, 256 210	410, 265 225
12	401 311 278	260 220		385, 312 212		420, 260 220

*Incomplete data given for 400 nm peak

a See reference 6
b See reference 12
c See reference 13
d See reference 6
e See reference 5

Again, there is a close similarity between this compound and Fluorescence I. Pyoverdine cannot be compared here due to lack of data.

The previously reported compounds also differ with regard to the changes in intensity and tone of the fluorescence with pH. This compound shows a very large change in intensity at about pH 5. Osawa's "C" substance shows a similar large change at pH 6 to pH 7, but the intensity of "C" substance remains high at higher pH's where the compound here drops in intensity to approximately the same level on either side of pH 5. Fluorescence I was reported to change in intensity about pH 5 but no figures were given. Compound I had an increase of intensity at a pH of 8 to 9, but again no figures on the relative amounts of change were given.

In this compound the four amino acids of Compound I and Pyoverdine were also found and in addition three other amino acids were found. The decomposition point of the compound was lower by 20° C (150° C versus 170° C) compared to Compound I. Chromatograms in the acid solvent gave R_f values of 0.48 for Compound I and 0.64 for this compound. The dialysis of the compound shows it to be a small compound which disagrees with Hulcher's suggestion of a large molecular weight. No data is available for Fluorescence I or II.

In conclusion, the compound reported here appears to be

a different fluorescent pigment than those compounds that have been previously reported. It appears to be most similar to Fluorescence I. It differs markedly from Fluorescence II, Compound I, "C" substance and Pyoverdine. Since only spectral data was given for Fluorescence I, it is somewhat difficult to say definitely that the compound isolated here is different, but there are several differences in the spectra which would indicate a different compound.

The function of the compound in the bacteria is still not known, but the fact that the fluorescence of the compound is quenched by iron and that the compound is only produced in iron-deficient media suggests that the compound is a precursor for an iron-containing compound. In iron-deficient media the pigment would not be used and might be released into the media. More would be produced in an attempt to synthesize the desired compound. The fact that the compounds produced show some biological activity (14) does not necessarily mean that the compound is a substitute for iron in the media.

Further work on the pigment is needed to determine its complete structure and its function in the Pseudomona. The most efficient means for future isolation of the compound would come from chromatographing the dialysate of the methanol-extracted agar plates. By this means several of

the fluorescent compounds could be isolated at the same time and the relationship between them can be studied. In addition, systems for chromatographing and eluting the complex should contain a minimum of acid and should be evaporated under very mild conditions. These precautions should be taken since the compound appears to be easily hydrolysed. Use of above method with the precautions should enable future workers to purify enough of the compound to crystalize it for more extensive analysis.

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