THE SPECTROFLUOROMETRIC DETERMINATION OF SOME MIXTURES OF ORGANIC COMPOUNDS

By

Glen A. Thornton

AN ABSTRACT

Submitted to the School of Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

A spectrofluorometer was constructed employing a Farrand UV monochromator for selecting the exciting radiation and a Beckman DU spectrophotometer with a photomultiplier attachment for measuring the fluorescence intensity. The excitation source was a Hanovia S-4 mercury lamp with the glass envelope removed.

By utilizing differences in fluorescence spectra under different conditions, methods for the quantitative analysis of the following mixtures of organic compounds were worked out.

1. Anthracene, Phenanthrene and Fluorene

Only anthracene fluoresces when a methyl alcohol solution of the hydrocarbons is exposed to 365 mu radiation and, therefore, may be determined directly in the presence of the other compounds.

Phenanthrene and fluorene are both excited by 265 mu radiation. The fluorescence spectrum of fluorene partially overlaps that of phenanthrene but at 316 mu it is free from interference and is, therefore, measured at this wavelength.

The phenanthrene fluorescence intensity is measured at 350 mu. At this wavelength the phenanthrene emission is free of fluorescence interference from anthracene but is subject to interference by the fluorescence spectrum of fluorene. Also the fluorescence at 316 mu and 350 mu is subject to absorption by anthracene and the 316 mu fluorene emission is subject to absorption by phenanthrene. Corrections are applied to the readings which are then used in a graphical method

of solution. The average error in this method is less than 5% over the concentration range 0 to 5.0 p.p.m.

2. Ortho, Meta and Para Hydroxybenzoic Acids

The ortho and meta isomers are fluorescent in aqueous solution, when excited by 314 mu radiation, but the para isomer is not. The fluorescence spectra of the ortho and meta isomers are of similar shape but that of the meta compound is slightly displaced towards longer wavelengths. At a pH of 5.5, only the ortho isomer fluoresces and therefore, it can be determined directly. In a solution having a pH of 12.0, the observed fluorescence is the sum of the fluorescence intensities of both acids. Simple subtraction of the contribution of the ortho isomer to the over-all fluorescence intensity of the pH = 12.0 solution, then yields a measure of the meta isomer.

The method is applicable over the concentration range 0 to 12 p.p.m., without interference from the para isomer, and yields results with an average error of less than 1% for both acids.

3. The Isomeric Aminobenzoic Acids

The ortho, meta and para aminobenzoic acids are all fluorescent under identical pH conditions, however, the fluorescence spectrum of the para isomer is isolated from those of the ortho and meta isomers. Consequently, mixtures of the ortho and para or the meta and para isomers are easily determined over the concentration range of 0 to 6 p.p.m. with an average error of less than 5%.

The fluorescence spectra of the ortho and meta isomers are similar in shape and their fluorescence maxima are only separated by 10 mu,

hence one must rely upon additivity of fluorescence intensities to resolve this pair. A method is presented for this resolution based upon the solution of simultaneous equations for fluorescence intensity measurements under two separate conditions of excitation. The results of this method are only accurate to ± 0.5 p.p.m.

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INTRODUCTION

Quantitative determination of a fluorescent species concentration is based upon the fact that in dilute solution the fluorescence intensity is directly proportional to the concentration, c, of the species present, that is:

$$1.) \quad I_f = Kc$$

It can be shown that the constant K is a function of the wavelength of the exciting radiation, the incident intensity of the exciting radiation, the length of light passage and the quantum efficiency of the absorbed radiation in producing fluorescent emission, that is:

$$2.) \quad K = QI_o\epsilon d$$

where;

Q = quantum efficiency = $\frac{\text{Intensity of radiation emitted}}{\text{Intensity of radiation absorbed}} = \frac{I_f}{I_a}$

I_o = intensity of exciting radiation

ϵ = molar absorbtivity

d = cuvette length

The validity of the above relationship can easily be shown in the following manner (10). If Beer's law is assumed to hold for the absorption process, the radiation absorbed by the solution is given by,

$$3.) \quad Ia = I_o (1 - e^{-\epsilon d c})$$

and the intensity of the fluorescent emission is then:

$$4.) \quad I_f = QIa = QI_o \ (1 - e^{-\epsilon d c})$$

For small values of c, equation 4 can be replaced by,

$$5.) \quad I_f = QI_o \epsilon d c = Kc$$

and the fluorescence intensity is shown to be a linear function of concentration, provided that the wavelength and intensity of the exciting radiation are maintained constant.

The extension of the fluorometric method of analysis to include binary mixtures of fluorescent species can be made in one of four ways, depending upon the fluorescence characteristics of the species in the mixture. Three of the four approaches to mixture resolution are similar in that they depend upon obtaining an independent measurement of the concentration of one or both of the species present. The four methods of resolution are listed below.

(1) If the fluorescence spectra of the two compounds are sufficiently separated, such that there are regions in each spectrum which do not overlap, independent measurements of the concentration of each species present can be made by obtaining fluorescence intensity measurements at wavelengths outside of the overlap area.

(2) In some cases the fluorescence spectra of the compounds can be selectively excited by a particular wavelength. Thus, by selecting the wavelength of excitation, it is possible to

obtain a fluorescence intensity measurement indicative of the concentration of one of the species without interference from the other. Then at another wavelength the spectrum of the other species or possibly those of both can be excited.

(3) The next method depends upon controlling the form of the species in solution. For example, if the compounds under consideration are acids the fluorescence spectra of the undissociated and dissociated forms will be different or, what is more likely, one form will exhibit a fluorescence spectrum and the other will not. Then if the two acids under consideration have sufficiently different dissociation constants it would be possible, by controlling the acidity of the solution, to maintain one in the dissociated form and the other in the associated form. This situation results in maximum separation of spectra, if both species are fluorescent, or it may cause one to be fluorescent and the other not.

(4) The fourth method of resolution depends upon obtaining additivity of fluorescence intensities in much the same manner as absorbances are found to be additive in adsorption spectroscopy. The principle of additivity must be applied when two compounds exhibit similar fluorescence spectra which can not be made mutually independent in one of the above ways. If this situation obtains, then the fluorescence intensity of the mixture, read at some wavelength, λ_R, and resulting from irradiation by a wavelength, λ_I, would be given by:

6.) I_f^T = Total fluorescence intensity = $K_A C_A + K_B C_B$

then remembering that $K = f(\lambda_I)$, another value of the total fluorescence intensity can be obtained by changing the wavelength of irradiation to $\lambda_{I'}$, then,

7.) $I_f^{T'} = K_A' C_A + K_B' C_B$

and simultaneous solution of the two equations 6 and 7 will yield the concentrations of the components A and B.

In the past very little attention has been devoted to mixture analysis by fluorometric procedures. The primary cause of the slow development of this technique of analysis has been that the basic instrumentation in this field has consisted of a filter fluorometer employing the 365 mu mercury line for fluorescence excitation. Thus, with this type of instrument, the analyst has been limited to those compounds whose fluorescence spectra can be excited by 365 mu irradiation and has, therefore, been confined to a study of compounds showing visible fluorescence. Also, the use of filters to isolate the fluorescent emission makes only minimal use of the differences in fluorescence spectra as a technique in resolving mixtures.

In recent years an increasing awareness of the importance of fluorescence spectra as a tool for structure studies, qualitative identification and quantitative application has become apparent. This awareness is evidenced by the advances made in spectrofluorometric instrumentation. A number of authors (3,9,10,11) have constructed instruments

employing the monochromator of the Beckman DU spectrophotometer to resolve fluorescent emission and thus obtain fluorescence spectra. They have, however, maintained the use of a filtered light source. Collat and Rogers (4) have recently constructed what might be characterized as a complete spectrofluorometer by making use of two Beckman DU monochromators, one to obtain monochromatic radiation for excitation of the sample and one to analyze the fluorescent emission.

Employing the instrument described above, Rogers and co-workers have been successful in analyzing mixtures of aluminum and gallium oxinates (4) and thiamine and riboflavin (12) fluorometrically. Unfortunately, however, they were unable to obtain a clear cut resolution of the mixtures and were forced to resort to a rather complex method of calibration in order to carry out the determinations.

The object of the work to be discussed herein, was to ascertain whether or not the principles of spectrofluorometry, as outlined above, could be practically and simply applied to the determination of mixtures of organic compounds, and in particular to isomeric mixtures which are exceedingly difficult to analyze by conventional methods.

INSTRUMENTATION

INSTRUMENTATION

The spectrofluorometer assembled for use in this work is shown schematically in Figure 1. The component parts used in the assembly of this instrument are:

A.) Commercially available parts:

1.) A Beckman DU spectrophotometer with a photomultiplier attachment.

2.) A Farrand grating type monochromator covering the range 220-440 mu.

3.) A fused silica lens.

4.) A Hanovia S-4 mercury arc equipped with a 115 volt constant voltage transformer.

B.) Constructed Parts:

1.) Light channel.

2.) Cell compartment.

3.) Lens mount.

4.) Arc housing.

The light channel and the arc housing were constructed of galvanized sheet metal while the lens mount and cell compartment were machined from aluminum stock. Figure 2 shows sketches and dimensions of these parts.

The S-4 mercury arc served as the source for excitation in all the experimental work that will follow. However, the effectiveness of a Xenon arc, supplied by the Hanovia Company, was also tested. This arc gives continuous radiation from 220 mu into the near infrared.

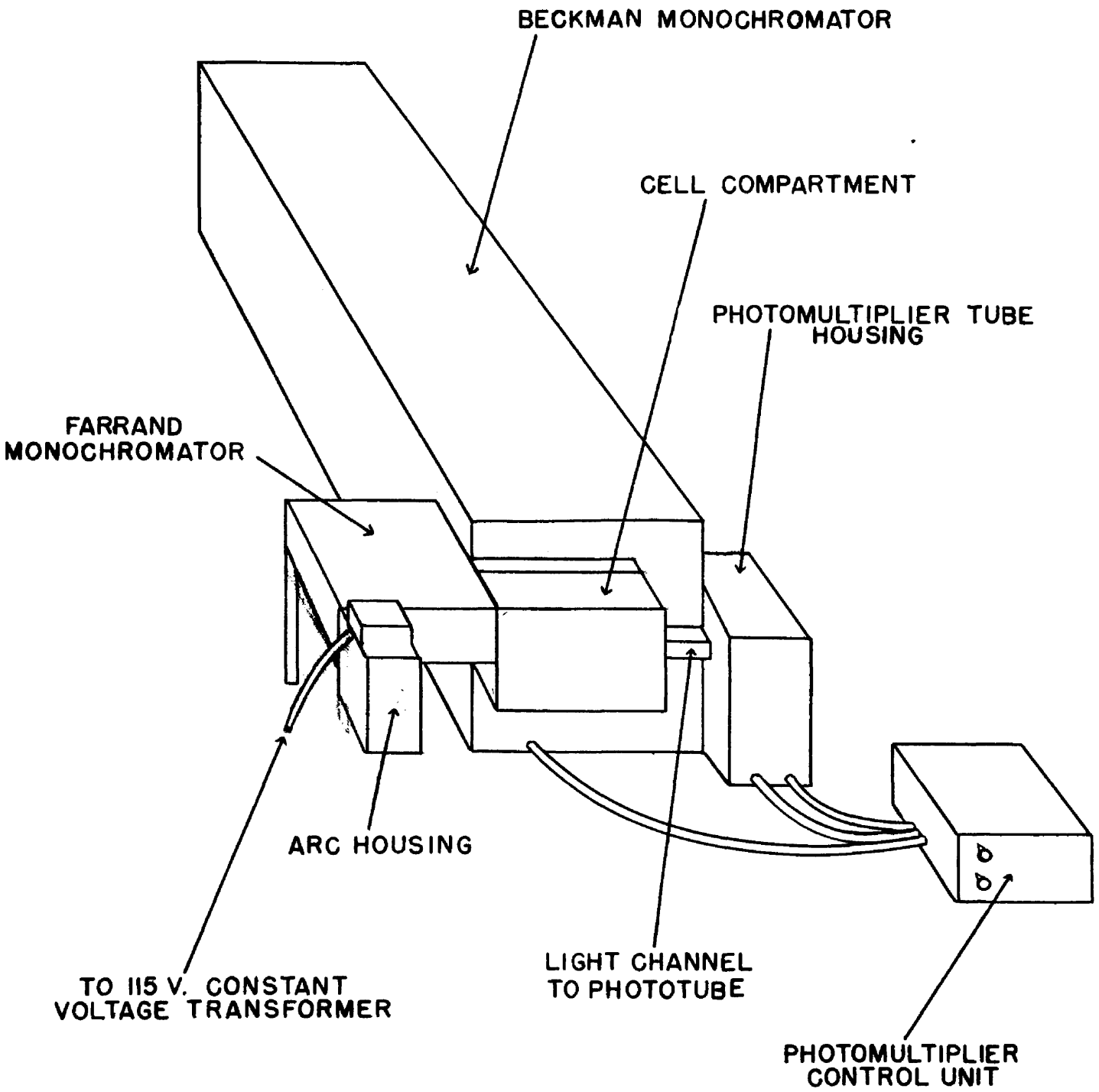


FIGURE I. BLOCK DIAGRAM OF SPECTROFLUOROMETER

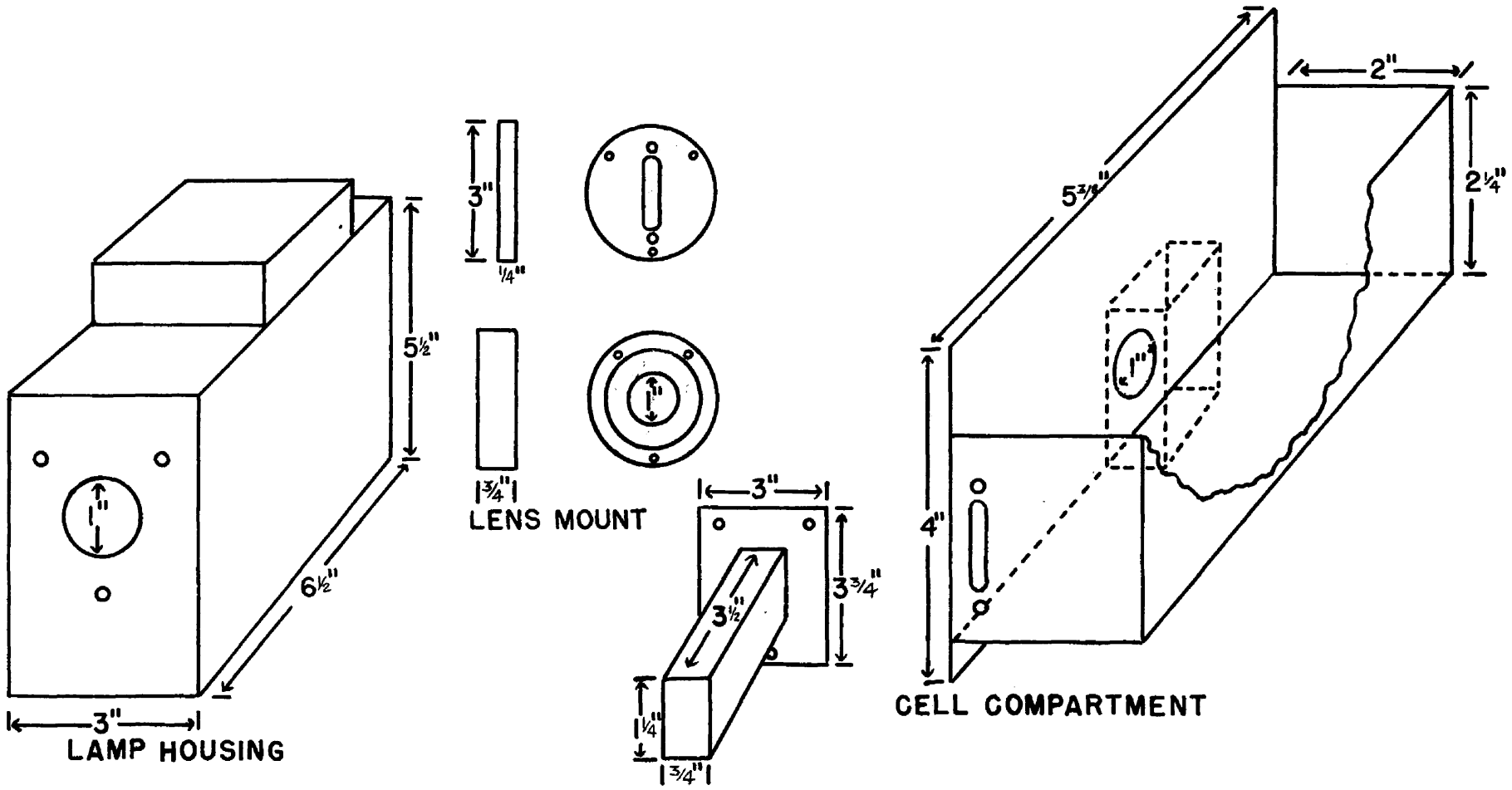


FIGURE 2. CONSTRUCTED PARTS

Unfortunately though, the intensity level of the arc, at wavelengths less than 300 mu, was too low to make the arc practical for ultraviolet fluorescence excitation. At wavelengths above 300 mu, however, the intensity of the arc increases rapidly and it was effective in producing visible fluorescence from fluorescent compounds which absorbed above 300 mu. The S-4 mercury arc, however, has spectral lines both above and below 300 mu which are sufficiently intense for fluorescence excitation. Consequently, it was selected as the excitation source. The mercury arc, as it comes from the manufacturer, is enclosed in a glass envelope. Since glass is opaque to wavelengths less than 316 millimicrons, the glass envelope was removed in order to make the short wavelength output of the arc available.

The arc housing is connected through the lens mount to the entrance slit of the Farrand monochromator and the exit slit of the monochromator is coupled to the cell compartment. The cell compartment is attached to the face plate of a Beckman monochromator from which the normal cell compartment and phototube housing have been removed. The compartment is constructed in such a manner that the cell holder maintains the cell in a position in line with the normal exit slit of the Beckman monochromator. The light channel, which is connected to the photomultiplier tube housing, is brought to bear, in a light tight seal, against the side of the Beckman faceplate and surrounds the normal light entrance aperture. Thus, fluorescent emission from the solution in the cuvette traverses the Beckman monochromator in the reverse direction of that encountered when it is in normal use. The light paths of the exciting

radiation and the fluorescent emission are shown schematically in Figure 3.

All parts constructed for use in the spectrofluorometer assembly were machined in such a manner that coupling of the parts to the Beckman, the Farrand monochromator and the photomultiplier tube housing could be made to screwtaps already present. Therefore, the spectrofluorometer can be readily dismantled and the Beckman instrument converted for absorption work in a matter of a few minutes.

For practical use there are seven mercury lines which are sufficiently intense for excitation use. These are; the 436, 405, 365, 314, 303, 265 and 254 mu lines. Experimentation has shown that the use of a 1.0 mm entrance slit and a 0.5 mm exit slit on the Farrand monochromator permits resolution of the 314 and 303 mu lines and passes a sufficiently high intensity for efficient excitation. Consequently, this combination of slit widths was selected for use with the source monochromator.

The cuvettes used with the instrument must be of fused silica or quarts if it is desired to work with compounds which are excited by wavelengths less than 316 mu. Clear window silica cuvettes, 10 x 20 x 50 mms., were obtained from the Farrand Optical Company and proved highly satisfactory for fluorescence work in the ultraviolet and visible spectral regions.

Regular Beckman absorption cells, composed of fused silica, were also used with the instrument. Fluorescent solutions in these cells were irradiated through one of the clear window faces and the fluorescent emission was then measured from one of the frosted window faces.

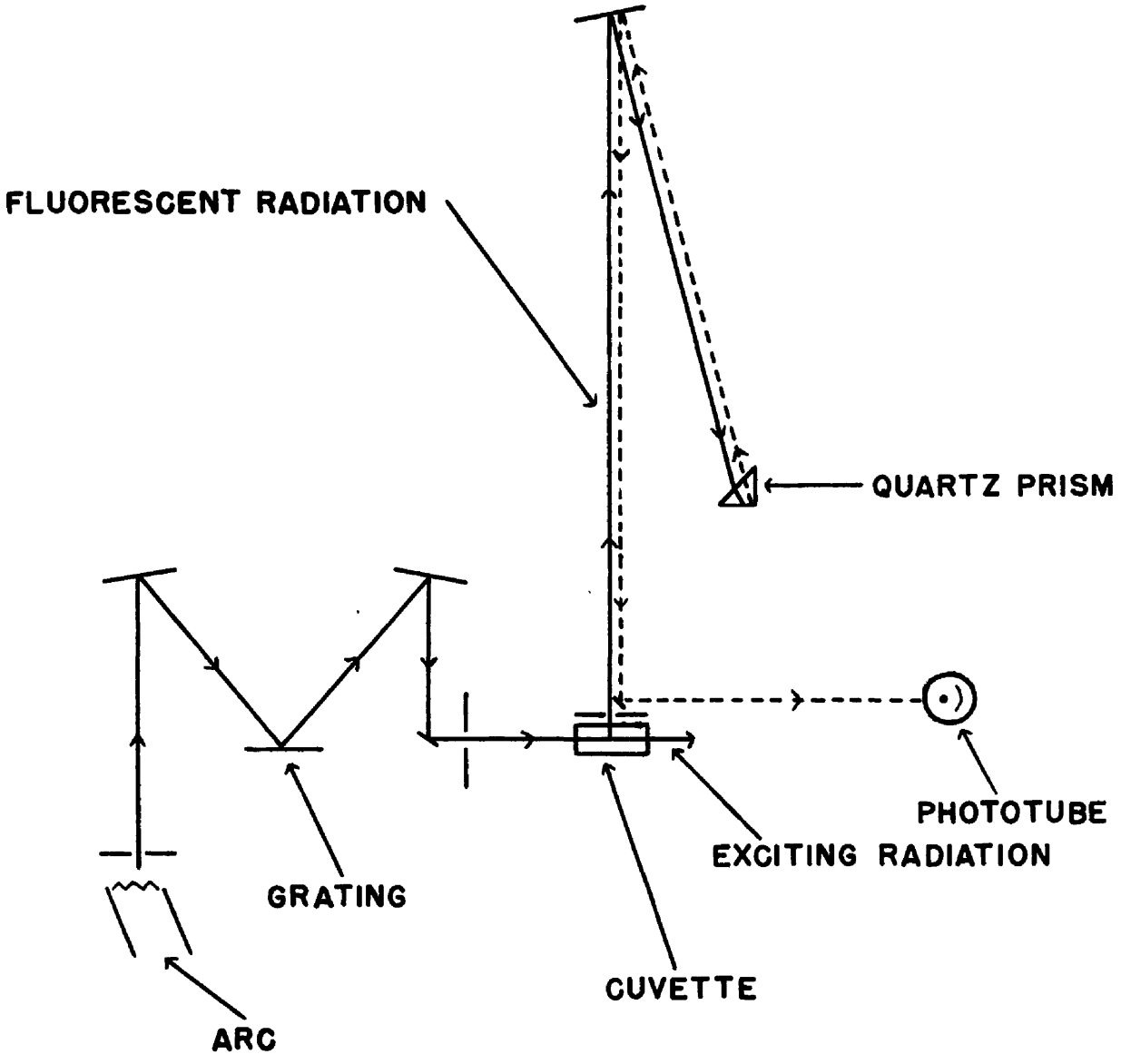


FIGURE 3. RADIATION PATHS IN SPECTROFLUOROMETER

Readings obtained with the Beckman cells and the Farrand cells were quite comparable except with short wavelength emission, where the clear Farrand cells were much more efficient in passing the fluorescent emission. This effect is particularly apparent in the case of the hydrocarbon fluorene. With this compound, the use of the clear cells gives a fluorescence intensity reading at 316 mμ which is approximately twice that of the reading obtained with the frosted Beckman cells.

The method of obtaining fluorescence intensity measurements with the spectrofluorometer is as follows:

1.) The mercury lamp and the Beckman instrument should be allowed to warm up for fifteen to twenty minutes before taking measurements, to insure maximum stability.

2.) The Beckman instrument is set for maximum sensitivity, that is, the sensitivity control knob is turned to its counter clockwise limit.

3.) The Beckman instrument is zeroed with the dark current control and the instrument is calibrated with a standard solution. This standard solution is preferably prepared from one of the compounds being studied, if it is sufficiently stable. In the calibration procedure the cuvette, containing the standard solution, is placed in position in the cell holder and the excitation wavelength is selected on the source monochromator. The Beckman is then set to give the selected standard reading and the monochromator is adjusted to the wavelength at which the emission is to be read. This done, the phototube

shutter is opened and the Beckman slit width is adjusted to obtain balance.

4.) Finally, the unknown solution is placed in the cuvette, the excitation wavelength and the wavelength at which the emission is to be read are selected and the fluorescence intensity reading is taken.

EXPERIMENTAL

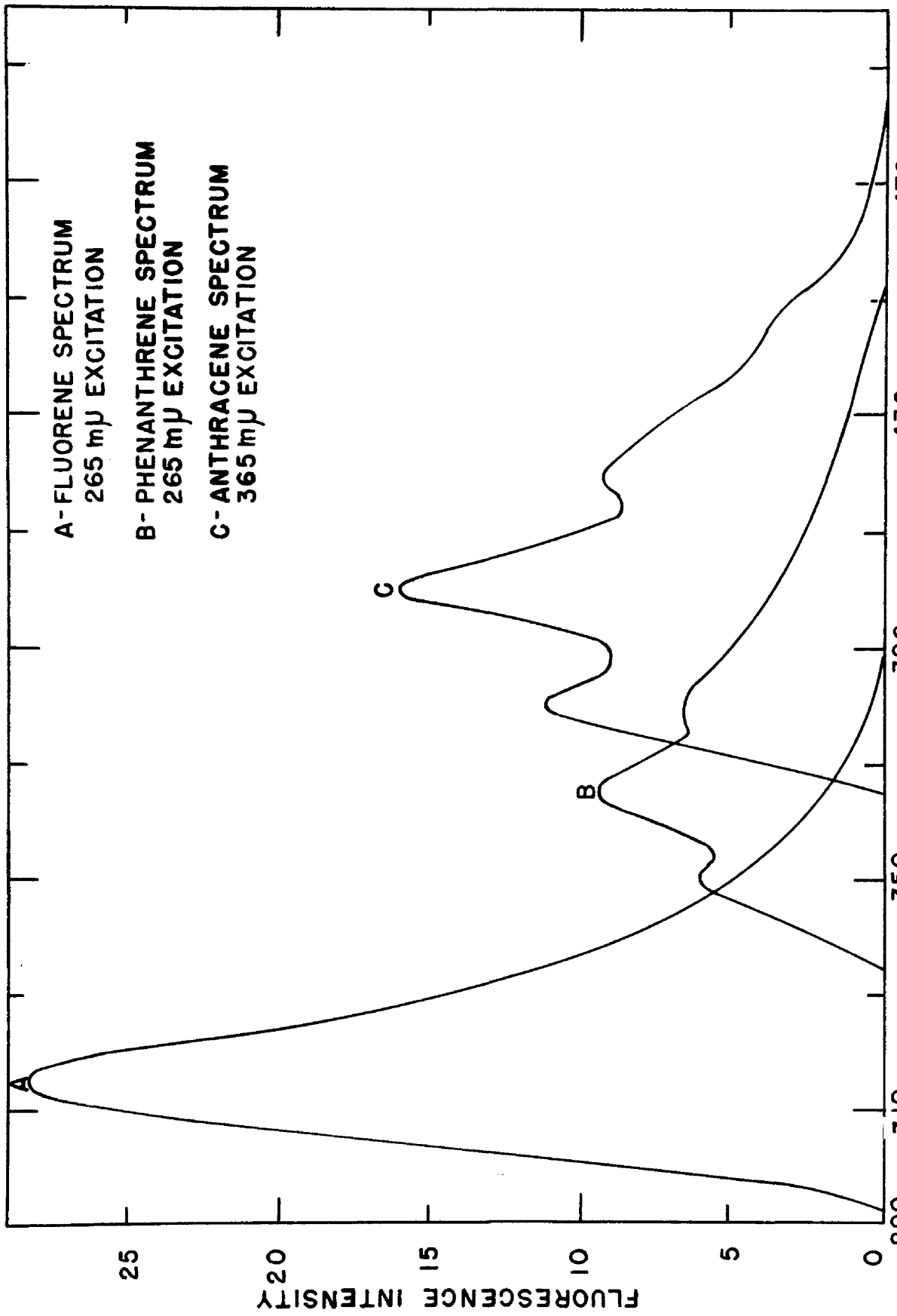
EXPERIMENTAL

A. Hydrocarbon Mixtures

1. Introduction

The compounds chosen for the initial mixture study were the coal tar hydrocarbons; anthracene, fluorene and phenanthrene. This system was chosen because it appeared to be a relatively simple mixture to attack and seemed to be an ideal pilot mixture.

The fluorescence spectra of these compounds, in absolute methyl alcohol, are shown in Graph 1. The spectra show fair separation, but also show overlap regions and selectivity of response to the wavelength of the exciting radiation. This mixture is, therefore, one which permits practical testing of three of the methods of approach to mixture analysis outlined in the introduction of this dissertation. That is; (1) the isolated 316 mu fluorene peak can be employed as a direct measure of the fluorene concentration in mixtures. (2) A fluorescene intensity measurement at 350 mu can be tested for additivity of fluorene and phenanthrene emission intensities. (3) Activation with 365 mu radiation can be employed to excite the emission spectrum of anthracene but not those of fluorene and phenanthrene and thus gives a direct measure of the anthracene concentration. Activation with radiation of 265 mu wavelength causes excitation of the spectra of all three compounds. The anthracene spectrum excited by 265 mu radiation is identical in form with that excited by 365 mu radiation, the only difference being one of intensity. The spectrum excited by 265 mu irradiation is



GRAPH I. FLUORESCENCE SPECTRA OF HYDROCARBONS

considerably more intense, as would be expected, since the anthracene absorption at 265 mu is greater than at 365 mu.

The qualitative data indicate that it should be possible to resolve mixtures of these compounds in the following manner:

1.) Anthracene-Phenanthrene mixture:

Activate a methyl alcohol solution of the hydrocarbons with 365 mu radiation and take fluorescence intensity readings at 400 and 420 mu. These wavelengths correspond to two peaks in the anthracene fluorescence spectrum and give two direct checks on the anthracene concentration. The anthracene concentration reported would then be the average concentration obtained from these two readings. Then activation with 265 mu radiation and a fluorescenne intensity measurement at 350 mu should give a reading indicative of the phenanthrene concentration.

2.) Anthracene-Fluorene Mixture:

Determine the anthracene concentration by excitation with 365 mu radiation and fluorescence intensity measurements at 400 and 420 mu. The fluorene concentration would then be determined by excitation with 265 mu radiation and a fluorescence intensity measurement at 316 mu.

3.) Fluorene-Phenanthrene Mixture:

Activate with 265 mu radiation and obtain the fluorene concentration directly from a fluorescence intensity measurement at 316 mu. Another fluorescence intensity measurement

is then made at 350 mu. This reading should be the sum of the 350 mu emission of the fluorene present and the 350 mu emission of the phenanthrene present. Since the concentration of fluorene is known from the 316 mu reading, its contribution to the 350 mu emission can be calculated and subtracted from the 350 mu reading. The difference between the two readings obtained should be indicative of the phenanthrene concentration.

4.) Anthracene-Fluorene-Phenanthrene Mixture:

The anthracene concentration would be determined directly from readings at 400 and 420 mu under 365 mu irradiation. The concentrations of fluorene and phenanthrene would be determined as described in (3) above.

The experimental work described below was conducted in an effort to apply the above generalizations to the development of an analytical procedure for the analysis of these hydrocarbon mixtures.

2. Experimental

The anthracene and phenanthrene used in this study were purified by azeotropic distillation, under reduced pressure, with ethylene glycol and diethylene glycol according to the procedure of Feldman et al. (5). The fluorene used was purified by repeated vacuum sublimation. The starting materials in all of the purification procedures were Eastman White Label Chemicals. The purification procedures were repeated until melting points, absorption spectra and fluorescence spectra of the compounds indicated a high grade of purity. Baker's analyzed absolute

methanol was used as the solvent throughout the work with the hydrocarbons.

Stock solutions of the three purified hydrocarbons were prepared which contained 0.5 milligram of hydrocarbon per milliliter of solution. All subsequent solutions prepared and used in the remainder of this work were prepared by direct dilution of the aforementioned stock solutions.

The concentration range, over which the fluorescence intensity remained directly proportional to the concentration of hydrocarbon present, was determined in the following manner. Solutions containing known varying concentrations of the hydrocarbons were prepared and their fluorescence intensities measured. These solutions were prepared by diluting aliquot portions of the stock solutions with methyl alcohol. The range over which linearity was observed for the three hydrocarbons, was found to be from 0 to 5 parts per million, or what is equivalent, 0 to 5 micrograms per milliliter. That is, plots of fluorescence intensities versus concentrations for anthracene activated with 365 mu radiation and fluorescence intensity read at 400 and 420 mu, phenanthrene activated with 265 mu radiation and read at 350 mu and fluorene activated with 265 mu radiation and read at 316 and 350 mu, displayed linearity over a concentration range of 0 to 5 parts per million.

The ranges having been thus determined, calibration curves, that is, plots of fluorescence intensity versus concentration, were prepared for the three hydrocarbons.

In obtaining the readings for the calibration curves the instrument was calibrated by assigning a fluorescence intensity reading of 17.0 to a solution containing 3.50 parts per million of anthracene when the fluorescence was activated by 365 mu radiation and the intensity was read at 400 mu. This reading was selected for the standard because it permitted the use of an approximately 1.0 mm. slit width on the Beckman monochromator. This slit width has previously been designated (4,12) as an optimum for spectrofluorometric work and it was, therefore, considered desirable to take readings at slit widths as close to 1.0 mm. as possible.

The methanol solution of anthracene was chosen as the calibration standard, rather than some common secondary standard such as quinine sulfate, because of the well known oxygen quenching of the fluorescence of polynuclear hydrocarbons (1,13,14,15). Oxygen has an appreciable solubility in methanol, 0.175 cc/ml of methyl alcohol at 18°C. Therefore, the concentration of oxygen present in the hydrocarbon solutions must be compensated for in some fashion if it is not constant from day to day. Since the solubilities of gases in liquids are quite highly temperature dependent, the concentration of oxygen present could not be expected to remain constant except under constant temperature conditions. Consequently, there were three possible ways to control the oxygen quenching; (1) Remove the oxygen by flushing with nitrogen and maintain a nitrogen atmosphere during the time required to take readings. (2) Thermostat the cell compartment and thus maintain a constant temperature during the time of measurement. (3) Employ a

standard which is also subject to fluctuations in oxygen concentration and thus compensate for any day to day change in oxygen concentration. The third method mentioned presented the least number of difficulties and was, therefore, selected as the method of controlling the degree of oxygen quenching.

The data shown in Table I are that taken for the preparation of calibration curves. The standard solutions, used in obtaining this data, were prepared by methyl alcohol dilution of aliquots of the stock solutions. The standards were prepared and measured on two different days to obtain a check on the reproducibility of the readings. The average values obtained from these measurements were then used for the preparation of the calibration curves shown in Graphs 2, 3 and 4.

With the calibration curve data now available, a group of synthetic mixtures of all four types, three binary and one ternary, were prepared. When these mixtures were analyzed by the procedure outlined above, it was found that the phenanthrene and fluorene results were always low, at times by as much as 25-50%, while the anthracene results checked out with the theoretical within 2-4%. The magnitude of the negative deviations in the fluorene concentrations when in a mixture with anthracene showed a dependence on the anthracene concentration. That is, the negative deviation increases with increasing anthracene concentration. The same phenomenon is observed with the deviation of phenanthrene concentration from the theoretical when it is in a mixture with anthracene. With phenanthrene-fluorene mixtures an analogous behavior is noted for the 316 mu fluorene emission, that is, it decreases with

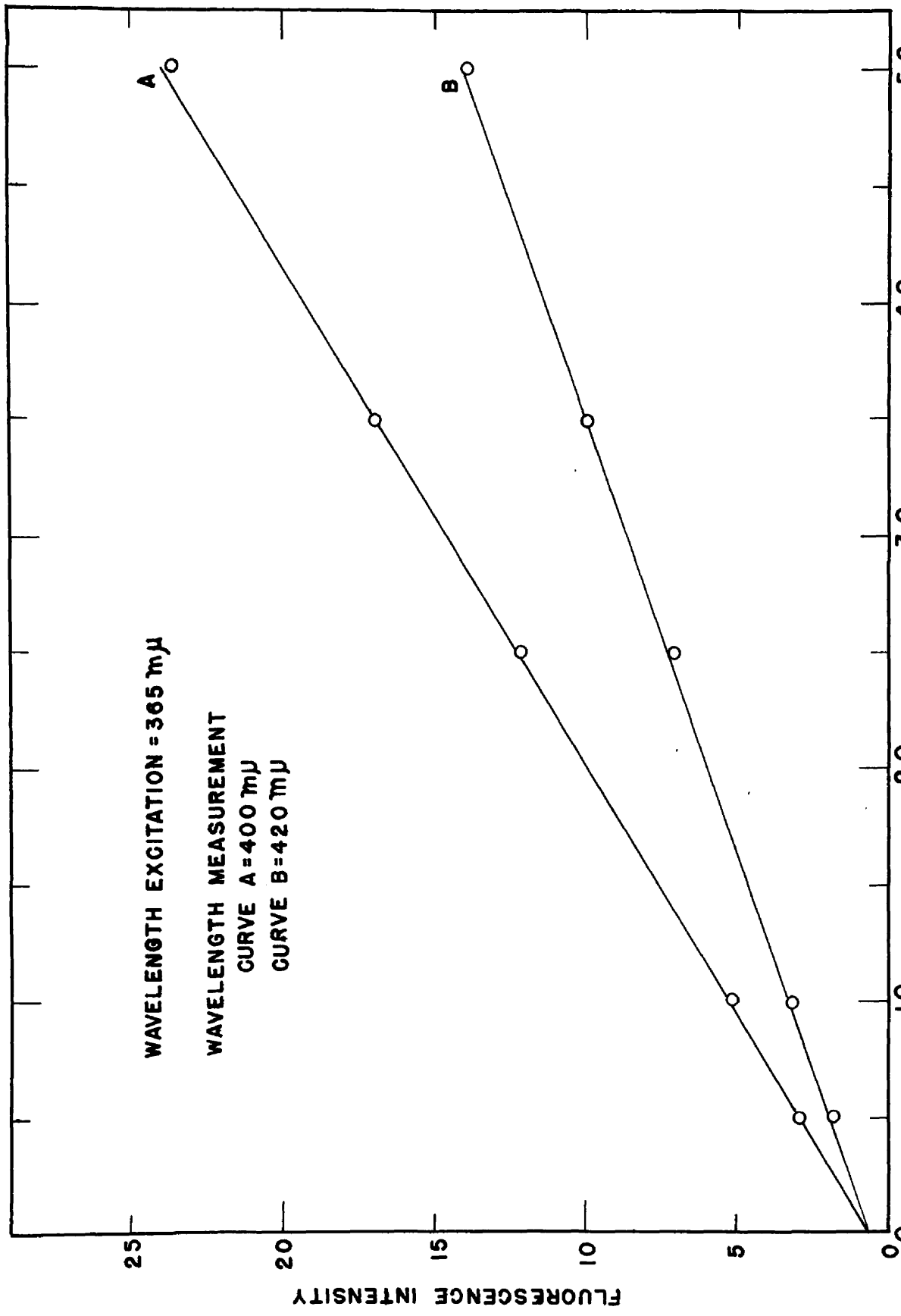
TABLE I

HYDROCARBON CALIBRATION CURVE DATA

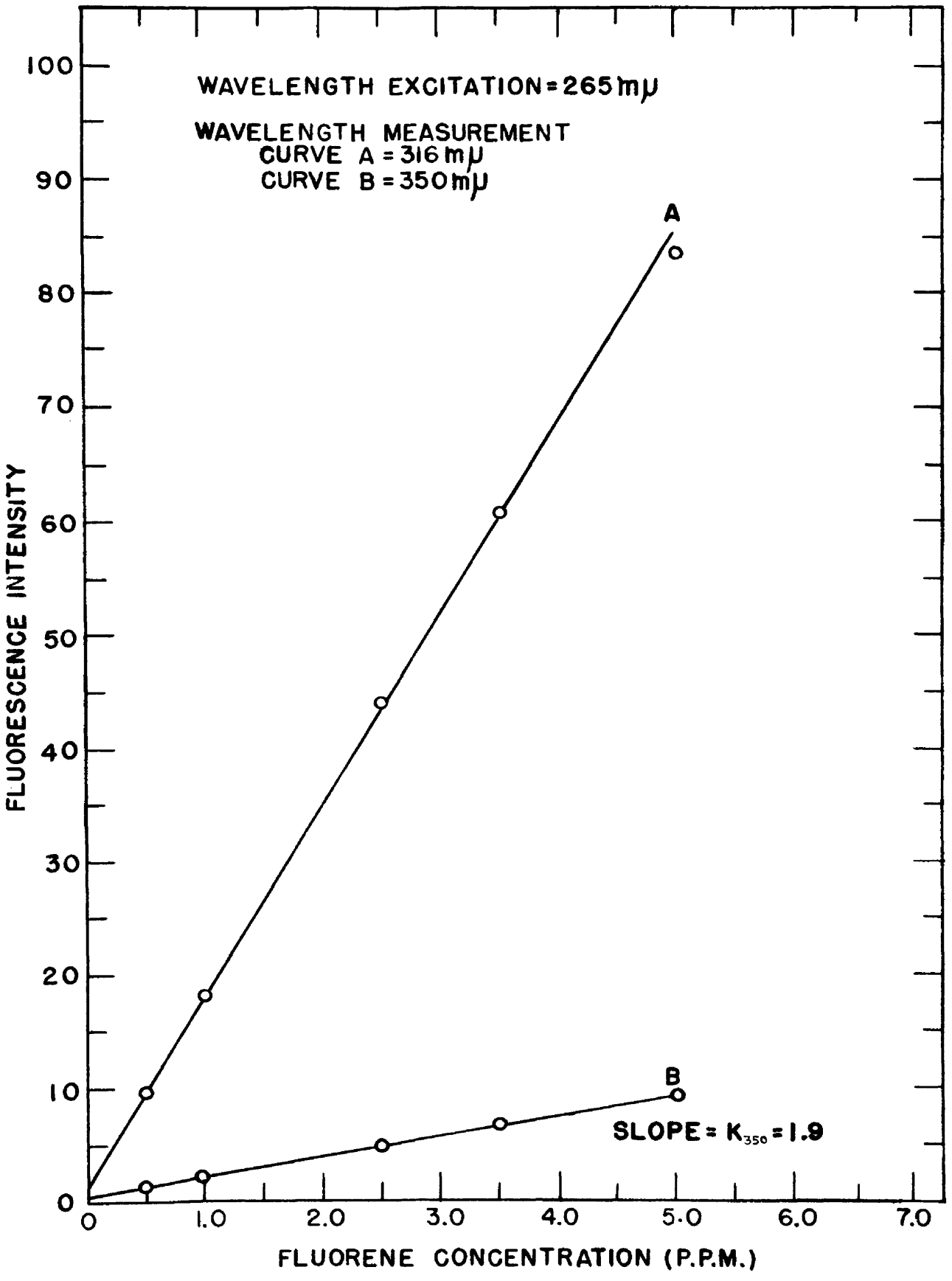
Anthracene (365 mu irradiation)						
Anthracene Concentration in p.p.m.	Wavelength of Measurement					
	400 mu			420 mu		
	1	2	Ave.	1	2	Ave.
0.5	2.88	2.90	2.89	2.00	1.80	1.90
1.0	5.00	5.20	5.10	3.10	3.10	3.10
2.5	12.1	12.1	12.1	7.00	7.20	7.10
3.5	17.0	17.0	17.0	10.0	10.0	10.0
5.0	23.8	23.6	23.7	13.0	14.0	13.9

Phenanthrene (265 mu irradiation)			
Phenanthrene Concentration in p.p.m.	Wavelength of Measurement		
	350 mu		
	1	2	Ave.
0.5	1.20	1.20	1.20
1.0	2.10	2.16	2.13
2.5	4.40	4.60	4.50
3.5	6.00	6.30	6.15
5.0	8.20	8.60	8.40

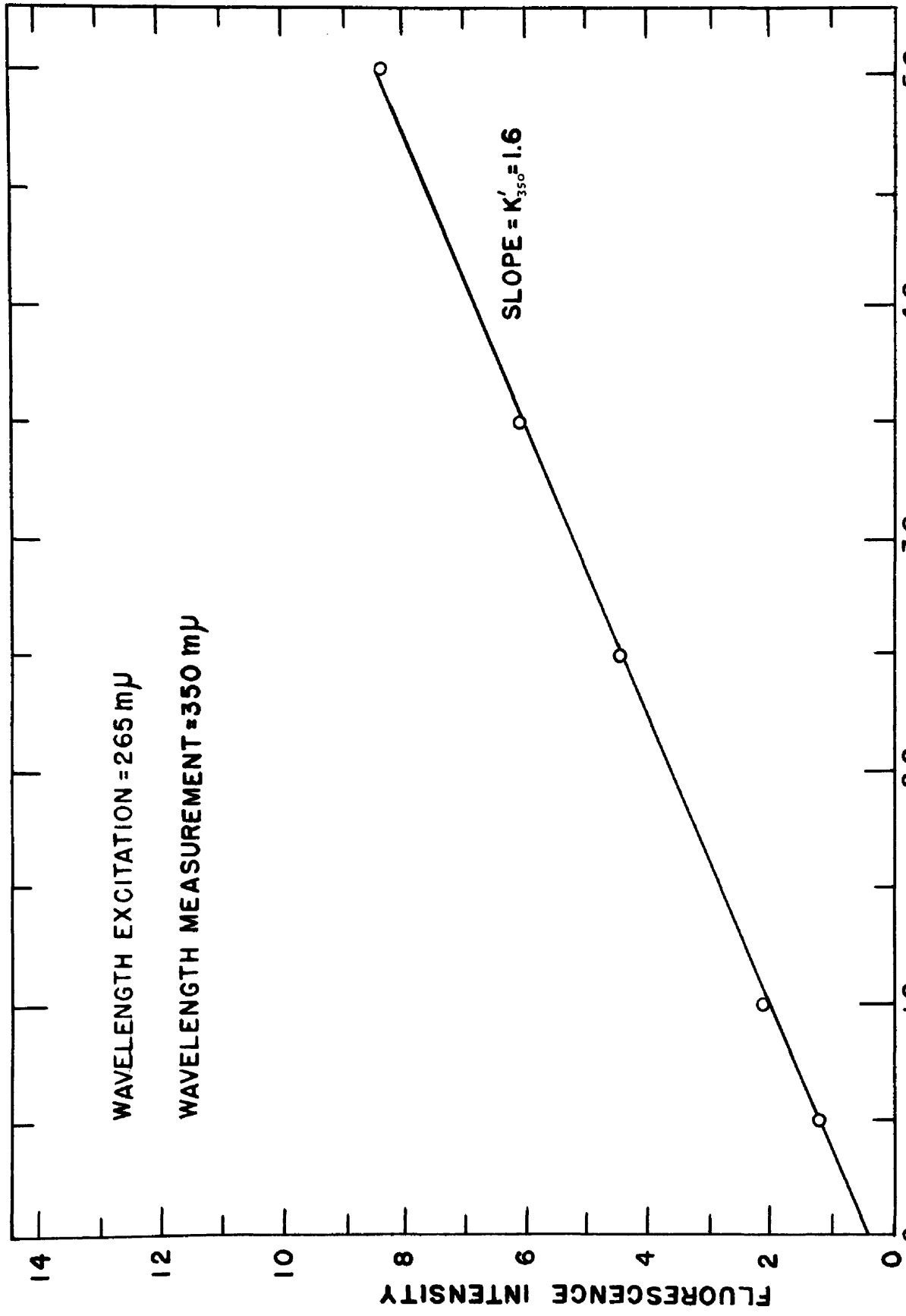
Fluorene (265 mu irradiation)						
Fluorene Concentration in p.p.m.	Wavelength of Measurement					
	316 mu			350 mu		
	1	2	Ave.	1	2	Ave.
0.5	9.60	9.70	9.65	1.20	1.30	1.25
1.0	18.3	18.3	18.3	2.20	2.20	2.20
2.5	44.0	44.5	44.3	4.90	5.10	5.00
3.5	60.6	60.7	60.7	6.70	6.70	6.70
5.0	83.0	84.0	83.5	9.00	9.30	9.15



GRAPH 2. ANTHRACENE CALIBRATION CURVES



GRAPH 3. FLUORENE CALIBRATION CURVES



GRAPH 4. PHENANTHRENE CALIBRATION CURVE

increasing phenanthrene concentration. These deviations become more intelligible when the absorption spectra of the three hydrocarbons are considered. Graphs 5, 6 and 7 show the experimentally determined absorption spectra of anthracene, phenanthrene and fluorene, in methyl alcohol solution, which duplicate those of Friedel and Orchin (8). On comparison of the absorption spectrum of anthracene with the fluorescence spectrum of fluorene, overlap areas are observed and it is seen that the fluorene emission at 316 mu and 350 mu will be subject to anthracene absorption when it is a component of the mixture. Similarly, the absorption spectrum of phenanthrene overlaps the fluorene fluorescence spectrum and phenanthrene would, therefore, be expected to partially absorb the fluorene 316 mu emission when they are simultaneously present in a mixture. Then, for example, when the fluorene emission is read at 316 mu in the presence of anthracene, the apparent fluorescence intensity, I_f^a, read is not that which would be read for an equivalent amount of fluorene present in the solutinn alone, I_f^o, but is rather;

$$I_f^a = I_f^o - \text{intensity absorbed by anthracene}$$

or

$$I_f^a = I_f^o - I_f^o (1-10^{-K_A C_A}) = I_f^o 10^{-K_A C_A}$$

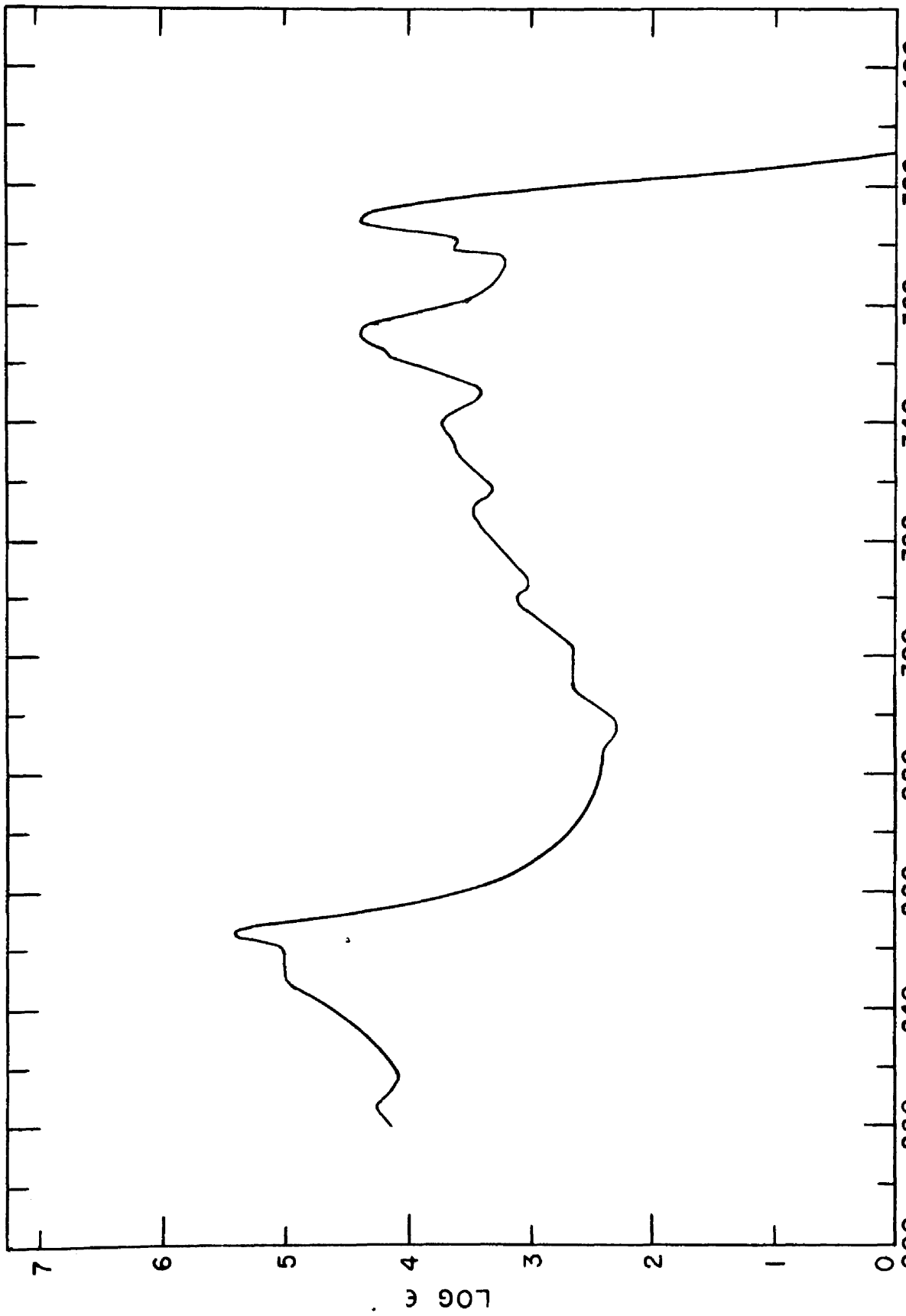
which on rearranging becomes

$$\log I_f^o - \log I_f^a = K_A C_A$$

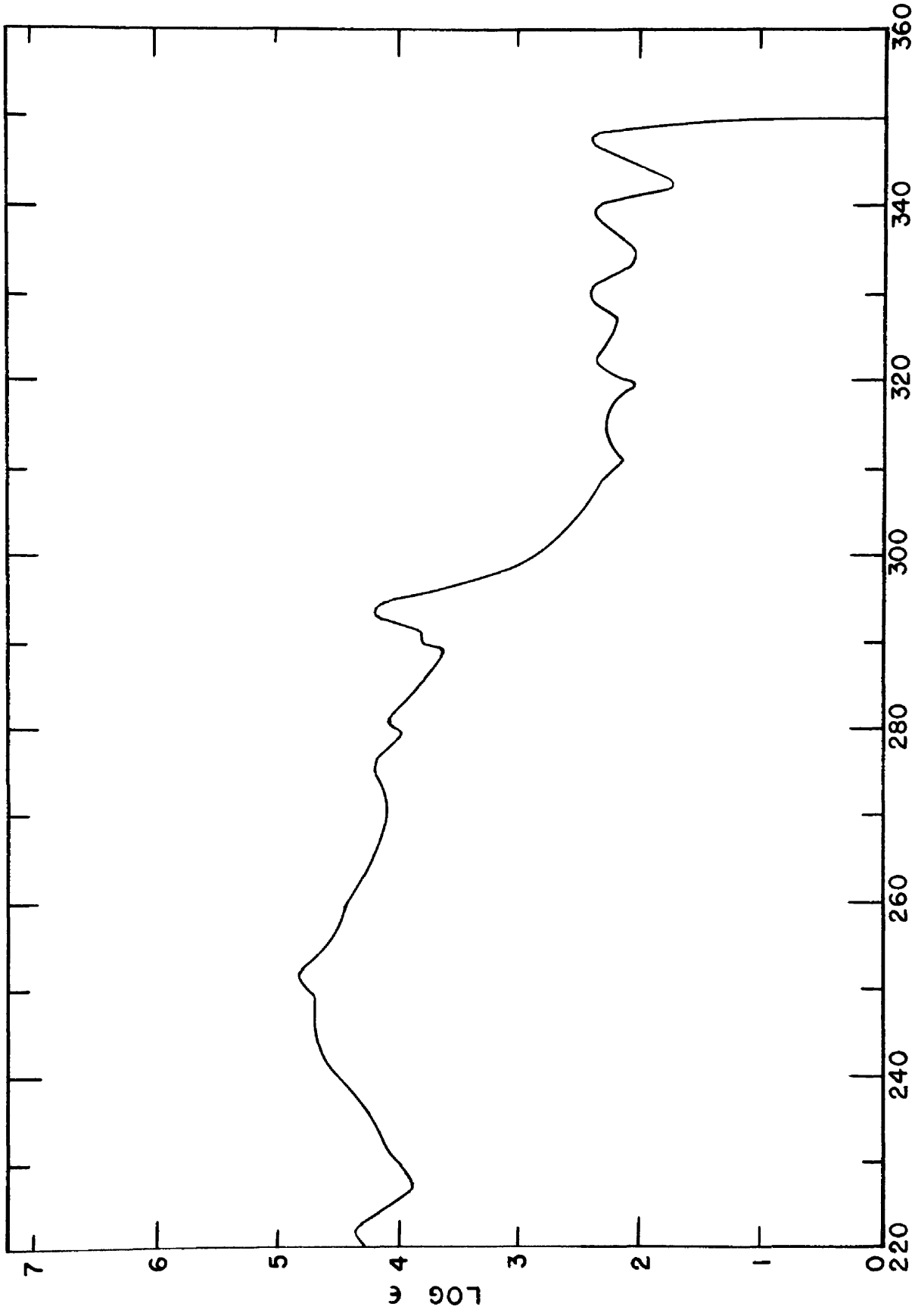
where $K_A = \epsilon d$

ϵ = molar absorbtivity of anthracene at 316 mu

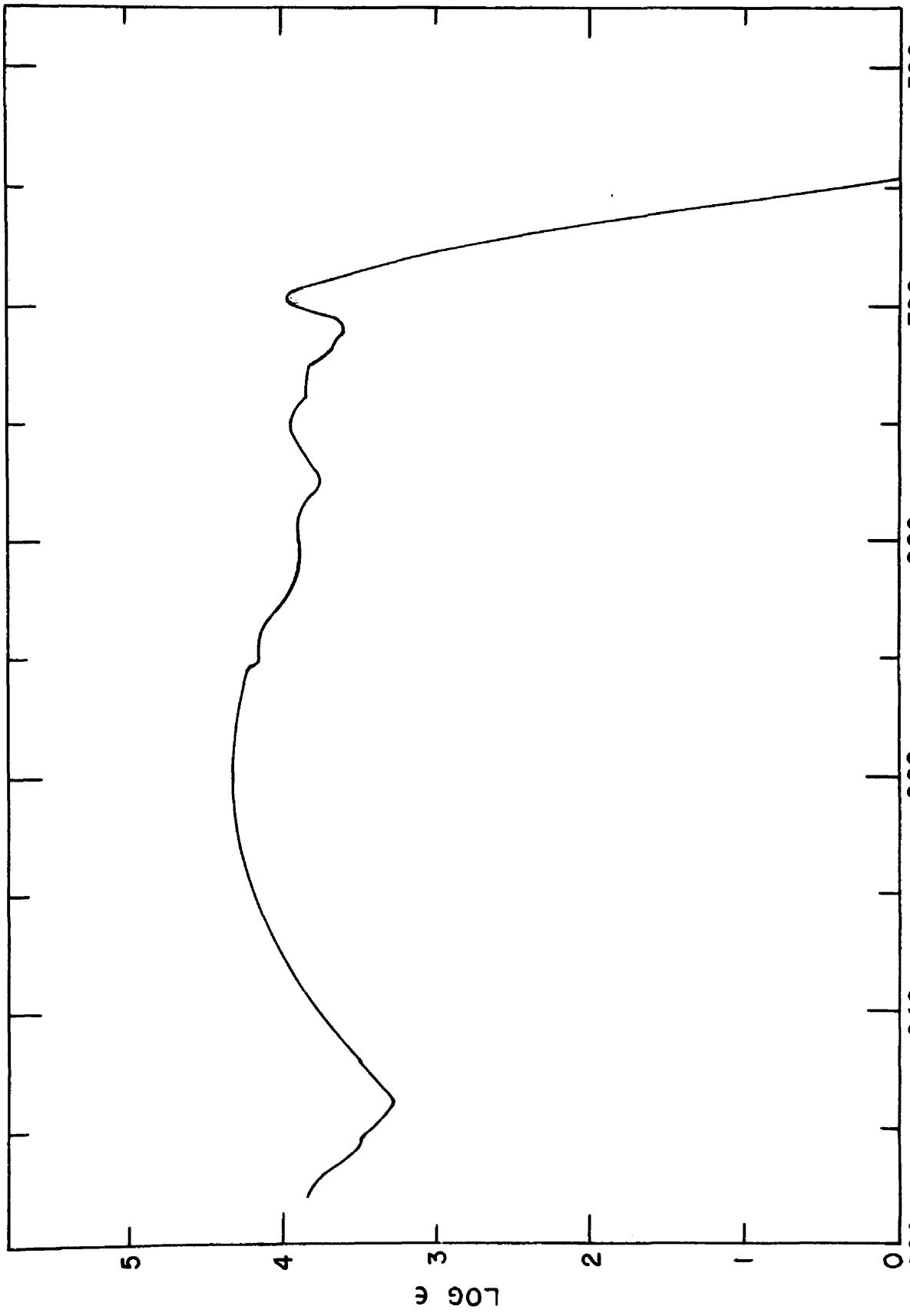
d = apparent "cell length" through which the fluorene 316 mu emission must pass.



GRAPH 5. ANTHRACENE ABSORPTION SPECTRUM



GRAPH 6. PHENANTHRENE ABSORPTION SPECTRUM



GRAPH 7. FLUORENE ABSORPTION SPECTRUM

Then if to some particular concentration of fluorene, varying concentrations of anthracene are added and apparent fluorescence intensities, I_f^a, are taken, a plot of log I_f^o - log I_f^a versus concentration of anthracene should be approximately linear. This procedure was carried out experimentally in the following manner. Five solutions containing 0.5, 1.0, 2.5, 3.5 and 5.0 parts per million of fluorene were prepared and then to each one 1.0 p.p.m. of anthracene was added. These solutions were activated with 265 mu radiation and their fluorescence intensities, I_f^a, at 316 mu were measured. From the calibration curve for pure fluorene solutions, Graph 3, the corresponding values of I_f^o for 316 mu emission are obtained and the values of $K_A C_A$ or log I_f^o - log I_f^a are calculated. Since in all of these solutions the concentration of anthracene is the same, the value of log I_f^o - log I_f^a obtained for all the solutions should, theoretically, be constant. The values obtained for log I_f^o - log I_f^a for the five solutions are not absolutely constant, because the precision of the measurement procedure is not absolute. However, if the high and low value of log I_f^o - log I_f^a are eliminated and the middle three values are averaged, a fairly reproducible value of $K_A C_A$ is obtained for a concentration of 1.0 p.p.m. of anthracene. The above procedure was repeated three times using 2.5, 3.5, and 5.0 p.p.m. of anthracene as contaminant. Average values of log I_f^o - log I_f^a for four concentrations of anthracene were thus obtained. Fluorescence intensity readings were also taken at 350 mu on these solutions so that the effect of anthracene absorption on 350 mu emission was determined at the same time.

This process was repeated with mixtures of fluorene and phenanthrene to determine the effect of phenanthrene absorption on the fluorene 316 mu emission. The average results obtained by this method are tabulated in Table II.

The results, shown in Table II, plotted as ($\log I_f^o - \log I_f^a$) versus concentration are shown in Graphs 8, 9 and 10. The plots are not completely linear as predicted by theory but taper off and show curvature at higher concentrations of absorbing material. Although the plots do not show complete adherence to theory, the anthracene absorption plots can still be used as semi-empirical correction curves. As shown before the concentration of anthracene can be determined independently by activation with 365 mu radiation and is not in any way dependent upon the concentration of phenanthrene or fluorene present. Therefore, in anthracene-phenanthrene or anthracene-fluorene mixtures, the phenanthrene 350 mu emission or the fluorene 316 mu emission can easily be corrected for anthracene absorption.

$$\log I_f^o = \log I_f^a + \Delta$$

where, $\Delta = \log I_f^o - \log I_f^a$, and is the correction term found from the absorption plots for the concentration of anthracene present. The value of I_f^o, obtained in this way, is then used to obtain the concentration of phenanthrene or fluorene, whichever is present, from the pure hydrocarbon calibration curves.

When mixtures of phenanthrene and fluorene are to be analyzed, the situation is complicated by the fact that no independent method is

TABLE II

ABSORPTION CORRECTION DATA

Anthracene Absorption of 316 mμ Emission

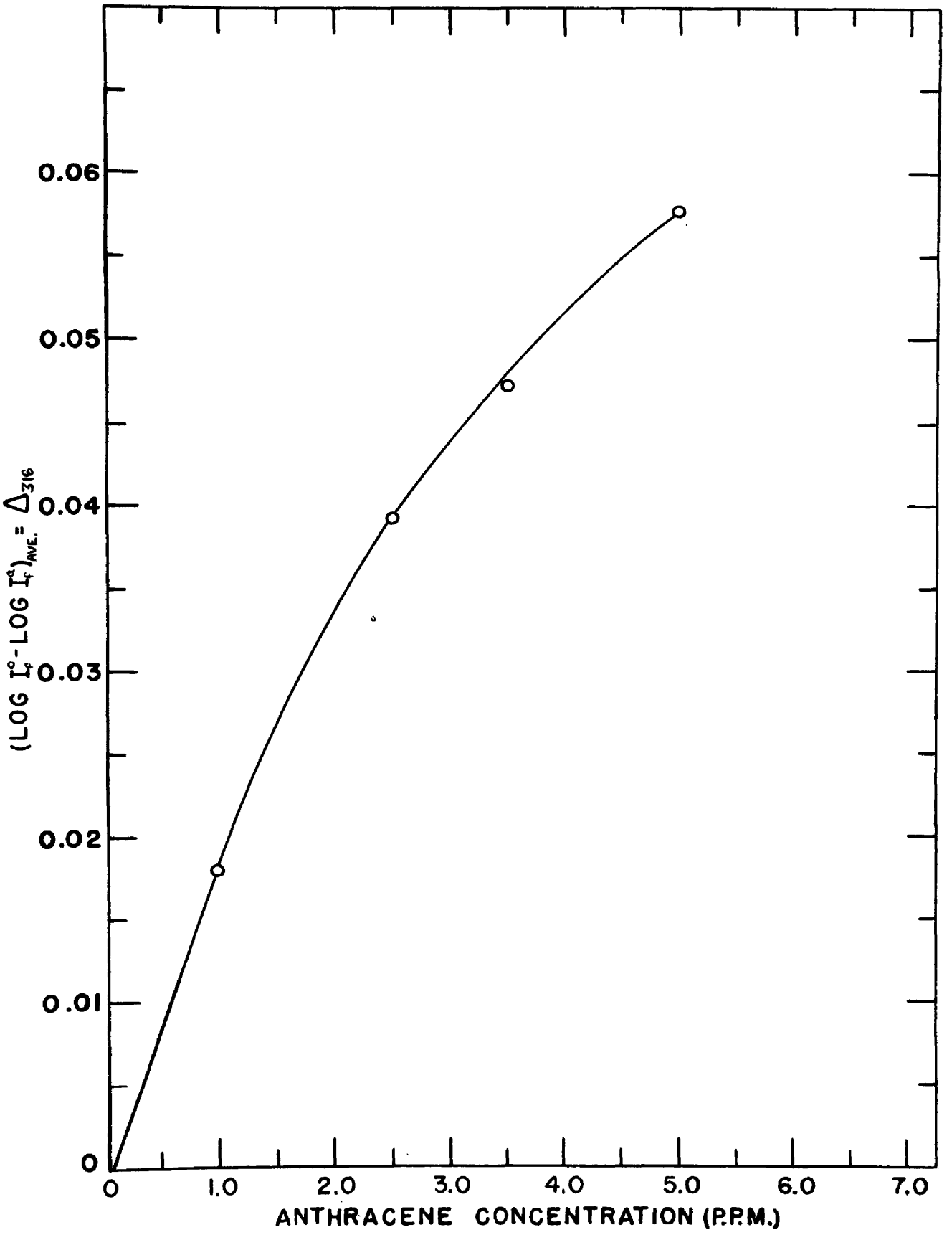
Concentration Anthracene	$\log I_f^o - \log I_f^a$			
	1	2	3	Average
0 p.p.m.	0	0	0	0
1.0	0.015	0.009	0.030	0.018
2.5	0.046	0.040	0.032	0.039
3.5	0.039	0.044	0.059	0.047
5.0	0.052	0.049	0.072	0.058

Anthracene Absorption of 350 mμ Emission

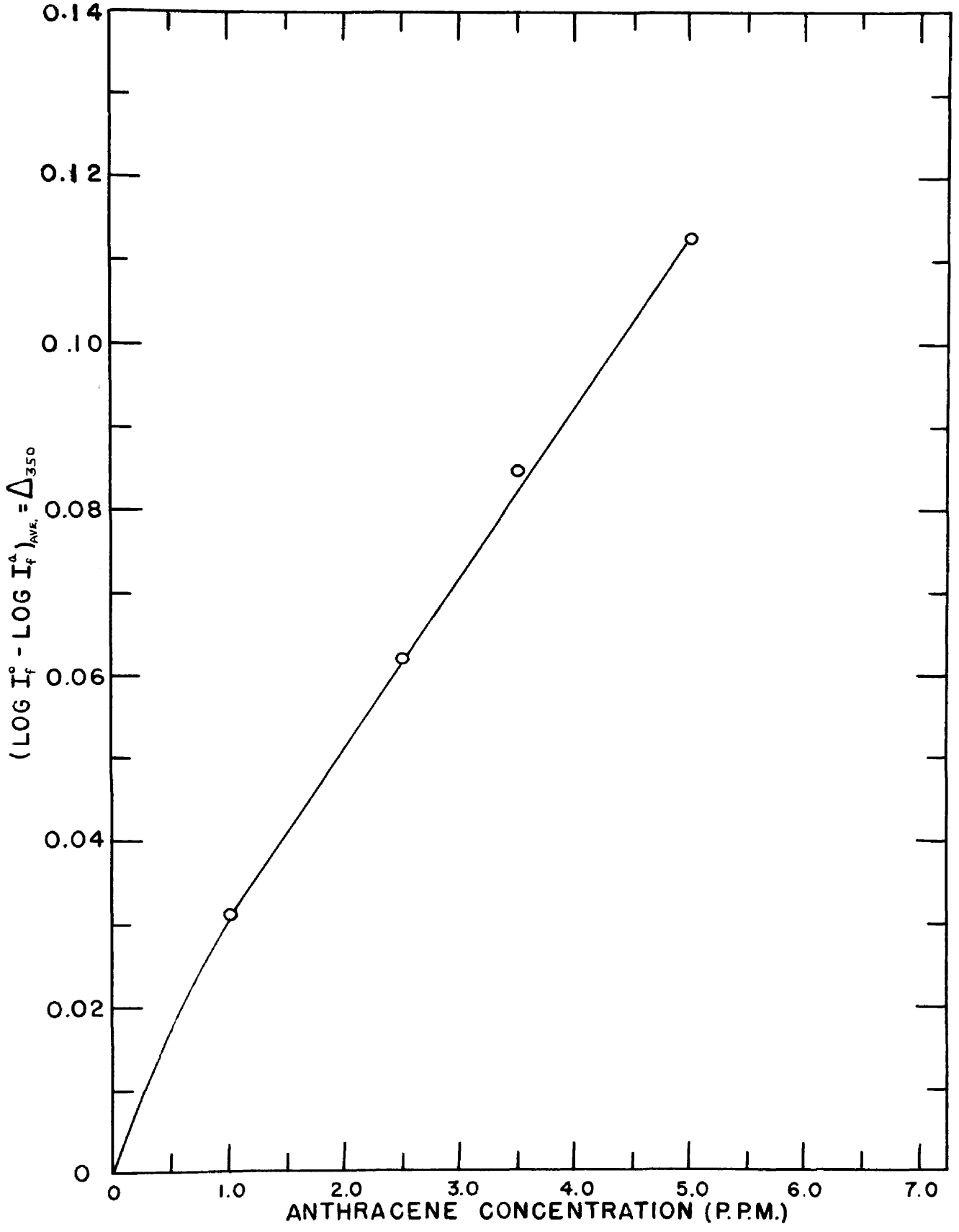
Concentration Anthracene	$\log I_f^o - \log I_f^a$			
	1	2	3	Average
0 p.p.m.	0	0	0	0
1.0	0.033	0.026	0.036	0.032
2.5	0.067	0.058	0.062	0.062
3.5	0.095	0.074	0.087	0.085
5.0	0.108	0.118	0.112	0.113

Phenanthrene Absorption of 316 mμ Emission

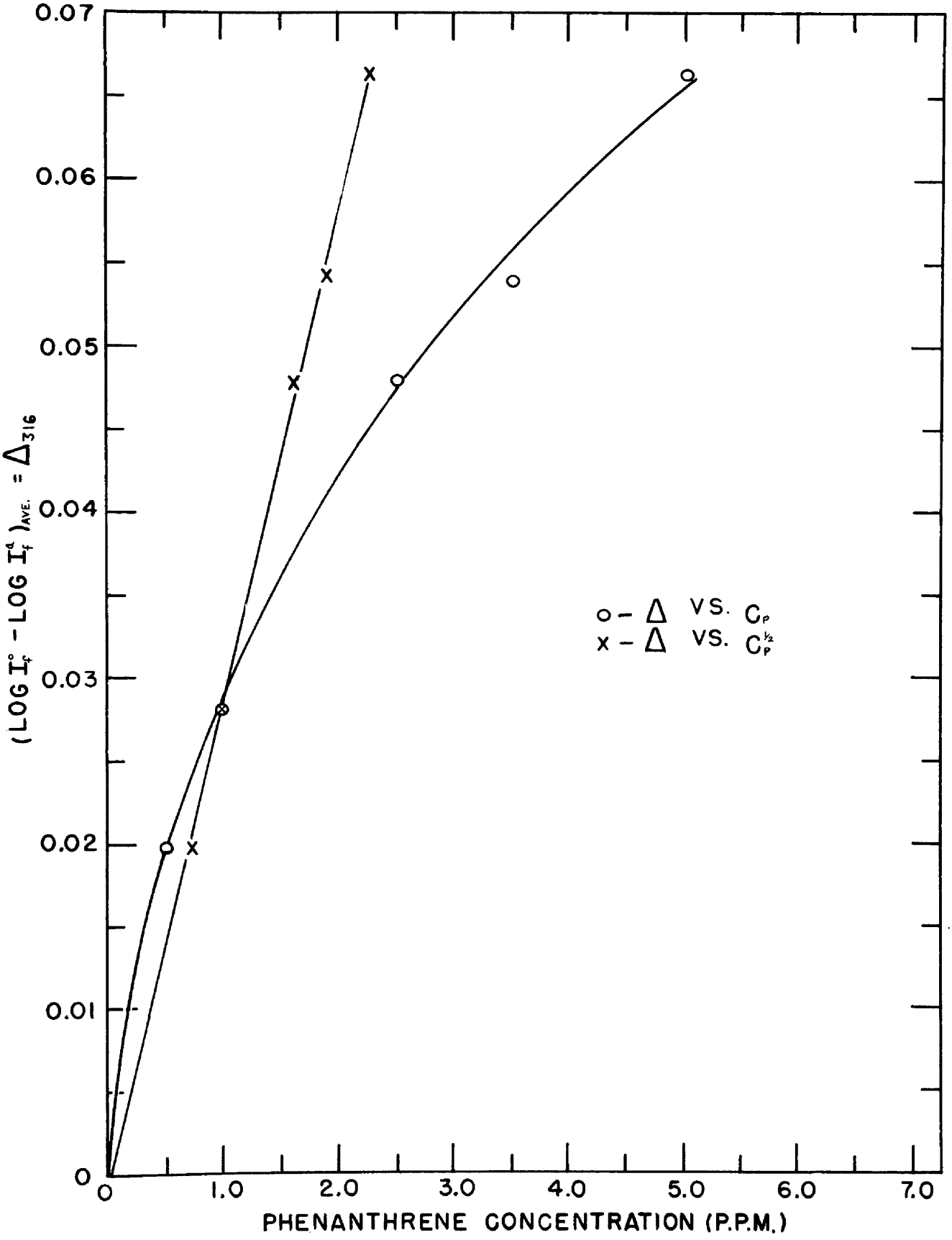
Concentration Phenanthrene	$\log I_f^o - \log I_f^a$			
	1	2	3	Average
0 p.p.m.	0	0	0	0
0.5	0.015	0.016	0.028	0.020
1.0	0.030	0.015	0.039	0.028
2.5	0.040	0.044	0.060	0.048
3.5	0.044	0.050	0.069	0.054
5.0	0.059	0.063	0.078	0.067



GRAPH 8. ANTHRACENE ABSORPTION OF 316 mμ EMISSION



GRAPH 9. ANTHRACENE ABSORPTION OF 350mμ EMISSION



GRAPH 10. PHENANTHRENE ABSORPTION OF 316 mμ EMISSION

available for the determination of either component. That is, the 316 mu fluorene emission must be corrected for phenanthrene absorption and the 350 mu reading is indicative of the sum of the fluorene and phenanthrene 350 mu emissions. It appears then, that the only solution to the problem is to obtain two equations, one describing the 316 mu emission and one describing the 350 mu emission, in terms of the phenanthrene and fluorene concentrations and then solve them simultaneously.

The equation for the 316 mu fluorene emission is given by

$$\log I_f^a = \log I_f^o - \triangle = \log K_{316} C_F - \triangle$$

where I_f^a and I_f^o are as described before.

$\triangle = f(C_p) = \log I_f^o - \log I_f^a$ = absorption correction term

K_{316} = slope of pure fluorene calibration curve

C_F = fluorene concentration in p.p.m.

The absorption term, $\triangle$, cannot be expressed absolutely by Beer's law, as was predicted, but if one plots $\triangle$ versus $C_p^{\frac{1}{2}}$ rather than versus C_p a fairly straight line results (see Graph 10) and thus we can say:

$$\triangle = kC_p^{\frac{1}{2}}$$

No theoretical explanation for this expression is obvious but it fits the experimental facts and permits the fluorene 316 mu emission to be expressed as:

$$\text{(a.)} \quad \log I_f^a = \log K_{316} C_F - kC_p^{\frac{1}{2}}$$

The fluorescence at 350 mu is indicative of the sum of the fluorescence intensities of the phenanthrene and fluorene present. This

reading should be, ideally, given by:

$$I_f = K_{350}C_F + K'_{350}C_p + I_s$$

where -

K_{350} = slope of pure fluorene calibration curve, I_f read at 350 mu.

K'_{350} = slope of pure phenanthrene calibration curve, I_f read at 350 mu.

I_s = sum of phenanthrene and fluorene calibration curve intercepts.

In reality, however, the readings obtained for phenanthrene-fluorene mixtures at 350 mu do not correspond to this equation. The readings always tend to be less than the calculated value.

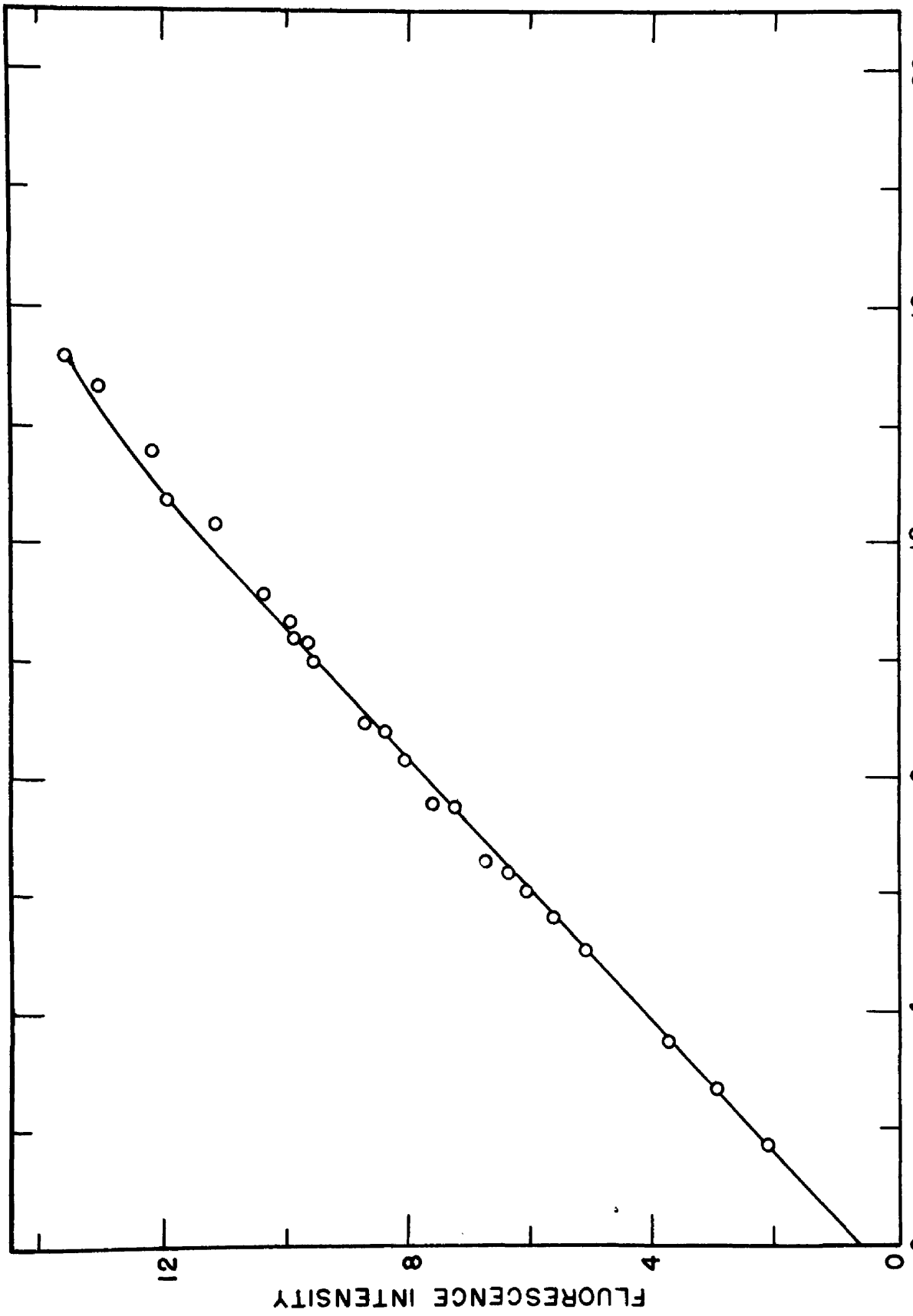
This difficulty was quite easily circumvented by defining a quantity, S, the theoretical sum of the fluorescence intensities, ignoring the intercepts.

$$S = K_{350}C_F + K'_{350}C_p$$

or

$$(b.) \quad S = 1.9C_F + 1.6\ C_p$$

The quantity, S was calculated for a group of mixtures containing varying amounts of phenanthrene and anthracene and the fluorescence intensities of the mixtures at 350 mu were obtained. A plot of the fluorescence intensity at 350 mu versus the value of S for the mixture yielded the curve shown in Graph 11. Thus, from the fluorescence intensity reading at 350 mu and the plot shown in Graph 11, a value of S is obtained which can be expressed by equation (b).



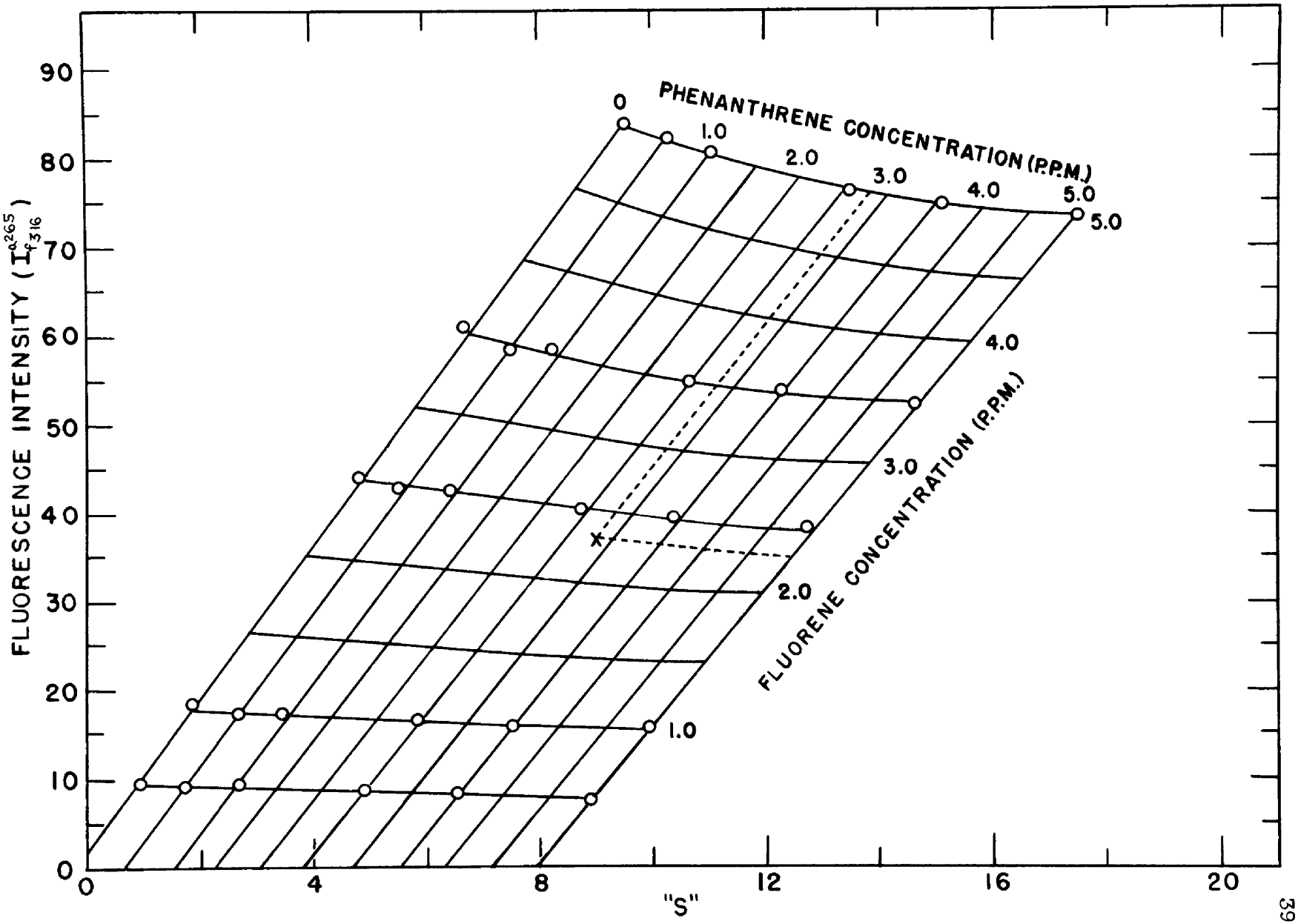
GRAPH II. "S" FUNCTION PLOT

$S = 1.6C_p + 1.9C_f$

The two equations (a) and (b) and fluorescence intensity readings at 316 mu and 350 mu now permit the resolution of phenanthrene-fluorene mixtures.

The above process, demanding the solution of simultaneous equations to obtain the concentrations of phenanthrene and fluorene, is difficult because it requires the solving of a cubic equation and a simpler method of resolving the mixture would be desirable. A simple graphical method of accomplishing this resolution becomes obvious when it is realized that the combination of an apparent fluorescence intensity reading at 316 mu and an S value obtained from the 350 mu reading, for a mixture of phenanthrene and fluorene, must be unique for the concentrations involved. This becomes more apparent when the value of the 316 mu fluorescence intensity is plotted versus the value of S for a series of mixtures. When this is done and the points corresponding to identical fluorene concentration are joined, and the points corresponding to identical phenanthrene concentration are joined, the grid shown in Graph 12 is obtained. This grid then serves as a standard calibration curve for both phenanthrene and fluorene.

The use of the calibration grid is best explained by considering a specific example. Consider an unknown mixture containing phenanthrene and fluorene which gives fluorescence intensity readings of 37.0 at 316 mu and 8.7 at 350 mu under 265 mu irradiation. From Graph 11 it is found that the reading of 8.7 corresponds to an S value of 9.0. Then the point corresponding to $I_f{}^a{}_{316} = 37.0$ and $S = 9.0$ is located on the grid of Graph 12. This point, marked by an "x" on the grid, is seen to



GRAPH 12. FLUORENE-PHENANTHRENE CALIBRATION GRID

fall on a line of constant phenanthrene concentration corresponding to 2.8 p.p.m. and a line of constant fluorene concentration corresponding to 2.3 p.p.m. Thus the concentrations of the two components in the mixture have been determined directly by graphical solution of the pair of simultaneous equations and the difficult process of arithmetic solution has been avoided.

All of the information necessary, for the resolution of all possible combinations of mixtures of the three hydrocarbons, is now available. The general procedure to be followed is as follows. Anthracene is in all cases determined directly by excitation with 365 mu radiation and fluorescence intensity measurements at 400 and 420 mu. The concentration reported is then the average found from the anthracene calibration curves shown in Graph 2. If fluorene is present with anthracene, then the fluorene concentration is determined by obtaining the fluorescence intensity at 316 mu under 265 mu irradiation, correcting it for anthracene absorption, by use of Graph 8, and then obtaining the fluorene concentration from Graph 3, the fluorene calibration curve. When phenanthrene is present with anthracene, the 350 mu reading obtained under 265 mu irradiation is corrected for anthracene absorption, by use of Graph 9, and is then used to obtain the phenanthrene concentration from Graph 4, the phenanthrene calibration curve. Mixtures of phenanthrene and fluorene are, of course, determined by use of the calibration grid as described previously. When all three hydrocarbons are present in a mixture, the anthracene concentration is determined in the usual manner. Then the 316 mu and 350 mu readings, obtained under 265 mu

irradiation, are corrected for anthracene absorption and are used to determine the concentrations of phenanthrene and fluorene from the calibration grid.

A group of synthetic mixtures of all four types, three binary and one ternary, were prepared and analyzed by the procedure described above. The results obtained are shown in Table III.

The average errors found in the determinations of the three hydrocarbons were: =

Anthracene, $E_{ave.}$ = 3.1%

Phenanthrene, $E_{ave.}$ = 4.6%

Fluorene, $E_{ave.}$ = 4.1%

The maximum error observed was 15%; however, errors of this order of magnitude are exceptional and in the majority of determinations an error of approximately $\pm$5% should be expected.

TABLE III

RESULTS OF HYDROCARBON MIXTURE ANALYSIS

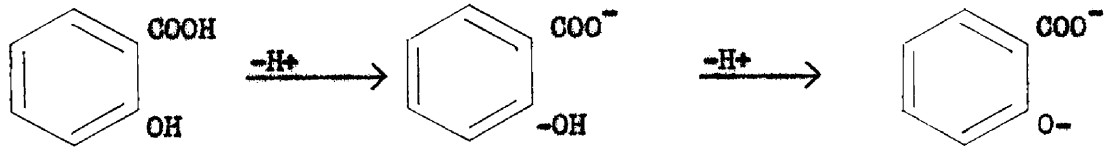
Anthracene					Phenanthrene			Fluorene		
p.p.m. Taken	p.p.m. Found (400mμ)	p.p.m. Found (420mμ)	Ave.	Percent Error	p.p.m. Taken	p.p.m. Found	Percent Error	p.p.m. Taken	p.p.m. Found	Percent Error
0.75	0.73	0.71	0.72	-4.0	4.00	4.06	+1.5	—	—	—
1.00	1.00	0.98	0.99	-1.0	1.00	1.05	+5.0	—	—	—
1.50	1.44	1.40	1.42	-5.3	—	—	—	3.00	3.07	+2.3
2.00	1.95	1.97	1.96	-2.0	—	—	—	2.00	2.14	+7.0
3.00	2.95	2.80	2.88	-4.0	—	—	—	1.00	1.05	+5.0
—	—	—	—	—	1.00	0.85	-15.0	3.00	3.04	+1.3
—	—	—	—	—	2.00	1.90	-5.0	2.00	2.05	+2.5
—	—	—	—	—	3.00	2.92	-2.7	1.00	0.98	-2.0
—	—	—	—	—	3.50	3.75	+7.1	1.00	1.00	0
—	—	—	—	—	3.50	3.50	0	2.50	2.52	+0.8
—	—	—	—	—	5.00	5.05	-1.0	3.80	3.45	-1.4
1.00	0.97	0.93	0.95	-5.0	2.00	1.78	-11.0	3.00	3.15	+5.0
2.00	1.97	1.95	1.96	-2.0	3.00	2.93	-2.3	2.00	2.20	+10.0
2.00	1.97	1.97	1.97	-1.5	2.00	2.10	+5.0	2.00	2.20	+10.0
3.00	2.93	2.85	2.89	-3.7	1.00	0.99	-1.0	1.00	1.02	+2.0
3.50	3.50	3.52	3.51	+0.3	3.00	3.00	0	3.00	3.20	+6.7
1.50	1.52	1.58	1.54	+2.7	4.00	4.34	+8.5	1.00	1.05	+5.0

B. The Hydroxybenzoic Acids

1. Introduction

In the second system of compounds to be studied it was desired that the further variable of acidity control be coupled with those of wavelength of exciting radiation and wavelength at which the emission intensity is measured.

Salicylic acid, the ortho isomer of the hydroxybenzoic acids, is a fluorescent compound and its fluorescence intensity is affected by acidity changes. This is to be expected since the fluorescence spectrum of a compound, like the absorption spectrum, is characteristic of the species present. Thus depending upon the pH of a solution, one or two of the following species could be present in solution.



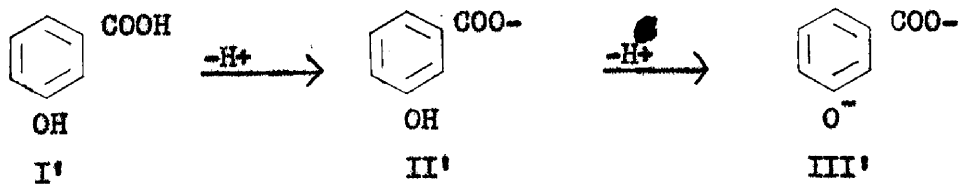
I II III

It is, therefore, theoretically possible that all three species would show distinct fluorescence spectra. With salicylic acid it is found from qualitative pH studies, that species I is not fluorescent but that species II and III are fluorescent under excitation with 314 mu radiation. It should be stated here that when a species is said to be non-fluorescent, the meaning intended is that under the available conditions of excitation no fluorescence is observed. The mercury arc, used as an excitation source, has a limited number of spectral lines sufficiently

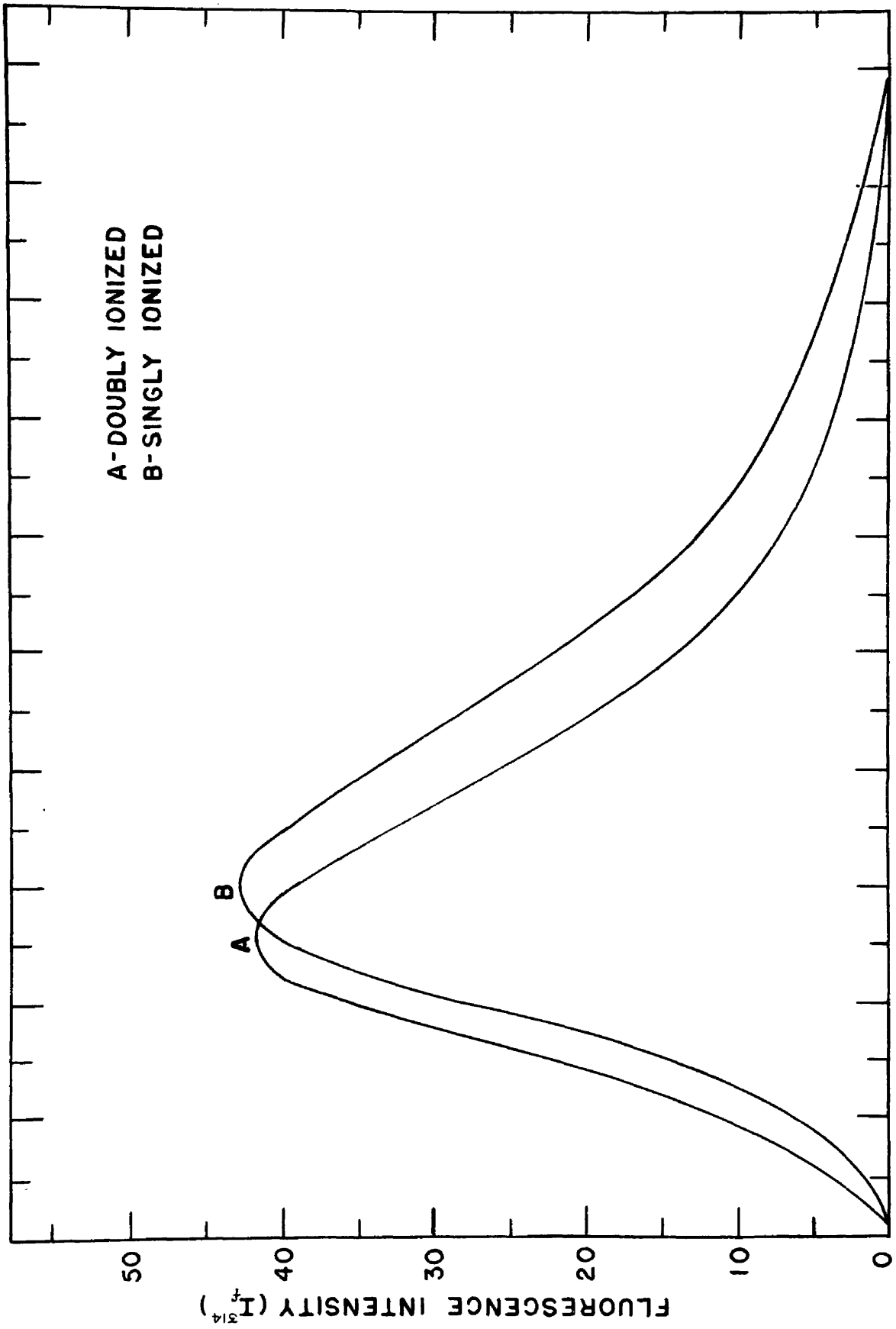
intense for excitation. Therefore, the fact that none of these wavelengths are capable of producing fluorescence from a species does not exclude the possibility that the species may be fluorescent under excitation by some other wavelength. In all subsequent discourse the statement that a species is non-fluorescent will be subject to the above conditions, that is, it is non-fluorescent under the excitation conditions available.

The fluorescence spectra of species II and III are shown in Graph 13. The spectra are very similar, the only difference being that the maximum of the fluorescence band of species III occurs at 400 mu while the maximum of species II occurs at 410 mu. Although the fluorescence spectra are markedly similar the species react quite differently under 314 mu irradiation. Species II is completely stable under prolonged irradiation, as shown by the fact that its fluorescence intensity remains constant, while species III is decidedly unstable, as shown by the fact that its fluorescence intensity falls off rapidly under prolonged irradiation. Consequently, for analytical work, the fluorescence of species II should be utilized since it is completely stable.

The meta isomer of the hydroxybenzoic acids can be considered in the same manner as was salicylic acid. Again there are three possible forms which can exist in solution, depending upon the acidity.



I' II' III'



GRAPH 13. FLUORESCENCE SPECTRA OF SALICYLIC ACID

As stated before, it is possible that all three of these species could exhibit fluorescence spectra. Under the conditions of excitation available, however, only species III' is found to be fluorescent.

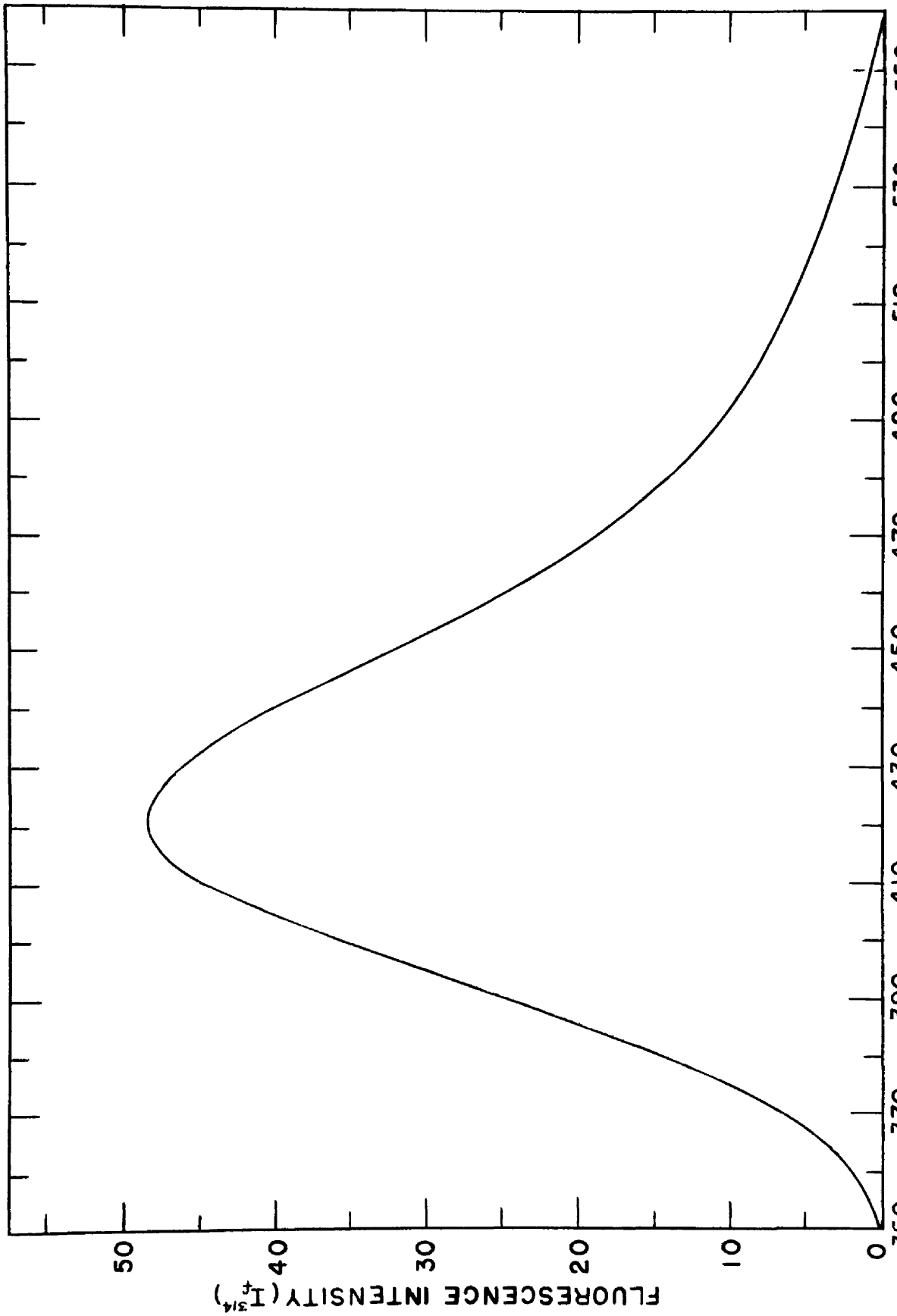
The fluorescence spectrum of species III, under 314 mu excitation, is shown in Graph 14. It is seen to be very similar to the spectra observed for the two fluorescent forms of salicylic acid. The shapes of the three spectra are all identical and only differ in the wavelength of maximum fluorescence intensity. The fluorescence maximum of the fluorescent form of m-hydroxybenzoic acid occurs at 420 mu as compared to 410 mu for the stable form of salicylic acid and 400 mu for the unstable form.

When p-hydroxybenzoic acid is investigated in the above manner it is found that none of the three forms is fluorescent under 314 mu excitation. Under 265 mu excitation the doubly ionized form of p-hydroxybenzoic acid is found to be very weakly fluorescent, showing a maximum at 340 mu. This emission is, however, too weak to be employed on a quantitative basis.

For analytical purposes then, it should be possible to employ the fluorescent emission of singly ionized salicylic acid and doubly ionized m-hydroxybenzoic acid while no interference should be expected from the para isomer.

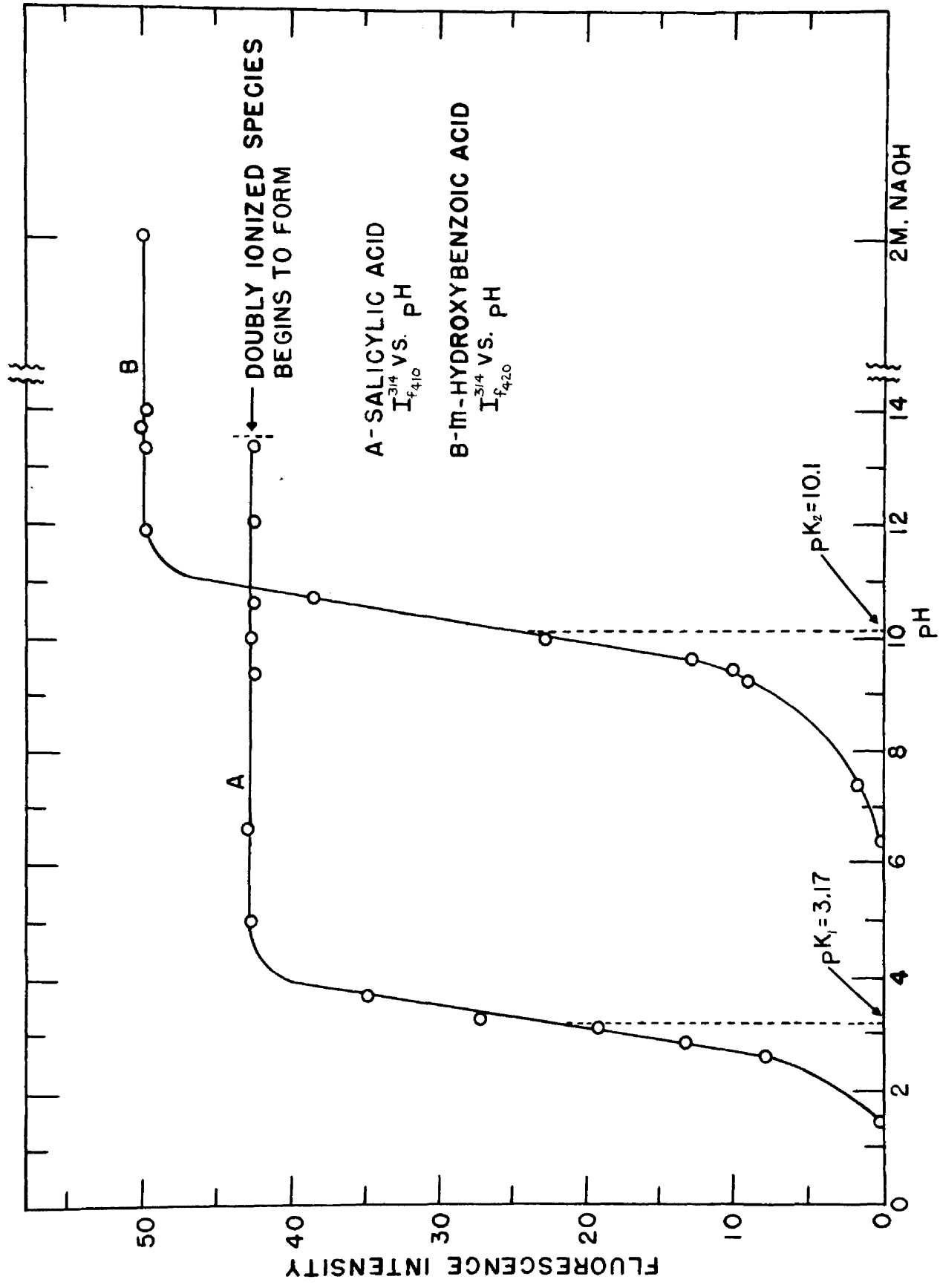
2. pH Studies

Since the fluorescence spectra of the ortho and meta isomers are characteristic of specific ionic forms, it is necessary to know the solution conditions under which these forms are present. This



GRAPH 14. FLUORESCENCE SPECTRUM OF m-HYDROXYBENZOIC ACID

information is readily obtained from a study of the fluorescence characteristics under variable pH conditions. For salicylic acid it has been stated that the stable, singly ionized form shows a fluorescence maximum at 410 mu under 314 mu excitation. Therefore, a group of solutions containing identical concentrations of salicylic acid were prepared and adjusted to various hydrogen ion concentrations, either by the addition of hydrochloric acid or sodium hydroxide. Then these solutions were activated with 314 mu radiation and their fluorescence intensities were read at 410 mu. The readings obtained in this fashion were then plotted against pH. The curve which resulted is shown in plot A of Graph 15. The plot shows that below a pH of 1.5 no fluorescence is noted. This indicates that all of the salicylic acid is present in the undissociated form. As the pH is increased above 1.5, fluorescence at 410 mu is detected and the intensity increases rapidly with increasing pH until it reaches a maximum at a pH of approximately 4.5. The observed fluorescence intensity then remains constant until a pH of approximately 13.5 is reached. Therefore, above a pH of 1.5 the fluorescent, singly ionized species begins to form. As the pH is increased the concentration of this species increases, thus causing an increase in the fluorescence intensity. Finally at a pH of 4.5, all of the salicylic acid present is converted to the singly ionized form and the fluorescence intensity remains constant until the hydroxide ion concentration becomes great enough to begin neutralizing the phenolic hydrogen ion. This process begins at a pH of approximately 13.0. Therefore, the pH range over which the salicylic



GRAPH 15. pH-FLUORESCENCE CURVES

acid is present completely in the singly ionized form is 4.5 to 13.0 and is the pH range, within which, quantitative fluorometric work could be conducted.

It is interesting to note that the ionization constant for the dissociation of the carboxylic hydrogen ion can be obtained from the plot (2). At the pH where the fluorescence intensity has reached one half of its maximum value, the acid exists half in the dissociated form and half in the associated form. Therefore, the pH at the half-intensity point should be the same as the pKa of the acid. From the plot the pH at the half-intensity point is found to be 3.17 and Fieser (6) lists the pKa for the first dissociation of salicylic acid as 3.00. Consequently, fair agreement is obtained between the literature value of the constant and the experimentally determined constant.

The same procedure outlined above, was carried out substituting m-hydroxybenzoic acid for salicylic acid and measuring the fluorescence intensity at 420 mu under 314 mu excitation. Plot B shown in Graph 15 resulted from this study and shows that the doubly ionized form of the meta isomer is completely formed at a pH of 12.0 and that the fluorescence intensity remains constant in increasingly alkaline solutions, even in 2 molar sodium hydroxide. The observed half-intensity value from the plot, indicates that the pKa for the second dissociation of m-hydroxybenzoic acid should be 10.1. However, no literature value of the second dissociation constant of m-hydroxybenzoic acid could be found to compare with this value.

Comparing the two fluorescence versus pH plots, plots A and B of Graph 15, it can be seen that in the pH region 4.5 to 6.0 the ortho

isomer will be fluorescent while the meta isomer will not. However, at a pH of 12 to 13, both isomers would be fluorescent.

Consequently, even though both isomers exhibit essentially identical fluorescence spectra and show no selectivity of response to different wavelengths of excitation, mixtures of the two should be resolvable through pH control.

If both the ortho and meta isomers were present in a solution, it should be possible to adjust the pH of the solution to 5.5, activate with 314 mu radiation and obtain two indications of the o-hydroxybenzoic acid concentration by fluorescence intensity measurements at 410 and 430 mu. These measurements should be completely independent of the concentration of the meta isomer present. Then, if the pH is adjusted to 12.0, the fluorescence intensity measurements at 410 and 430 mu should be indicative of the sum of the ortho and meta isomers fluorescence intensities at these wavelengths. Since the fluorescence intensity of the ortho isomer should be the same at a pH of 12.0 as it is at a pH of 5.5, simple subtraction of the pH = 5.5 readings from the pH = 12.0 readings should give difference readings indicative of the m-hydroxybenzoic acid concentration.

3. Preparation of Calibration Curves

Aqueous stock solutions of ortho, meta and para hydroxybenzoic acids were prepared which contained 1.5 milligrams of organic acid per milliliter of solution. All subsequent solutions, prepared for use in this study, were prepared by dilution of known volumes of these stock solutions. The acids used to prepare the stock solutions were

Eastman White Label chemicals which were recrystallized from absolute methyl alcohol.

An acetic acid-sodium acetate buffer solution was prepared and used as the diluent for the solutions which were measured at a pH of 5.5. The pH = 5.5 buffer was prepared as follows: 4.67 gms. of sodium acetate and 0.6 gm. of acetic acid were dissolved and diluted to one liter with distilled water.

The pH = 12.0 solutions were prepared by the addition of 2 ml. of approximately 2 molar sodium hydroxide to the stock solution aliquot and subsequent dilution to 100 ml. with distilled water.

The range, over which the fluorescence intensity is a linear function of the concentration, was determined for salicylic acid in pH = 5.5 solution and for salicylic acid and m-hydroxybenzoic acid in pH = 12.0 solution. Linearity of response was observed for both acids up to a concentration of 12 p.p.m.

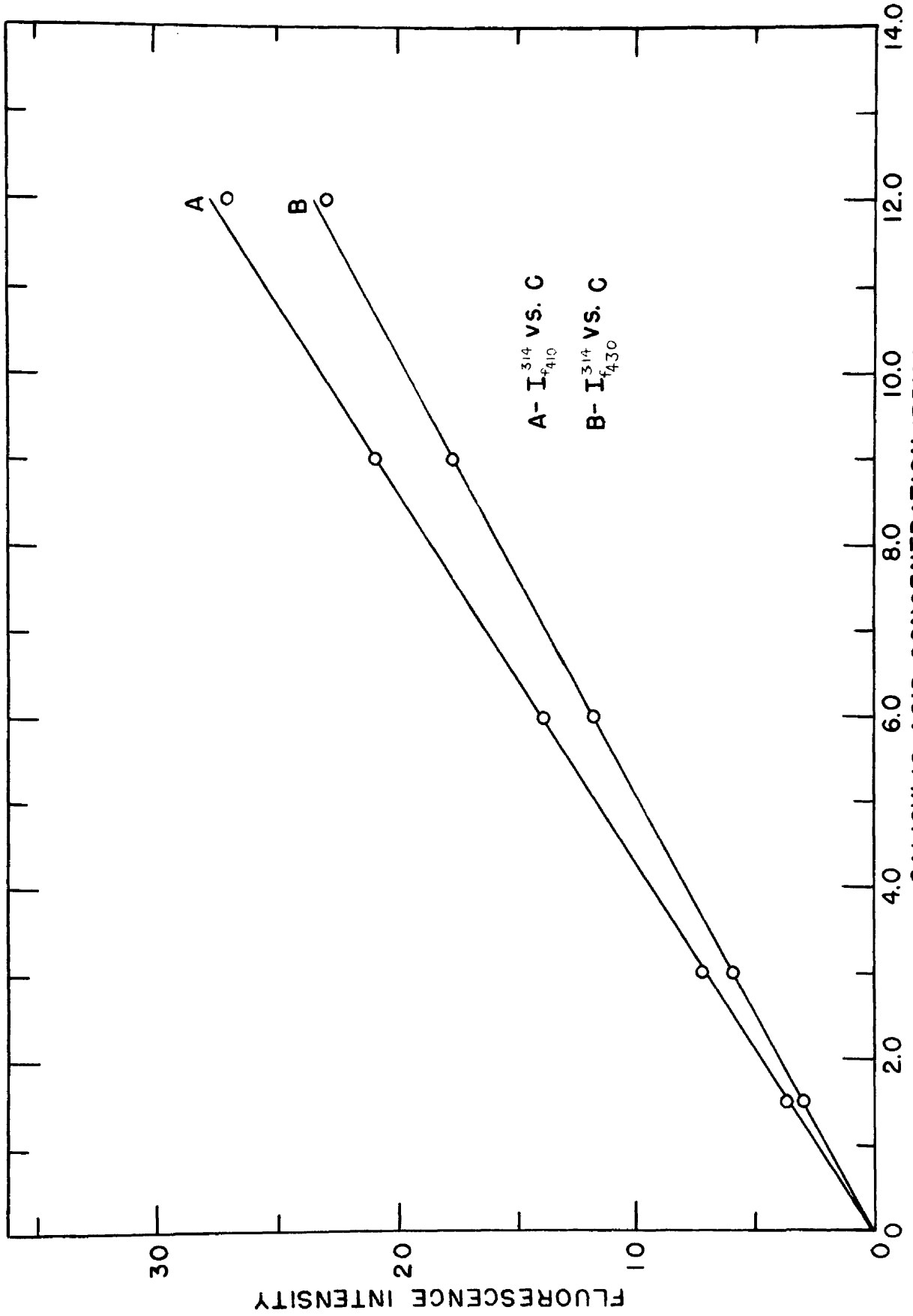
Calibration curves for both acids were prepared which consisted of plots of fluorescence intensity versus concentration. Actually two calibration curves were prepared for each acid, one corresponding to fluorescence intensity measurements at 410 mu and the other to measurements at 430 mu. Therefore, when unknown solutions are run, the two measurements at 410 mu and at 430 mu yield two values of the concentration. The concentration reported is, consequently, the average of these two values.

Instrument calibration, for the quantitative work with the hydroxybenzoic acids, was effected by using a standard salicylic acid solution

which contained 6.0 p.p.m. of the acid in a solution having a pH of 5.5. This standard solution was assigned a reading of 14.0 at 410 mu under 314 mu excitation. All subsequent measurements are, therefore, relative to instrument calibration with this standard solution. The standard solution was prepared fresh each day to insure against possible deterioration and to obtain maximum reproducibility of measurements.

The calibration curves for salicylic acid, shown in Graph 16, were obtained in the following manner. Two groups of standard solutions, containing 0 to 12 p.p.m. of salicylic acid, were prepared. One group of standards was adjusted to a pH of 5.5 by dilution of the aliquots, from the salicylic acid stock solution, to 100 ml with acetate buffer solution. The other group was adjusted to a pH of 12.0 by the addition of 2.0 ml. of 2 molar sodium hydroxide solution to the aliquots and subsequent dilution to 100 ml. with distilled water. According to the results of the pH studies, the fluorescence intensities exhibited by both the pH = 5.5 and the pH = 12.0 solutions should be identical for identical concentrations of salicylic acid. This was found to be true, within the limits of experimental error, and the average values of the fluorescence intensities for the pH = 5.5 and the pH = 12.0 standards were used in the preparation of the salicylic acid calibration curves. The results employed in the preparation of the salicylic acid calibration curves are tabulated in Table IV.

The m-hydroxybenzoic acid calibration curves were prepared in a manner similar to that described above except that both groups of standards were adjusted to a pH of 12.0 with sodium hydroxide.



GRAPH 16. SALICYLIC ACID CALIBRATION CURVES

TABLE IV

CALIBRATION CURVE DATA FOR SALICYLIC ACID

Concentration Salicylic Acid in p.p.m.	$I^{314}_{f_{410}}$			$I^{314}_{f_{430}}$		
	pH=5.5	pH=12.0	Ave.	pH=5.5	pH=12.0	Ave.
1.5	3.5	3.7	3.6	2.9	3.0	3.0
3.0	7.0	7.4	7.2	5.9	6.3	6.1
6.0	13.5	14.3	13.9	11.6	12.0	11.8
9.0	20.6	21.2	20.9	17.6	17.9	17.8
12.0	26.3	28.0	27.1	22.7	23.3	23.0

The results employed in the preparation of the m-hydroxybenzoic acid calibration curves, Graph 17, are shown below in Table V.

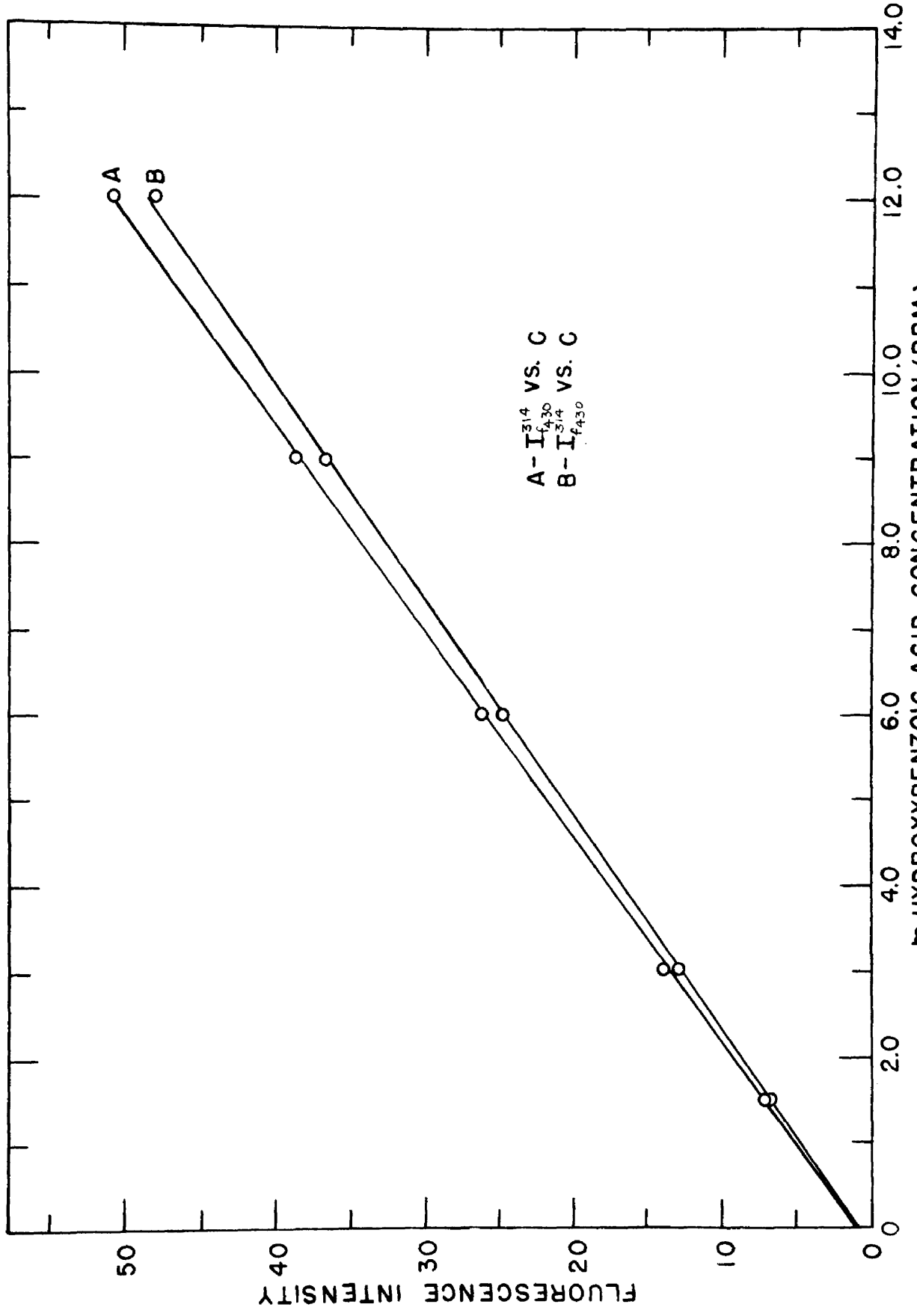
TABLE V

CALIBRATION CURVE DATA FOR m-HYDROXYBENZOIC ACID

Concentration m-Hydroxybenzoic Acid in p.p.m.	$I^{314}_{f_{410}}$			$I^{314}_{f_{430}}$		
	Group 1	Group 2	Ave.	Group 1	Group 2	Ave.
1.5	7.1	6.9	7.0	6.9	6.4	6.7
3.0	14.0	13.7	13.9	13.1	12.9	13.0
6.0	26.0	26.8	26.4	24.6	25.3	26.0
9.0	39.2	38.4	38.8	37.2	36.2	36.7
12.0	51.3	51.1	51.2	48.3	48.3	48.3

4. Results of Mixture Analysis

Five test solutions, containing both the ortho and meta hydroxybenzoic acid isomers in varying concentrations, were prepared. These solutions were prepared in duplicate, one being diluted with buffer solution and the other being diluted with distilled water after the



GRAPH 17. m-HYDROXYBENZOIC ACID CALIBRATION CURVES

addition of 2.0 ml. of 2 molar sodium hydroxide. Therefore, there are two solutions corresponding to each test mixture, one having a pH of 5.5 and one having a pH of 12.0. Then from fluorescence intensity measurements on the pH = 5.5 solutions and the calibration curves of Graph 16, the concentration of o-hydroxybenzoic acid can be determined directly. The fluorescence intensity readings obtained from the pH = 12.0 solutions are then the sums of the ortho and meta isomers fluorescence intensities at 410 and 430 mu. Therefore, if the readings obtained on the pH = 5.5 solutions are subtracted from the pH = 12.0 readings, the resulting differences are the contributions to the fluorescence intensities by the meta isomer. These differences and the calibration curves of Graph 17 can be used to obtain the concentration of the m-hydroxybenzoic acid present in the mixture.

The five test solutions, when analyzed by the outlined procedure, yielded the results shown in Table VI.

The results obtained indicate that, in mixtures of ortho and meta hydroxybenzoic acids, both components can be determined with an expected error of less than 5%. Since the upper limit of the range of the method, for either acid alone, is 12 p.p.m., the test solutions were prepared so that the sum of the ortho and meta acid concentrations did not exceed 12.0 p.p.m.

It was predicted, at a prior point in the discussion, that the para isomer would not interfere in the determination of the ortho and meta isomers. This prediction was tested, experimentally, by preparing test mixtures containing all three isomers in varying

TABLE VI

ANALYSIS RESULTS ON MIXTURES OF o- AND m-HYDROXYBENZOIC ACIDS

Mixture	Ortho Isomer					Meta Isomer				
	p.p.m. Taken	p.p.m. Found From 410 mμ Reading	p.p.m. Found From 430 mμ Reading	Ave. p.p.m. Found	Per-Cent Error	p.p.m. Taken	p.p.m. Found From 410 mμ Reading	p.p.m. Found From 430 mμ Reading	Ave. p.p.m. Found	Per-Cent Error
1	3.00	3.00	2.98	2.99	-0.3	3.00	3.00	2.95	2.98	-0.7
2	4.50	4.35	4.30	4.33	-3.8	1.50	1.55	1.50	1.53	+2.0
3	1.50	1.47	1.47	1.47	-2.0	4.50	4.54	4.45	4.50	0
4	3.00	2.95	2.95	2.95	-1.7	6.00	6.00	5.93	5.97	-0.5
5	6.00	5.75	5.75	5.75	-5.0	3.00	3.00	2.94	2.97	-1.0

concentrations and determining the concentrations of the ortho and the meta isomers present by the same procedure as above. The average results obtained, on a series of five test solutions containing all three isomers, are shown below in Table VII.

TABLE VII

ANALYSIS RESULTS ON MIXTURES OF o- AND m-HYDROXYBENZOIC ACIDS IN THE PRESENCE OF THE PARA ISOMER

Mixture	Ortho Isomer			Meta Isomer			Para Isomer
	Taken	Found	Percent Error	Taken	Found	Percent Error	Concentration Present
1	4.50ppm	4.54ppm	+0.9	4.50ppm	4.42ppm	-1.8	15.0ppm
2	1.50	1.49	-0.7	3.00	2.99	-0.3	4.50
3	3.00	2.90	-3.3	1.50	1.45	-3.3	6.00
4	3.00	2.96	-1.3	3.00	2.99	-0.3	1.50
5	4.50	4.51	+0.2	6.00	5.72	-4.7	3.00

The results shown above are the average concentrations found from fluorescence intensity measurements at 410 and 430 mμ.

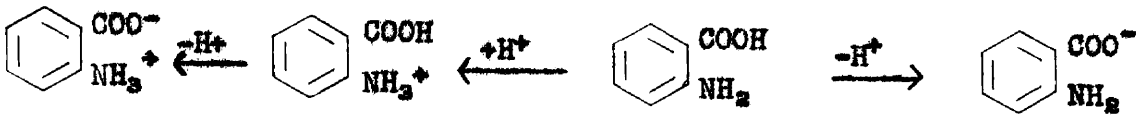
The results indicate that the presence of p-hydroxybenzoic acid, in concentrations up to 15.0 p.p.m., have no noticeable effect on the accuracy of the method.

C. The Aminobenzoic Acids

1. Introduction

The aminobenzoic acids are a group of compounds which one would expect to be quite similar in behavior to the hydroxybenzoic acids. With the aminobenzoic acids, there are, as with the hydroxybenzoic

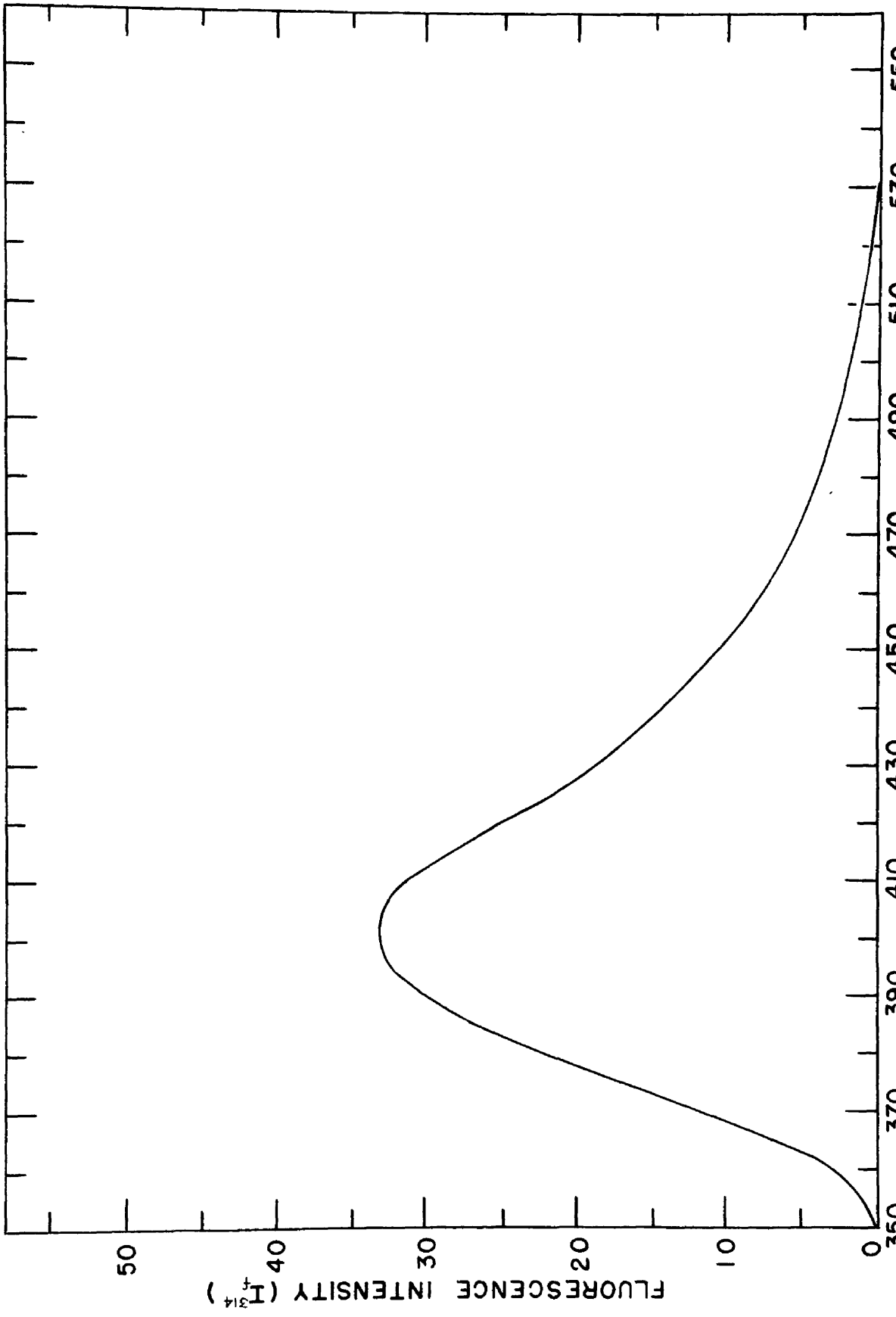
acids, several species possible in solution.



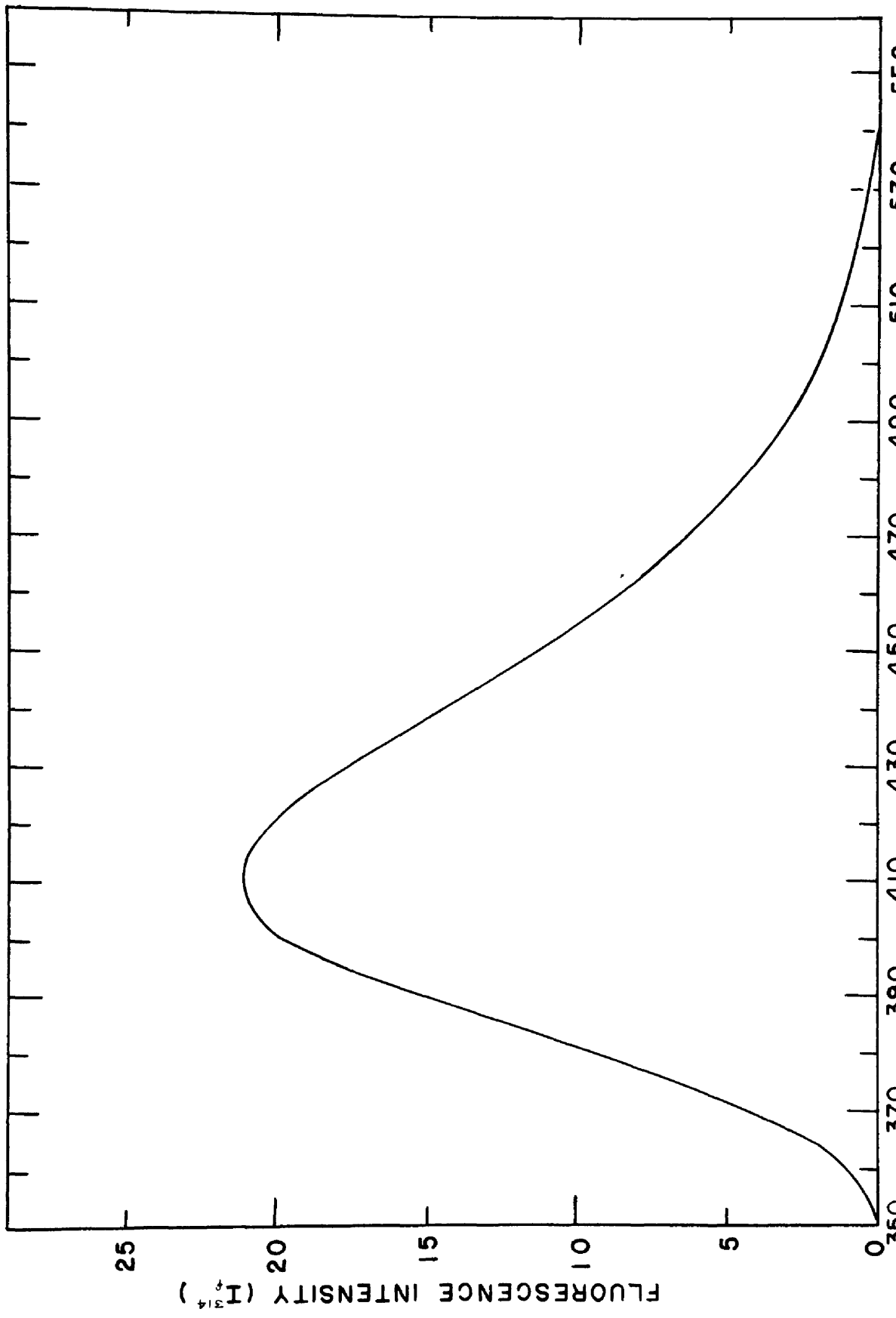
I II III IV

The form, or forms, which will be present in solution are, of course, dependent upon the pH of the solutions. Qualitatively, it was found that the three isomeric acids, ortho, meta and para, are all fluorescent in solution of pH approximately equal to 9.0. Graphs 18, 19 and 20 show the fluorescence spectra of the three isomers in a solution having a pH of 9.0. The ortho and meta isomers show fluorescence spectra which are activated by absorption of 314 mu radiation and have their emission maxima at 400 and 410 mu respectively. The fluorescence characteristics of the para isomer are radically different from those of the other two isomers. The fluorescence of this isomer is activated by absorption of 265 mu radiation and exhibits maximum fluorescence intensity at 345 mu.

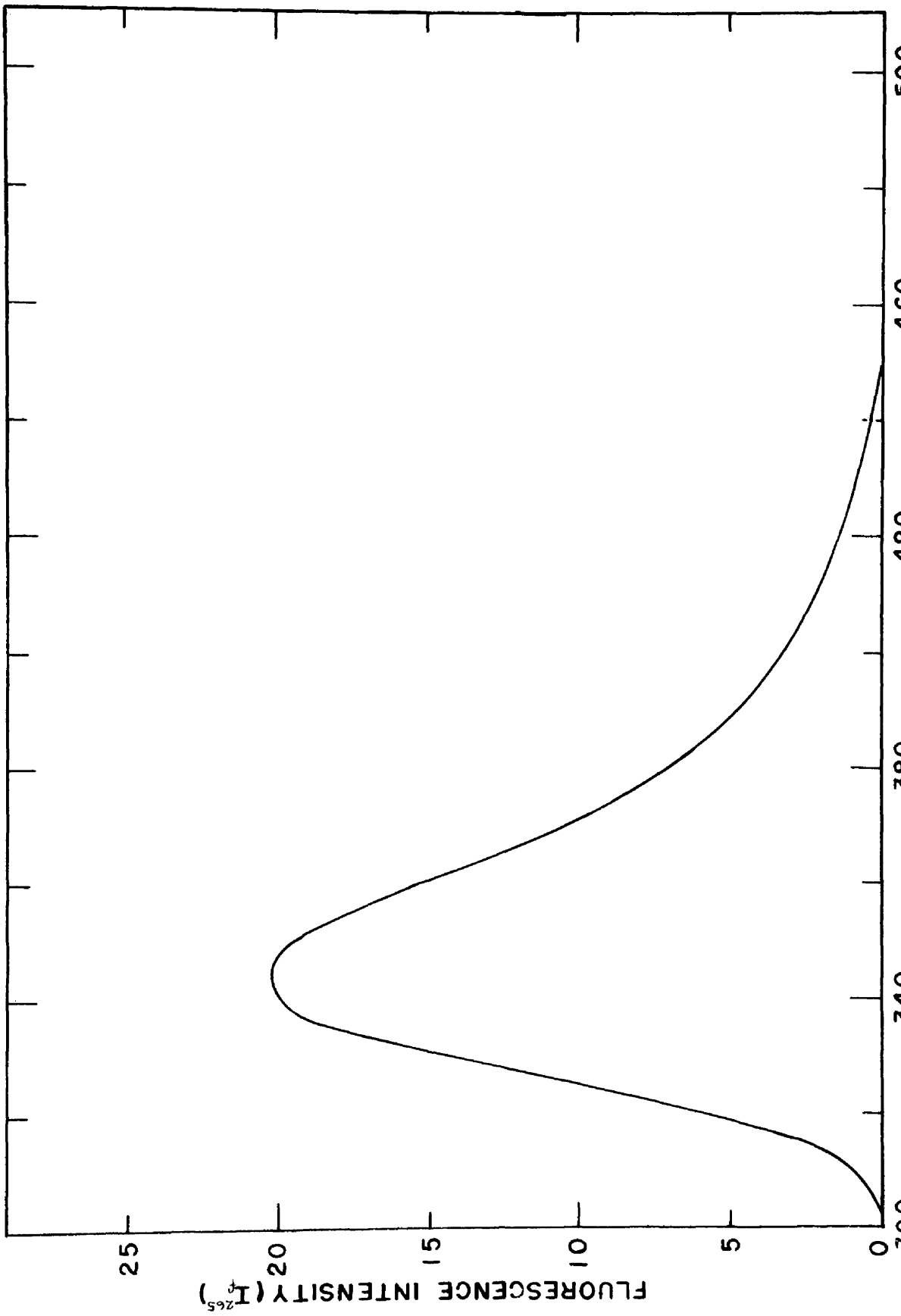
The fluorescence characteristics of the three acids were studied under variable pH conditions to discover what the optimum pH ranges were for fluorescence purposes and also so that the form of the fluorescent species could be elucidated. The fluorescence intensities were measured at the wavelength of maximum intensity for each isomer and the excitation was effected by 314 mu radiation in the case of the ortho and meta isomers, and 265 mu radiation in the case of the para isomer. The curves shown in Graphs 21, 22 and 23 resulted from the study. The curves all exhibit plateau regions, indicating that in high and low pH



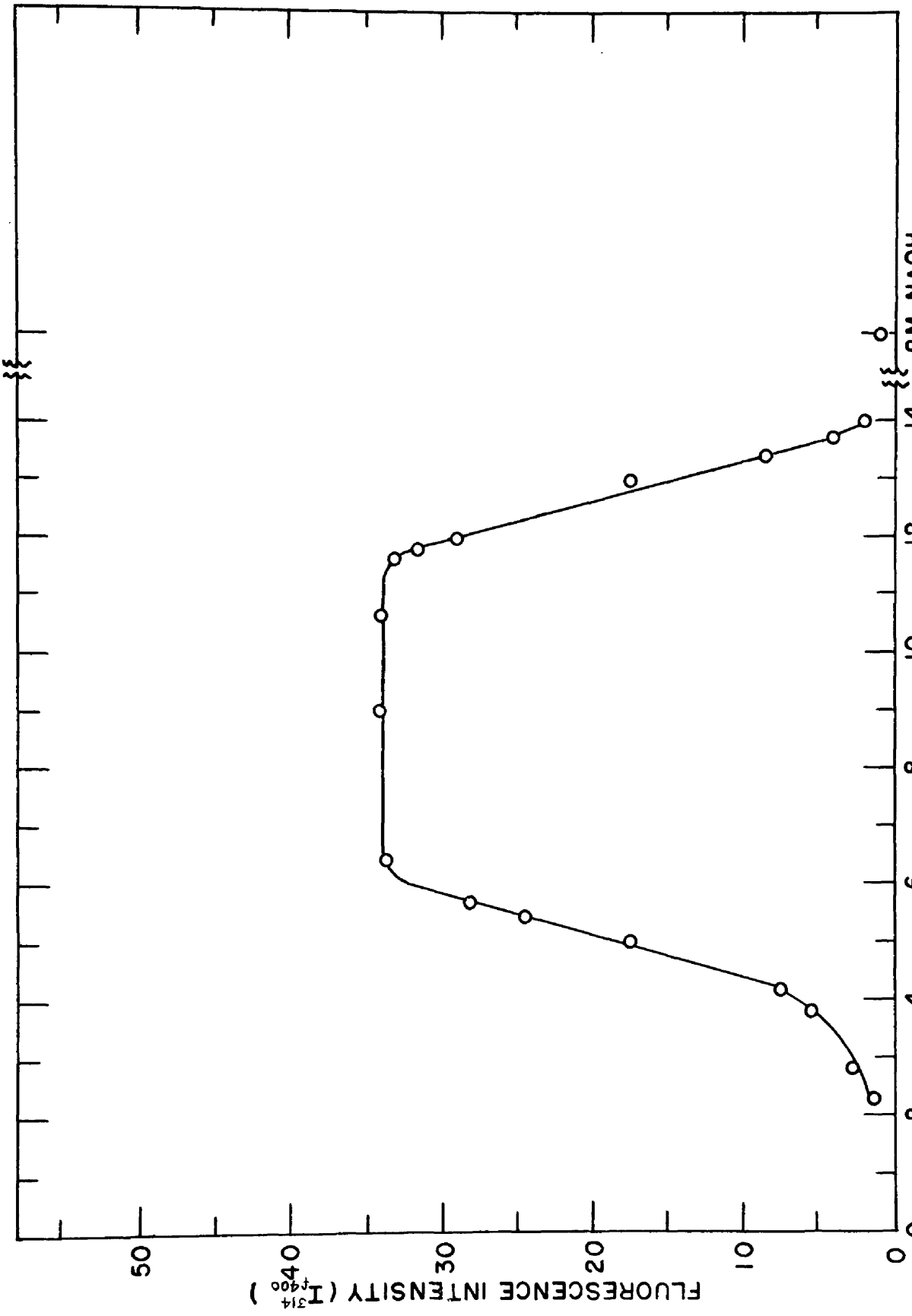
GRAPH 18. FLUORESCENCE SPECTRUM OF O-AMINOBENZOIC ACID



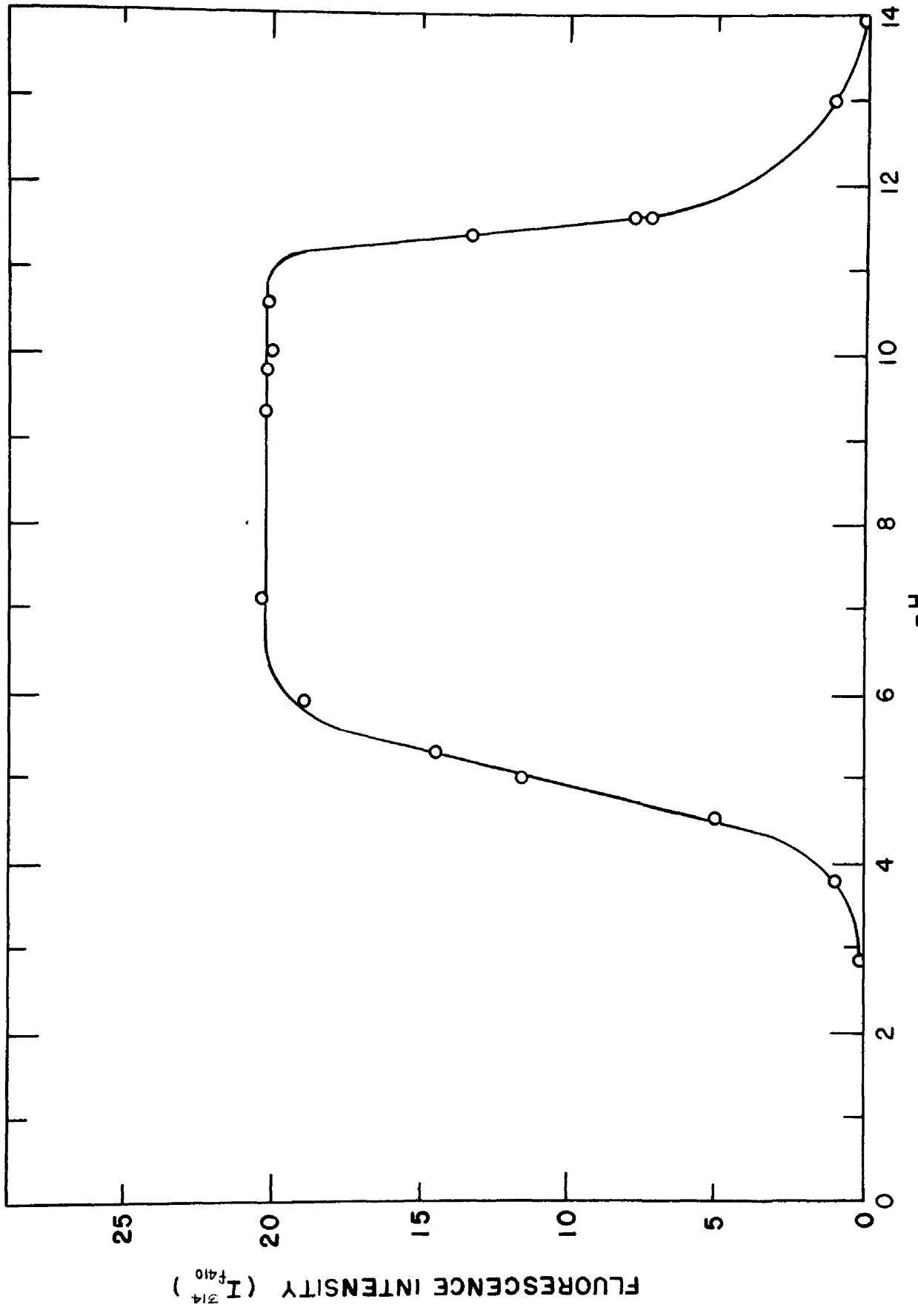
GRAPH 19. FLUORESCENCE SPECTRUM OF m-AMINOBENZOIC ACID



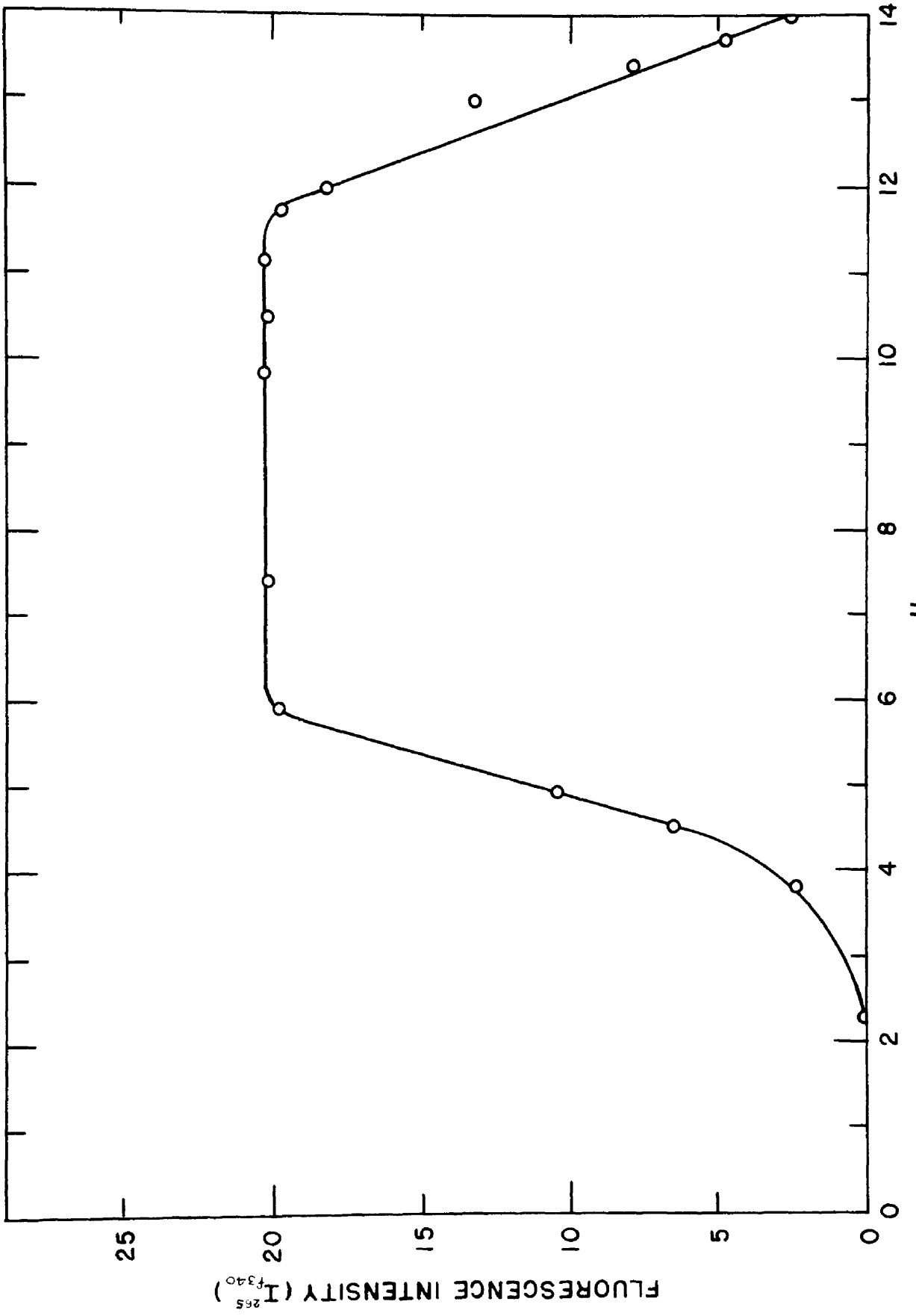
GRAPH 20. FLUORESCENCE SPECTRUM OF P-AMINOBENZOIC ACID



GRAPH 21. pH-FLUORESCENCE CURVE (O-AMINOBENZOIC ACID)

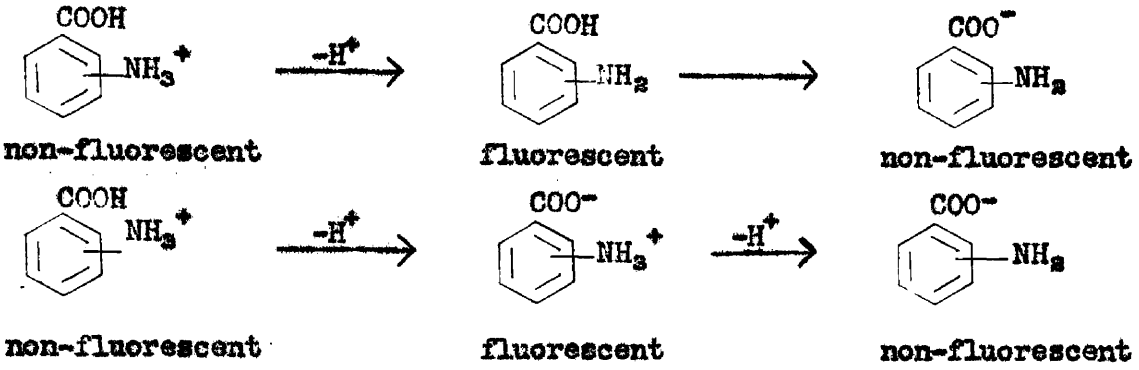


GRAPH 22. pH-FLUORESCENCE CURVE (m-AMINOBENZOIC ACID)

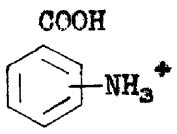


GRAPH 23. pH-FLUORESCENCE CURVE (P-AMINOBENZOIC ACID)

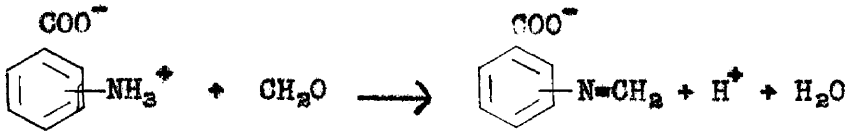
regions the solutions became non-fluorescent. At first observation it would seem then, that one of the following situations obtains



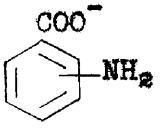
Both of these situations are, however, ruled out by the experimental facts. The first situation is realised to be nontenable when it is observed that the pH values at half-intensity for the rising legs of the curves, correspond exactly to the recorded (6) pKa values of the acids. Thus, the first process taking place, and that which leads to the fluorescent species, must be the neutralisation of the carboxyl group. The second possible process is also ruled out by the fact that Formal titrations of the amino acids indicate that all available acid is neutralized by a pH of 8.5. The formal titration procedure was carried out as follows. Known weights of the amino acids, 0.55 millimole, were dissolved in distilled water and a known volume of standard hydrochloric acid solution, 1.18 millimoles was added to each. The species present in solution was therefore:



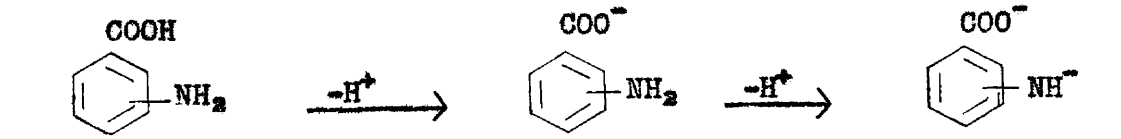
These solutions were titrated with standard sodium hydroxide solution and the neutralization process was followed by means of a Beckman model H pH meter. The titration was carried out to a pH of 8.5 and then 40 ml. of 20% formaldehyde solution, which had been adjusted to a pH of 8.5, was added, and the titration continued. The results of these titrations showed that no further hydrogen ion was released by the addition of formaldehyde.



The stoichiometry of the titration also showed that, at a pH of 8.5, all of the acid in the system had been neutralized. That is, 1.73 millimoles of base were required to reach a pH of 8.5. The conclusion drawn from these results is, therefore, that the species present, under the pH conditions in the plateau region, is:



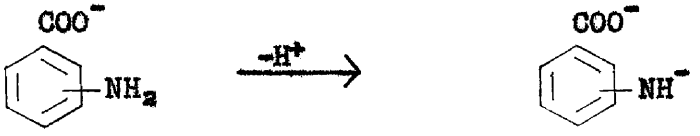
The difficulty in explaining the pH curves, now becomes one of defining the change which takes place causing the loss of fluorescence in strongly alkaline solution. If the amino group were to act as an acid in highly alkaline solution the process could be defined as:



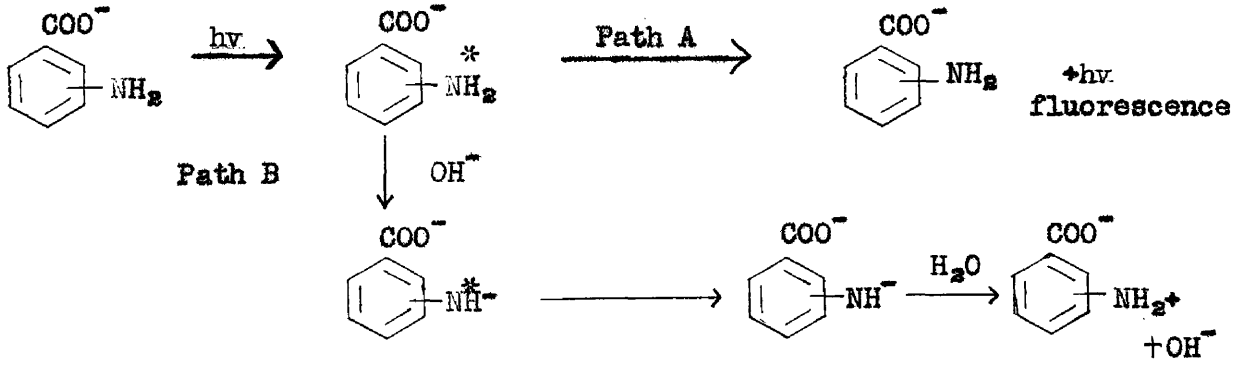
non-fluorescent I — fluorescent II — non-fluorescent III

This type of process seems highly improbable and by means of absorption spectra, has been shown to be not representative of the actual situation. Species II and III should exhibit different ultraviolet absorption spectra, however, the absorption spectra of the amino acids in pH = 9.0 solution and in 2 molar sodium hydroxide solution are identical. Consequently, the same species must be present in solution in the pH range of the plateau region and also beyond it, in more alkaline solution, where the fluorescence is lost. This fact forces one to the conclusion that the loss of fluorescence in strongly alkaline solution is due to a quenching process of some type. The quenching could be caused by trace quantities of a foreign material introduced by the sodium hydroxide or it could be caused by the sodium hydroxide itself. The latter, however, seems quite improbable since quenching is a process usually associated with trace quantities of an impurity. The possibility of quenching by trace quantities of a foreign material is also quite easily ruled out by the fact that the addition of hydrochloric acid and subsequent lowering of the pH of the solution, restores its fluorescent properties. This would only be possible if the addition of acid destroyed the quenching species. Carbonate ion is definitely an impurity in the sodium hydroxide solution and would be destroyed by the addition of acid. However, when carbonate ion is added to fluorescent solutions of the amino acids, it is found to have no quenching effect. It seems then, that all ordinary explanations of the loss of fluorescence in highly alkaline solution have been investigated and found lacking. Förster (7) has, however, noted

this complication before with the hydroxy- and aminopyrene sulfonates and has put forth what seems to be a logical explanation of the phenomenon. The essence of his explanation is that the excited state of a species will possess a different electrolytic dissociation constant from that of the ground state. Thus, a dissociation which does not take place when the species is in the ground state, may take place when it is in the excited state. With the amino acids it has been shown, by means of absorption spectra, that the dissociation



does not take place. However, the possibility does exist, following the reasoning of Förster, that in strongly alkaline solution the following process could take place.



That is, it is possible for the excited state, which has a different electrolytic dissociation constant from that of the normal state, to dissociate within the life-period of 10^{-8} seconds and thus kill the fluorescence by returning to the normal state by path B. No absolute

evidence can be put forth in support of the above hypothesis, but it is the only explanation which is consistent with all of the observed experimental facts.

Aside from the interesting behavior of the amino acids at higher pH's, the important information to be obtained from the pH curves is that the three isomers maintain constant fluorescence intensity over the pH range 7 to 10. Therefore, for quantitative work with these fluorescence intensities, it is desirable to work with solutions whose pH is within this range. A sodium hydroxide-boric acid buffer, having a pH of 9.0, was prepared and used in preparing all the amino acid solutions so that this condition would be fulfilled.

Referring again to the fluorescence spectra, Graphs 18, 19 and 20, it is obvious what approach must be used in an attempt at mixture analysis. The fluorescence maximum of the para isomer, under 265 mu excitation, occurs at 345 mu and hence is free from interference by the fluorescence spectra of the ortho and meta isomers. Therefore, an independent fluorescence intensity measurement, indicative of the para isomer concentration can easily be made. The fluorescence spectra of the ortho and meta isomers are, however, almost identical and are activated under the same conditions. Consequently, one must rely upon obtaining additivity of fluorescence intensities for the resolution of this pair. Activation of the ortho-meta pair can be effected by both 303 mu and 314 mu radiation. Therefore, the two measurements necessary for the calculation of the concentrations of the two isomers are readily obtained. Also, no interference from the para isomer is

encountered because the para isomer does not absorb at these wavelengths and hence, will not fluoresce.

2. Preparation of Calibration Curves

The amino acids employed in this study were Eastman White Label chemicals and were recrystallized from absolute methyl alcohol before use.

Aqueous stock solutions of the recrystallized acids were prepared which contained 1.5 milligrams of amino acid per milliliter of solution. All subsequent solutions prepared and used in the remainder of this work were prepared by dilution of aliquots of these solutions with borate buffer.

The borate buffer solution was prepared by dissolving 0.86 gm. sodium hydroxide and 3.1 gm. of boric acid in one liter of distilled water. Solutions of this composition give pH readings of 9.0 when they are measured on a Beckman pH meter.

The stock solutions, after standing several days, take on a yellow cast and on prolonged standing turn amber. This coloration is probably due to air oxidation of the amino group and indicates slow deterioration of the solutions. Although the solutions develop color within a few days, no significant difference in the fluorescence intensities, of solutions made by dilutions from them, could be detected over a period of two weeks time. However, as a precautionary procedure, fresh stock solutions were prepared whenever coloration was noted.

Because of the apparent instability of the amino acid solutions, it was decided to use a secondary standard for instrument calibration.

The order of magnitude of the fluorescence intensity observed for the aminobenzoic acids was approximately the same as that observed for the hydroxybenzoic acids. Consequently, a salicylic acid solution containing 6.0 p.p.m. of the acid in pH = 5.5 acetate buffer solution, was again used as the standard for instrument calibration. The standard solution, activated with 314 mu radiation, was assigned a reading of 14.0 at 410 mu. All subsequent measurements are, therefore, relative to instrument calibration with this standard solution. As before, the standard solution was prepared fresh each day to insure against possible deterioration and to obtain maximum reproducibility of measurements.

The range over which the fluorescence intensity was a linear function of the concentration was determined and found to be from 0 to 6 p.p.m. for all three acids. Calibration curves covering this concentration range were prepared for the three isomeric acids in the following manner.

(a.) o-Aminobenzoic acid: The fluorescence intensity, as a function of concentration, was measured at 400 and 410 mu under excitation by both 303 mu and 314 mu radiation. Readings were taken at 400 and 410 mu so that a double check could be made on the concentration of isomer present.

(b.) m-Aminobenzoic acid: The procedure here is identical to that listed above for o-aminobenzoic acid.

(c.) p-Aminobenzoic acid: The fluorescence was activated by absorption of 265 mu radiation and the fluorescence

intensity was determined as a function of concentration at 340 mu. The fluorescence intensity was measured at 340 mu, rather than at the 345 mu peak, as a precautionary measure against interference by the ortho and meta isomers. Their fluorescence spectra begin at 350 mu and should not interfere with the 345 mu para isomers emission. However, to insure against interference, the fluorescence intensity readings were taken at 340 mu.

Graphs 24, 25, 26, 27 and 28 show the calibration curves obtained in this manner.

Inspection of the o-aminobenzoic acid calibration curves, show that the fluorescence intensities as functions of concentration can be expressed by a slope-intercept linear equation. Thus, for 314 mu excitation of o-aminobenzoic acid solutions, the following expressions are true.

(a.) For 400 mu emission

$$I_{fo_{400}}^{314} = 9.2\ Co + 0.5$$

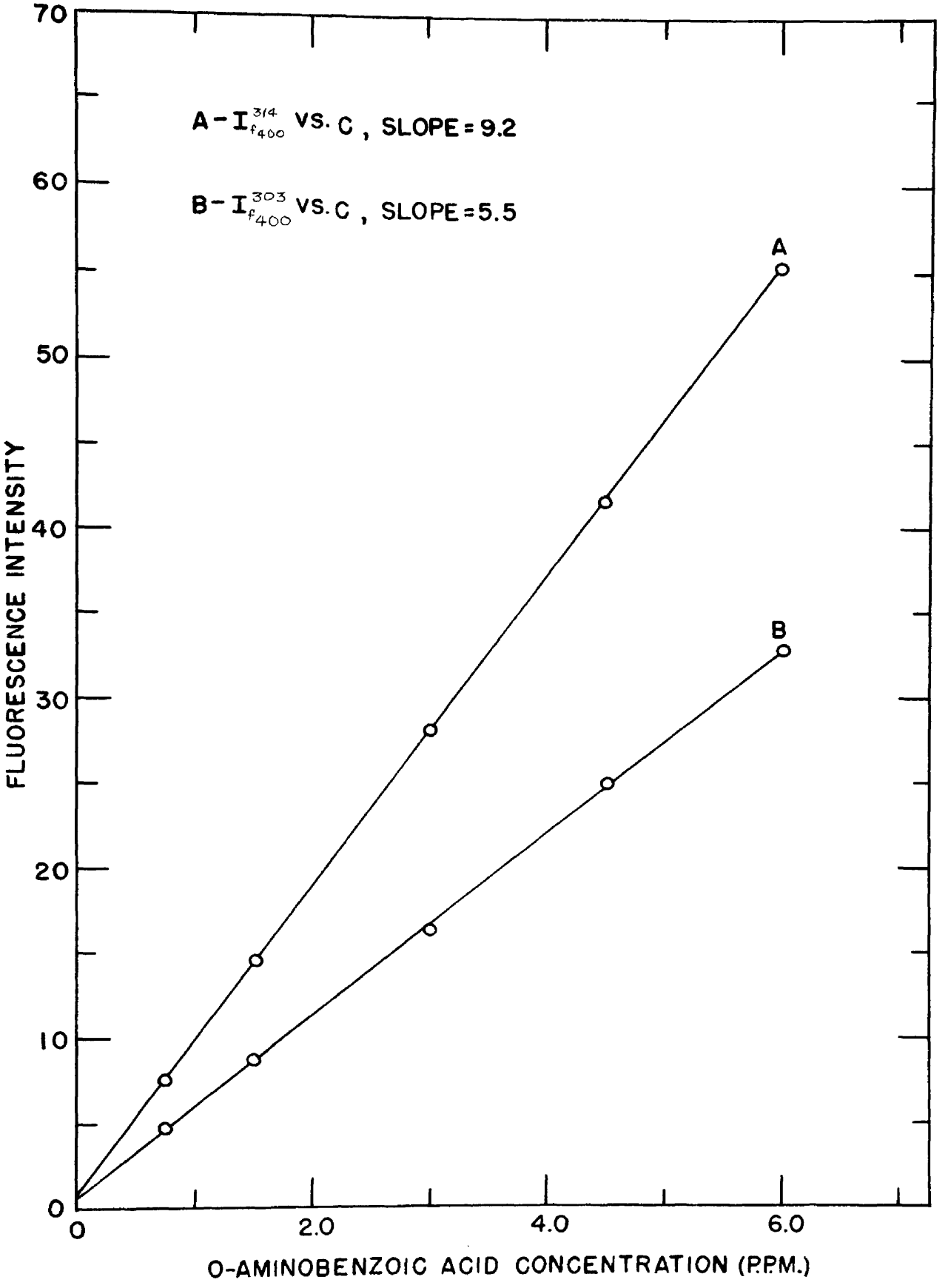
(b.) For 410 mu emission

$$I_{fo_{410}}^{314} = 8.6\ Co + 0.5$$

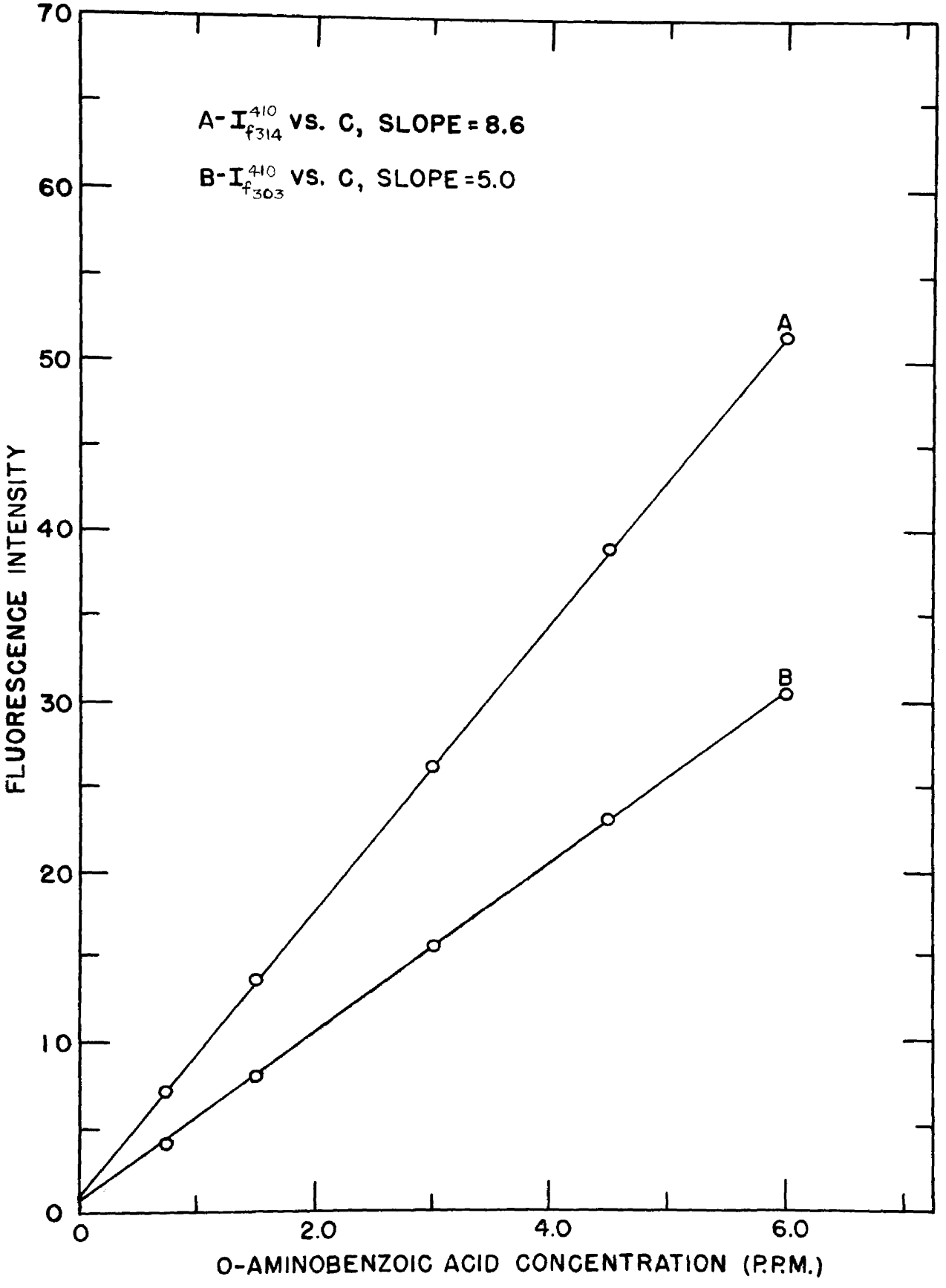
where I_f = fluorescence intensity

Co = concentration of o-aminobenzoic acid in p.p.m.

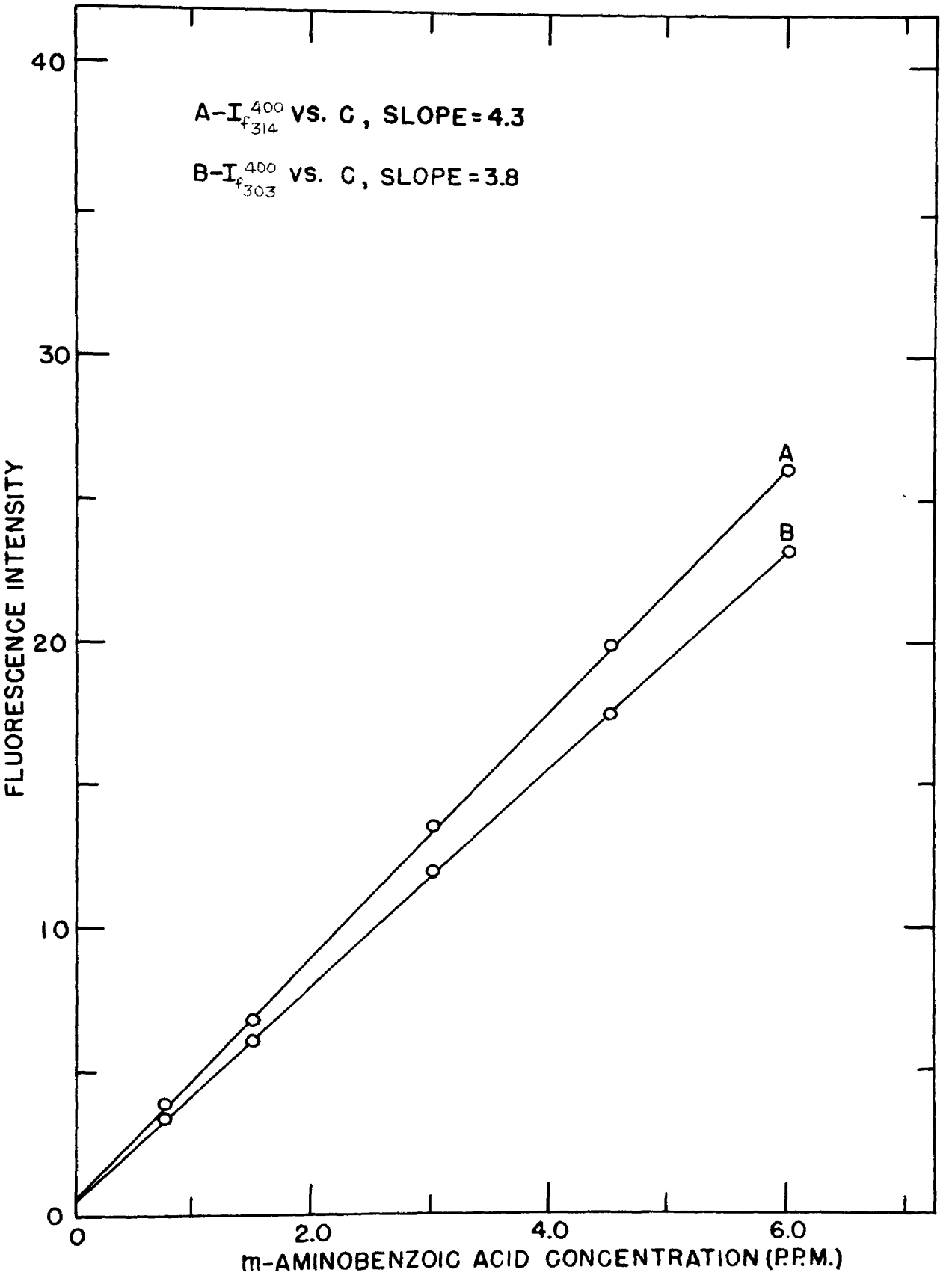
Similarly, for 303 mu excitation of o-aminobenzoic acid, the following expressions are true.



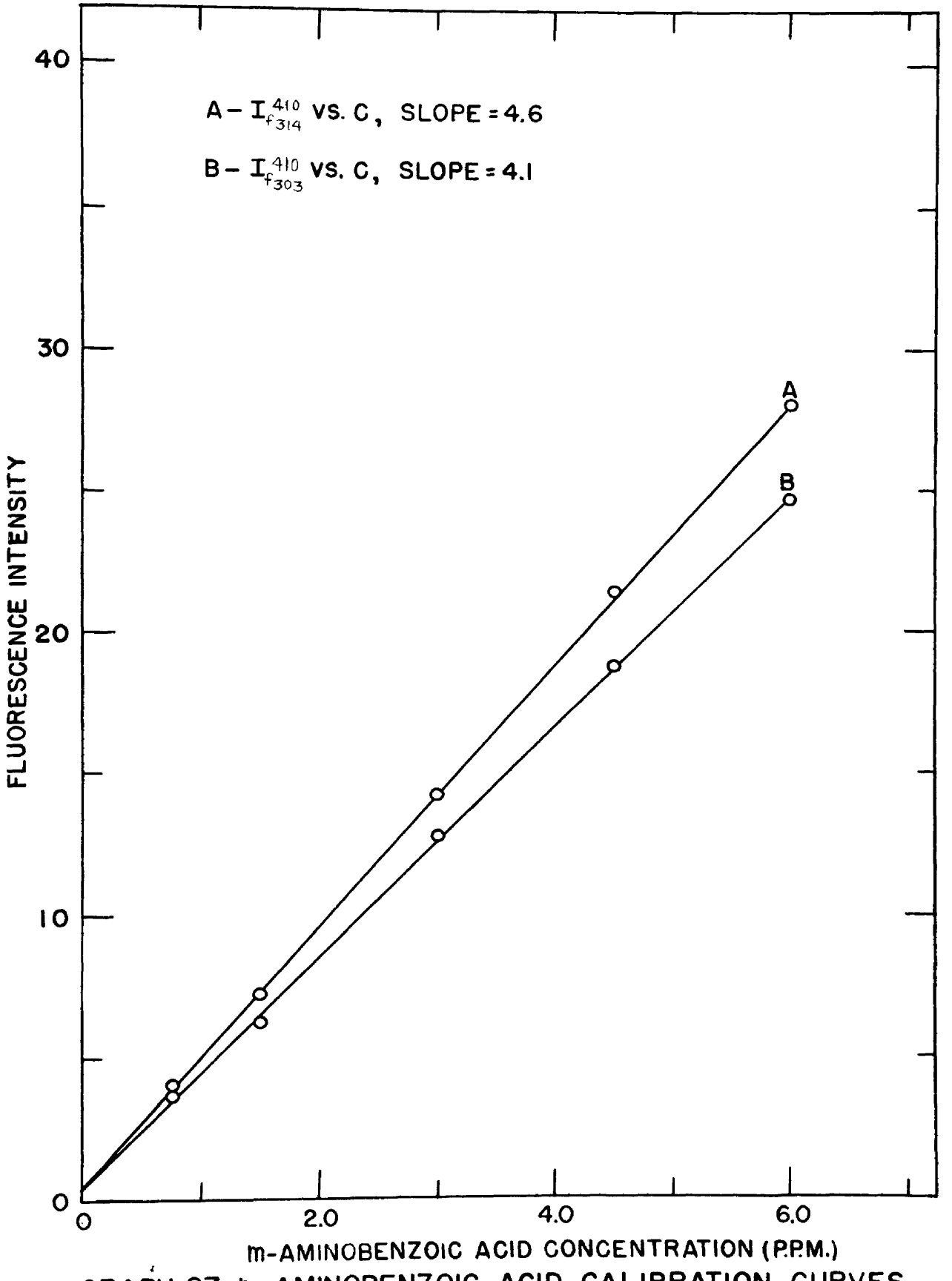
GRAPH 24. O-AMINOBENZOIC ACID CALIBRATION CURVES



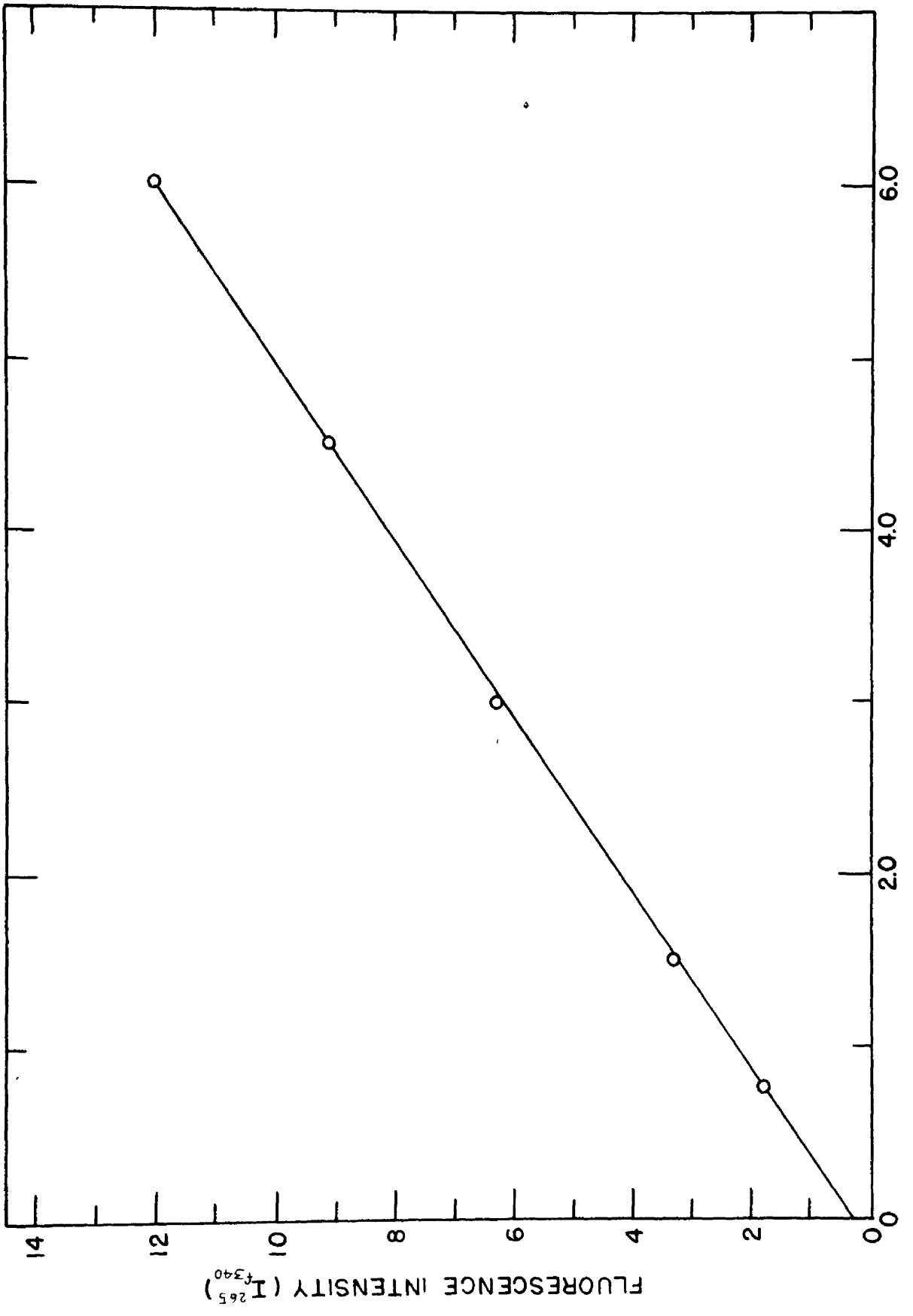
GRAPH 25. O-AMINOBENZOIC ACID CALIBRATION CURVES



GRAPH 26. m-AMINOBENZOIC ACID CALIBRATION CURVES



GRAPH 27. m-AMINOBENZOIC ACID CALIBRATION CURVES



GRAPH 28. P-AMINOBENZOIC ACID CALIBRATION CURVE

(c.) $I_{fo_{400}}^{303} = 5.5\ Co + 0.5$

(d.) $I_{fo_{410}}^{303} = 5.0\ Co + 0.5$

The calibration curves of m-aminobenzoic acid can be expressed in exactly the same manner.

(e.) $I_{fm_{400}}^{314} = 4.3\ Cm + 0.5$

(f.) $I_{fm_{410}}^{314} = 4.6\ Cm + 0.5$

(g.) $I_{fm_{400}}^{303} = 3.8\ Cm + 0.5$

(h.) $I_{fm_{410}}^{303} = 4.1\ Cm + 0.5$

When both the ortho and meta isomers are present in a solution, the fluorescence intensity measurements at 400 mu or at 410 mu, under 314 or 303 mu irradiation, should be the sum of the individual intensities. This can be illustrated with the readings taken at 400 mu.

(i.) $I_{s_{400}}^{314} = I_{fo_{400}}^{314} + I_{fm_{400}}^{314} = 9.2\ Co + 4.3\ Cm + 1.0$

(j.) $I_{s_{400}}^{303} = I_{fo_{400}}^{303} + I_{fm_{400}}^{303} = 5.5\ Co + 3.8\ Cm + 1.0$

Therefore, if the predicted additivity is a reality, simultaneous solution of equations (i.) and (j.) will yield values of the concentrations of the ortho and meta isomers present. The identical procedure can be carried out for the fluorescence measurements at 410 mu and another pair of values for the concentrations of the ortho and meta isomers can be obtained. The average values of the concentrations obtained from the 400 mu measurements, and those obtained from the 410 mu measurements,

should then be representative of the true situation if additivity of fluorescence intensities is obtained.

The calibration curve for the para isomer is used directly, since the reading at 340 mu, under 265 mu excitation, is independent of any interference by the ortho or meta isomers.

Similarly, if either the ortho or meta isomer is present alone or in a mixture with the para isomer their concentrations are determined directly from the calibration curves. It is only when the ortho and meta isomers are present at the same time that one must resort to calculation from simultaneous equations. When the ortho isomer is free from interference by the meta isomer or vice-versa, four fluorescence intensity measurements are taken which are indicative of its concentration. These are; $I_{f_{400}}^{314}$, $I_{f_{410}}^{314}$, $I_{f_{400}}^{303}$ and $I_{f_{410}}^{303}$, where the superscript refers to the wavelength employed for excitation and the subscript refers to the wavelength at which the fluorescence intensity is measured. Concentrations corresponding to these intensities, are obtained from the appropriate calibration curves and an average concentration is reported.

3. Results of Mixture Analysis

Test mixtures of the three possible binary mixtures and the one ternary mixture were prepared. The mixtures were prepared by taking aliquots from the stock solutions of the acids and diluting them to 100 ml. with borate buffer solution. Therefore, the final test solutions, upon which measurements were taken, were all at a pH of 9.0.

The measurement procedure on the test solutions was conducted as follows:

(1.) The test solution is activated with 265 mu radiation and a fluorescence intensity measurement is made at 340 mu. This reading will yield the concentration of the para isomer directly from Graph 28, the para isomer calibration curve.

(2.) The wavelength of the activating radiation is changed to 303 mu and fluorescence intensity measurements are made at 410 and 430 mu. This procedure is then repeated with 314 mu activation. The four readings, obtained in this manner, give four checks on the concentration of either the ortho or meta isomers if they are present alone. If the two isomers are present together, the four readings yield two sets of simultaneous equations from which the concentrations of the ortho and meta isomers can be calculated. The concentrations reported then, are the average concentrations found from the four measurements if either of the isomers is present alone; or if both are present, the concentrations reported are the averages of those obtained from the solution of the two sets of simultaneous equations.

The results obtained on the four possible types of mixtures are shown in Table VIII.

It was mentioned previously that the concentration of the ortho and meta isomers reported, when they are not simultaneously present, are the average concentrations obtained from the four readings;

TABLE VIII

RESULTS OF DETERMINATIONS OF MIXTURES OF AMINOBENZOIC ACID ISOMERS

Mixture Number	Ortho			Meta			Para		
	p.p.m. Taken	p.p.m. Found	Percent Error	p.p.m. Taken	p.p.m. Found	Percent Error	p.p.m. Taken	p.p.m. Found	Percent Error
1	0.75	0.78	+4.0	--	--	--	3.00	3.10	+3.3
2	4.50	4.43	-1.6	--	--	--	4.50	4.45	-1.1
3	1.50	1.47	-2.0	--	--	--	1.50	1.59	+6.0
4	--	--	--	4.50	4.52	+0.45	1.50	1.63	+8.7
5	--	--	--	3.00	2.97	-1.0	3.00	3.13	+4.3
6	--	--	--	0.75	0.76	+1.3	6.00	5.93	-1.2
7	1.50	1.58	+5.3	1.50	1.22	-18.7	--	--	--
8	0.75	0.90	+20.0	3.00	2.75	-8.3	--	--	--
9	4.50	4.41	-2.0	0.75	0.56	-25.3	--	--	--
10	1.50	1.59	+6.0	0.75	0.37	-50.6	6.00	5.98	-0.3
11	3.00	3.11	+3.7	3.00	2.62	-12.7	3.00	3.05	+1.7
12	4.50	4.35	-3.3	1.50	1.17	-22.0	1.50	1.50	0

$I_{f_{400}}^{303}$, $I_{f_{410}}^{303}$, $I_{f_{400}}^{314}$ and $I_{f_{410}}^{314}$. In order that the average concentrations, of the ortho isomer in mixtures 1, 2 and 3, and the meta isomer in mixtures 4, 5 and 6 be more meaningful, Table IX has been constructed to show the concentrations found from the four readings and averaged to give the values reported in Table VIII.

TABLE IX

RESULTS OBTAINED FROM INDIVIDUAL READINGS ON THE ORTHO AND META ISOMERS

	Concentration (p.p.m.) Ortho Isomer Found			
Mixture	$I_{f_{400}}^{303}$	$I_{f_{410}}^{303}$	$I_{f_{400}}^{314}$	$I_{f_{410}}^{314}$
1	0.79	0.77	0.79	0.77
2	4.45	4.40	4.43	4.44
3	1.44	1.48	1.47	1.49
	Concentration (p.p.m.) Meta Isomer Found			
Mixture	$I_{f_{400}}^{303}$	$I_{f_{410}}^{303}$	$I_{f_{400}}^{314}$	$I_{f_{410}}^{314}$
4	4.53	4.50	4.52	4.53
5	3.00	2.95	2.96	2.97
6	0.78	0.76	0.73	0.77

The values recorded above indicate that, on the average, all four measurements are equally accurate. Consequently, for practical application, it would only be necessary to take one of the four readings to obtain a representative measure of the concentration of whichever isomer is present.

Referring again to Table VIII, it is seen that in all cases the concentration of the para isomer is easily and accurately determined by the measurement at 340 mu. Similarly, if either the ortho or meta isomer is present alone with the para isomer, no difficulty is encountered in obtaining accurate measurements of their concentrations. However, when the ortho and meta isomers are present together and one must rely upon the solution of simultaneous equations to determine their concentrations, the results are not nearly as good. The error, percentage-wise, is considerably higher than that observed in mixtures where it is not necessary to rely upon additivity of fluorescence intensities. The percent error, as would be expected, is greater at lower concentrations and in the extreme case is approximately 50%. The resolution of the ortho-meta pair should, therefore, be classified as an approximation rather than a determination. The approximation should be valid, even in the extreme cases to approximately $\pm$ 0.5 p.p.m.

If a large number of approximations of the ortho and meta isomers concentrations were to be carried out, one could avoid the tedious process of arithmetic calculation of their concentrations by the preparation of a calibration grid similar to that described in a previous section of this work. This could be accomplished by plotting values of $I^{314}_{f_{400}}$ versus values of $I^{303}_{f_{400}}$ for a series of mixtures containing known quantities of the two isomers. If the points corresponding to constant ortho concentration were then joined and the same was done for points corresponding to constant meta concentration, a calibration grid would be obtained. A grid could be obtained in like manner for the fluorescent

emission at 410 mu. The two calibration grids would replace the two sets of simultaneous equations and make it possible to obtain the concentrations of the ortho and meta isomers, from the 400 and 410 mu readings, without calculation.

SUMMARY

SUMMARY

A spectrofluorometer has been constructed to facilitate the obtaining of fluorescence spectra under conditions of monochromatic excitation.

The instrument has been utilized to study the fluorescence characteristics of some mixtures of fluorescent organic compounds, which are chemically quite similar. These studies have shown that differences in the fluorescence characteristics of these compounds do exist and that they can be employed to analyze mixtures of these compounds.

The spectrofluorometric approach has been applied to the following mixtures of organic compounds and has proved to be a rapid and accurate analytical method.

1.) Hydrocarbon mixtures consisting of anthracene, phenanthrene and fluorene, in all possible mixture combinations have been determined. All three components can be determined individually with an average error of less than 5%. The concentration range, over which the method is applicable, is 0 to 5 p.p.m. (0 to 5 micrograms per milliliter). However, higher concentrations can be handled by diluting until the concentrations fall within this range.

2.) Isomeric mixtures of the hydroxybenzoic acids have been studied and it has been found that mixtures of the ortho and meta isomers can be quantitatively determined. Furthermore, the para isomer is non-fluorescent, under the conditions employed, and does not constitute an interference in the

determination of the ortho and meta isomers. The method developed is applicable over the concentration range of 0 to 12 p.p.m. and yields results, for both isomers, which are subject to an average error of less than 5%.

3.) Mixtures of the isomeric aminobenzoic acids have also been studied and analytical methods have been developed for the determination of the following mixture components:

(a.) Both the ortho and para isomers in the presence of one another.

(b.) Both the meta and para isomers in the presence of one another.

(c.) The concentration of the para isomer in the presence of both the ortho and meta isomers.

The above determinations can be carried out over the concentration range 0 to 6 p.p.m. with an expected average error of less than 5%.

A method has also been described for the approximation of the concentrations of the ortho and meta isomers in mixtures. The method is applicable over the concentration range 0 to 6 p.p.m. but yields results which are accurate to only $\pm$ 0.5 p.p.m.

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