FLUORIMETRIC ANALYSIS OF PYRIDINE AND ITS DERIVATIVES AFTER HYDROXYLATION WITH THE HAMILTON HYDROXYLATION SYSTEM

BY

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I am indebted to Professor Joseph R. Robinson for lending me the Orion Research digital pH meter and the high temperature water-bath.

I praise and thank God for the strength He has given me to persevere, His continual love, guidance, and discipline.

To My Dear Roommates

Chiew Hui, Estella, and Nora

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I. INTRODUCTION

One of the fruitful directions of organic analytical research is the development of a body of general reactions on which the analyst can draw when confronted with a particular problem. In this context, the ability of the Hamilton hydroxylation system, using catechol, ferric perchlorate, and hydrogen peroxide as reagent, to introduce a hydroxy group on an aromatic ring is analytically potentially useful. Among the various non-enzymatic hydroxylating systems, namely the Fenton, Hamilton, and Udenfriend, the Hamilton system appears simplest and its yields of hydroxylated product are highest.

Albert and Connors (1,2) have used this hydroxylation system as an analytical reagent for the analysis of aqueous solutions of aromatic compounds by their conversion to phenols, which were subsequently determined colorimetrically. The method as described has some disadvantages, including susceptibility to interferences, and the necessity to add a stabilizer (glucose or cyclodextrin).

The purpose of this research was to carry out the hydroxylation step and then measure the resulting hydroxylated product fluorimetrically, because most hydroxy-

compounds fluoresce strongly. This will eliminate the color development step. It may also eliminate the need for the stabilizer if polyhydroxylation, which had to be eliminated in the colorimetric method, does not seem to be a problem in the fluorimetric method. Attention was restricted to pyridine compounds because they do not fluoresce strongly whereas the hydroxy- pyridines do. For applicable compounds, fluorescence spectroscopy has the advantage of selectivity over absorption spectroscopy. It is useful to see if the analytical method could analyze mixtures of structurally similar compounds which would otherwise be very difficult.

A. Hydroxylation Systems

When an aromatic compound "foreign" to the organism is administered to animals, it is usually converted to hydroxy derivatives. In vivo hydroxylations generally result in a fairly random distribution of the hydroxy isomers (3). Similarly, nonspecific hydroxylation is brought about by an enzyme system in liver microsomes (4). In order to elucidate the mechanism of hydroxylation in the biological processes, simple chemical systems have been considered as models for such reactions. Among

the various non-enzymatic hydroxylation systems, those of Fenton (5-9), Udenfriend (10-14), and Hamilton (15-19) were extensively studied. Reviews of aromatic hydroxylation are also available (20-23).

Table I compares the percentage yield of phenol and phenol isomer distribution from the hydroxylation of anisole using the Hamilton, Fenton, and Udenfriend reagents (16).

Table I. Products from the Hydroxylation of Anisole[a] (16).

Reagents	% Yield of Phenols[b]	Phenol isomer Distribution %		
		o-	m-	p-
Hamilton system using Fe(III)-catechol-H_2O_2[c]	55	64	3	33
Hamilton system with Fe(III)-hydroquinone-H_2O_2[d]	58	65	5	35
Fenton system using Fe(II)-H_2O_2[e]	20	86	0	14
Udenfriend system with Fe(III)-EDTA-ascorbic acid-H_2O_2[f]	5	88	0	12

[a] Analyzed by gas chromatography.

[b] Yield based on initial amount of H_2O_2.

[c] Acetate buffer, 0.005 M, pH 4.3; $NaClO_4$, 0.15 M; anisole 0,01 M; catechol 1.5×10^{-4} M; $Fe(ClO_4)_3$, 7.9×10^{-5} M; H_2O_2, 1.75×10^{-3} M.

[d] same as ref. c but with hydroquinone, 1.5×10^{-4} M, instead of catechol.

[e] Acetate buffer, $NaClO_4$, anisole, and H_2O_2 same as ref. c and in addition $Fe(ClO_4)_2$, 2.01×10^{-3} M.

[f] Acetate buffer, $NaClO_4$, and anisole same as ref. c and in addition: $Fe(ClO_4)_3$, 2.17×10^{-3} M; EDTA, 8×10^{-3} M; L(+) ascorbic acid, 1.01×10^{-2} M; H_2O_2, 1.79×10^{-2} M.

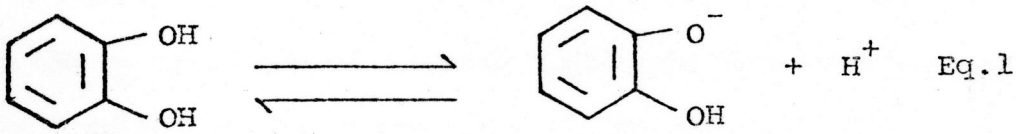
Of the several non-enzymatic hydroxylation systems compared, the Hamilton system affords a means of converting aromatic compounds to phenols in highest yield. The Hamilton systems using either hydroquinone or catechol give an isomer distribution of products very different from that observed in the Fenton or Udenfriend reagents. The Fenton reaction involves the hydroxyl radical as the hydroxylating agent (23). The similarity of products in the Udenfriend system suggests that the same hydroxylating species is involved (9). The higher yields of products and the different isomer distribution obtained from the Hamilton system indicate that a different hydroxylating agent might be involved.

Hamilton, et al. (17,18) studied the hydroxylation of some mono-substituted benzene derivatives. Products obtained from the hydroxylation were determined quantitatively by gas chromatography. The low intramolecular selectivity indicates a radical-like reagent, and the low substrate selectivity (anisole and nitrobenzene possess comparable reactivities) suggests a non-electrophilic reagent as shown in Table II.

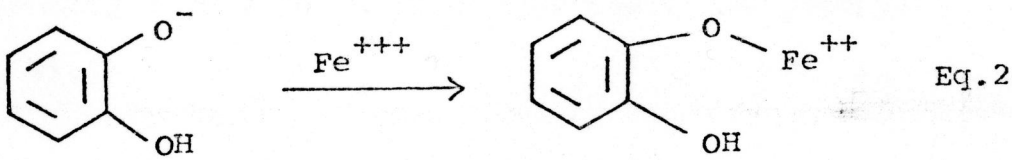
Table II. Product Distribution and Reactivity for the Hydroxylation of Aromatic Compounds by the Hamilton and Fenton Systems (18).

	Hamilton System				Fenton System			
X in PhX	% o	% m	% p	$\frac{k_{ArX}}{k_{ArH}}$	% o	% m	% p	$\frac{k_{ArX}}{k_{ArH}}$
H	-	-	-	1.0	-	-	-	1.0
OMe	64	3	33	1.4	84	0	16	6.4
Cl	45	15	40	0.6	42	29	29	0.6
NO_2	48	26	26	0.6	24	30	36	0.1

The following mechanistic scheme for hydroxylation with the Hamilton system has been suggested (34):

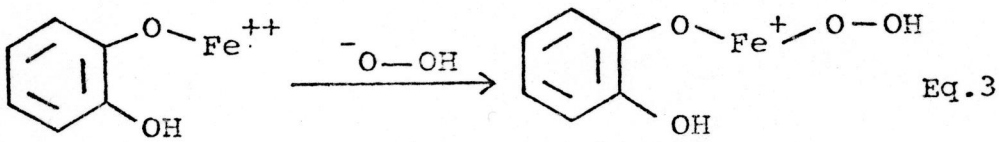


Eq.1



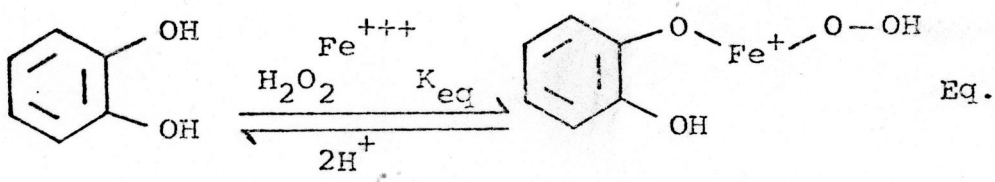
Eq.2

Complex I



Eq.3

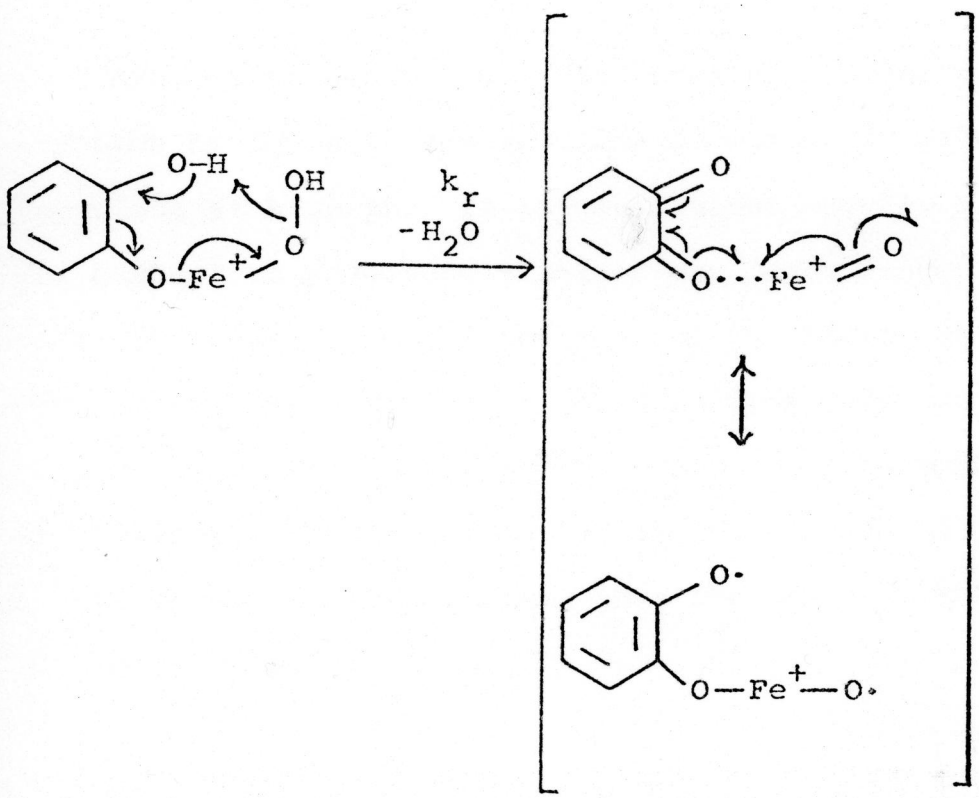
Complex II



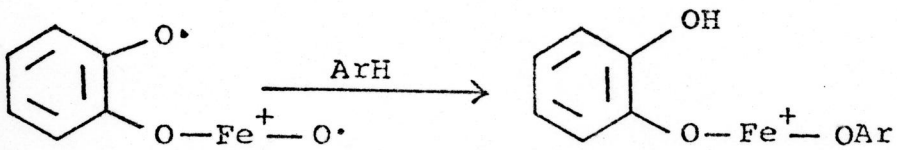
Eq.4

Complex II

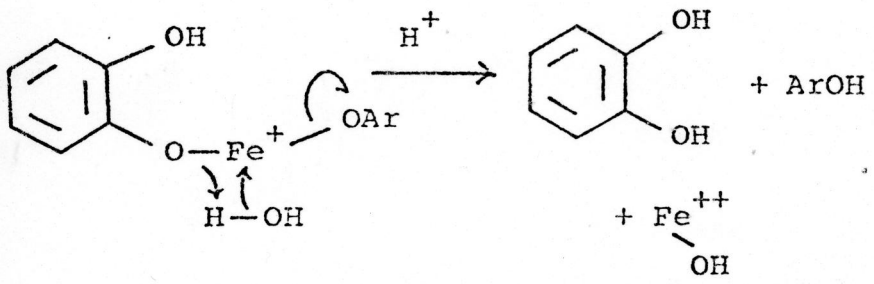
Equations 1, 2, and 3 can be summarized by Eq. 4. The complex formed by catechol, Fe(III), and H_2O_2 then loses a molecule of water as shown by Eq. 5. This was thought to be the rate-determining step. Since iron can transfer electrons by overlapping its d orbitals with the p orbitals of ligands (24), the transition state leading to the formation of the radical-like hydroxylating reagent is stabilized by resonance. This reagent then attacks an aromatic compound (Eq. 6) to eventually form a phenol and regenerate the catalyst (Eq. 7).


O-H
OH
O-Fe+
O
kr
-H2O
O
O···Fe+
O
O·
O—Fe+—O·

Eq.5

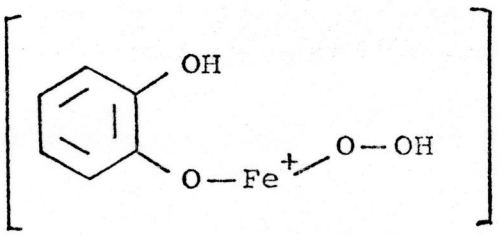

O·
O—Fe+—O·
ArH
OH
O—Fe+—OAr

Eq.6

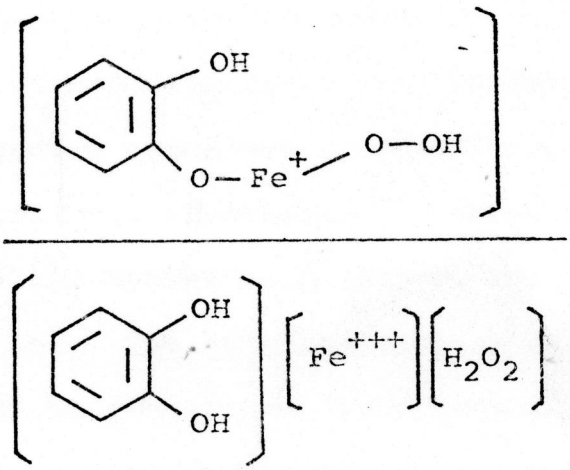

OH
O—Fe+
OAr
H—OH
H+
OH
OH
+ ArOH
+ Fe++
OH

Eq.7

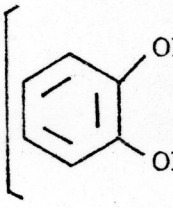
High pH seems to favor the formation of the complex as shown by Eq. 4, since equilibrium would be shifted to the right at high pH. It is not obvious why the yield was highest at pH around 4 unless the destruction of the catalyst occurs at a greater rate at the higher pH's. The isomer distributions obtained by hydroxylation at various pH values were similar. Presumably, reactions at different pH's undergo a similar mechanism. If Eq. 4 is the rate-determining step, then the rate of reaction can be described by Eq. 8.

rate of reaction = k_r  Eq.8

where k_r is the rate constant for the loss of a molecule of water from the complex of ferric, catechol, and H_2O_2.

Assume K_{eq} =  Eq.9

Substituting Eq. 8 into Eq. 9 yields Eq. 10

$$\text{rate of reaction} = k_r \times K_{eq} \left[\text{  } \right] \left[Fe^{+++} \right] \left[H_2O_2 \right] \quad \text{Eq.10}$$

Mechanistic studies of the Hamilton system reported the following (34):

1. The reaction is first-order in Fe(III).
2. If the enediol is omitted, H_2O_2 reacts much slower.
3. Fe(III) can be replaced by Fe(II), or less effectively Cu(II).
4. The reaction is inhibited by EDTA.

These features are in agreement with the proposed mechanism. Table I showed that the Hamilton system gives the highest yield of phenol on hydroxylation of anisole when compared with the Fenton or Udenfriend system. The mechanism described for the Hamilton hydroxylation system suggested a radical-like non-electrophilic reagent. Therefore, it seems appropriate to use the Hamilton system as a reagent for the hydroxylation of pyridine. The hydroxypyridines formed presumably can then be determined fluorimetrically, since hydroxypyridines fluoresce strongly while the parent

pyridine compounds do not.

B. Fluorimetric Determination of Final Products

Spectrofluorimetry in general is much more sensitive than absorption spectrophotometry. One reason is that absorbance values do not depend on the intensity of the incident light, whereas the intensity of fluorescence is directly proportional to the intensity of the exciting light source. The relation between fluorescence intensity and concentration of sample compound at low concentrations can be expressed by the following equation:

$$F = 2.3 \, \phi \, I_o \, \varepsilon \, b \, C$$

where I_o = intensity of incident light

b = thickness of cell

C = molar concentration of fluorescing species

ε = molar absorptivity

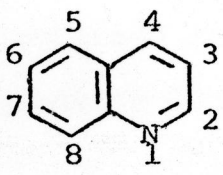
ϕ = quantum yield

From the above relation, fluorescence intensity, and hence sensitivity, could be increased if a high intensity light source is used. On the other hand,

this would affect fluorescence measurements if the lamp intensity is unstable. Fluorimetric measurement also has the advantage of being selective. Some compounds fluoresce strongly while others do not, depending on the quantum yield of the compound. Another advantage spectrofluorimeters offer is that only relative intensities of fluorescence are read from an arbitrary scale. Suitable instrumental adjustments allow the reading of solutions within a very wide range of fluorescence intensities.

Fluorescence was chosen as the final analytical finish because heterocyclic compounds usually have a low quantum efficiency whereas the hydroxy-heterocyclic compounds have a higher quantum efficiency. One example is shown in Table III.

Table III. Effect of the Position of Substitution on the Fluorescence Intensities of Some Hydroxyquinolines (25).

	Position of OH Substitution	Relative Intensity
 Quinoline	None	1.0
	2	40
	3	245
	4	6.0
	5	8.0
	6	207
	7	29
	8	8.0

II. EXPERIMENTAL

A. Materials

All inorganic chemicals were analytical reagent grade, and were used without further purification. Ferric perchlorate hexahydrate ($Fe(ClO_4)_3 \cdot 6H_2O$) was obtained from G. F. Smith Co. and was used directly. Potassium chloride, sodium hydroxide, and sodium borate were obtained from Mallinckrodt Chemical Works. Hydrogen peroxide 30% analytical reagent grade was obtained from Fisher Scientific Co. Perchloric acid, obtained from Baker Chemical Co. meets A.C.S. specifications.

Acetic acid, analytical reagent, was obtained from Mallinckrodt. Sodium acetate (Baker Chemical Co.) meets A.C.S. specifications. Pyridine was obtained from Merck Chemical Co., reagent grade, and from Fisher Scientific Co., Certified A.C.S. The following compounds obtained from Aldrich were used without further purification: o-hydroxypyridine, m-hydroxypyridine, p-hydroxypyridine, isoquinoline, 3-quinoline carbonitrile, 4-quinoline carboxaldehyde, 2-pyridinealdoxime, 2-aminopyridine, pyridoxine·HCl, 2,3-pyridine dicarboxylic acid, 2-chloro-6-methoxypyridine, 2,4,6-trimethylpyridine, 3-picoline, 4-picoline.

The following chemicals, listed according to source, were purified before use. 2-picoline (Aldrich) was distilled, b.p. 127 - 129° (lit. (26), 128.8°); quinoline (Aldrich) and quinaldine (Aldrich) were both double distilled at reduced pressure; 2,6-pyridine dicarboxylic acid (Aldrich) was recrystallized from water, m.p. 252° (lit. (27) 252°); 4-dimethyaminopyridine (Aldrich) was recrystallized from Skellysolve A, m.p. 113 - 114° (lit. (27) 114°); catechol (Eastman Organic Chemicals) was recrystallized twice from toluene, m.p. 103 - 104° (lit. (28) 104°).

Standard buffers were prepared according to Bates (29-31). All water used was purified by passage of once-distilled water through an ion-exchange column (Continental Deionized Water Service). pH 4.0 acetate buffer, in which hydroxylation reactions were run, was prepared to be 5×10^{-3} M in total buffer concentration, ionic strength 0.1 M. Ionic strengths were adjusted using potassium chloride. Borate buffer was prepared to be 0.4 M in total sodium borate and 0.2 M in sodium hydroxide. Aqueous catechol was 3×10^{-3} M. Ferric perchlorate stock solution (1.5×10^{-3} M) was prepared by diluting 10.0 ml of ferric perchlorate stock solution to 100.0 ml with water.

B. Apparatus

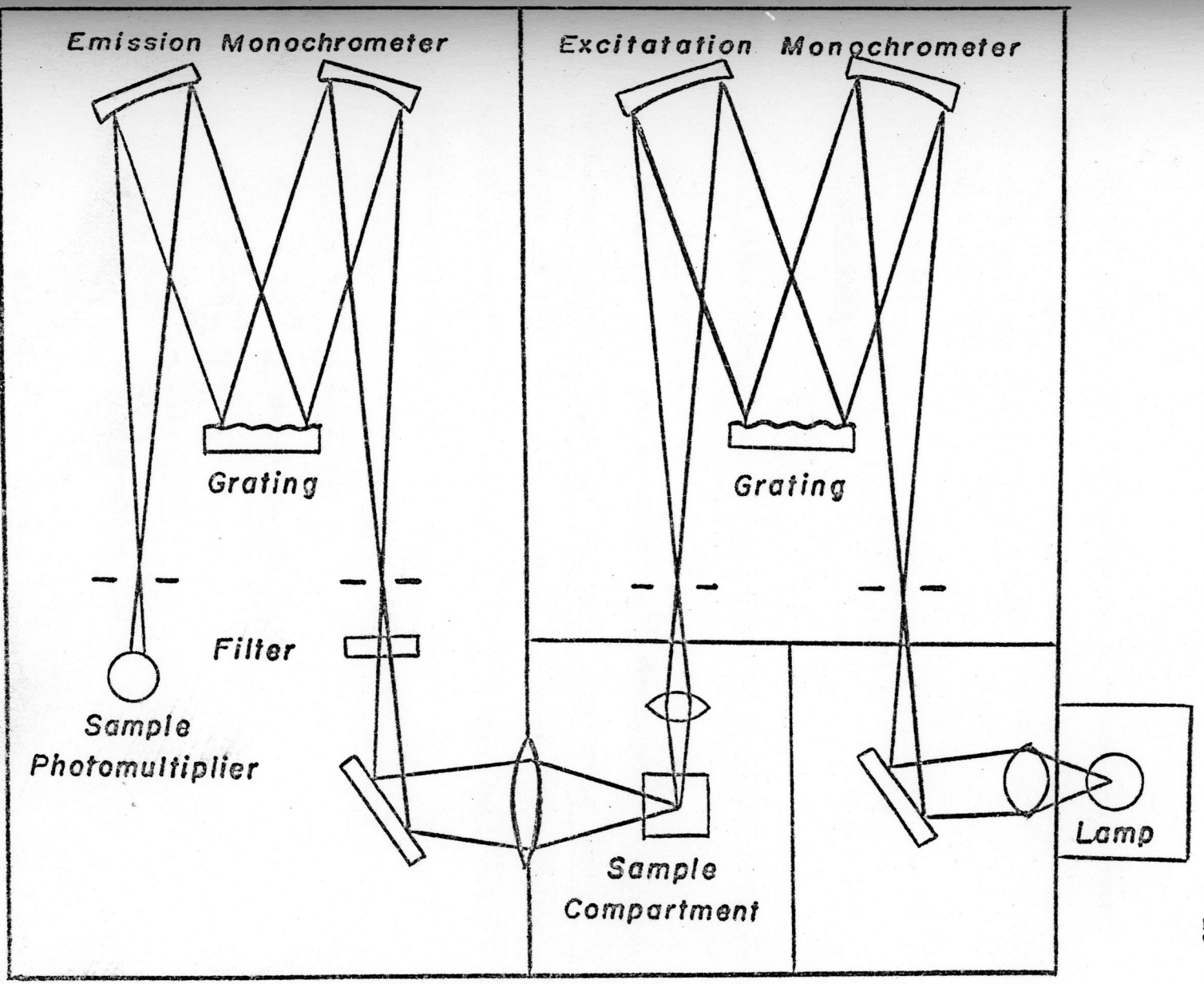
Fluorescence measurements were made with the Perkin-Elmer MPF-4 Fluorescence Spectrophotometer fitted with thermostated cell compartments that maintained temperature constant to $\pm$ 0.1°. An optical layout of the instrument is shown in Fig. 1.

pH measurements at 25.0°C were made with an Orion Model 801 digital pH meter.

Water bath temperature at 25.0° was maintained to $\pm$ 0.1° with a Sargent Thermonitor Electronic Relay. Water bath temperature at 27.5° was maintained to $\pm$ 0.1° with a "Temptrol 151" water bath (Precision Scientific Co.). Water bath temperature at 80.0° was maintained to $\pm$ 0.1° with a "Dual-Purpose" water bath system equipped with a relay, heater, stirrer, and circulator (American Instrument Co.) and regulated by a mercury column thermoregulator (Branwill Scientific Co.).

Melting points were determined on a Thomas-Hoover Capillary melting point apparatus.

Thermometers for use at 25.0° and 27.5° were calibrated against a thermometer carrying a National Bureau of Standards calibration certificate. Thermometers for


Emission Monochrometer
Excitatation Monochrometer
Grating
Grating
Filter
Sample
Photomultiplier
Sample
Compartment
Lamp

use at 80° were calibrated against a reference thermometer which had been calibrated against a thermometer carrying an ASTM certificate.

C. Procedures

1. Modification of the Hamilton Hydroxylation System

Hamilton (17,18) studied the hydroxylation of some monosubstituted benzene derivatives, measuring the kinetics of loss of peroxide when the aromatic substrate was in excess. The experimental conditions employed by Hamilton are listed below:

1.5×10^{-4} M catechol
4.0×10^{-5} M ferric perchlorate
2.0×10^{-3} M H_2O_2
1.0×10^{-2} M anisole
pH 4.3 in 0.005 M acetate buffer

Since the Hamilton system is to be used as an analytical reagent, the hydrogen peroxide in the Hamilton system must be in excess over the aromatic sample. Hence, Connors and Albert varied the concentrations of

the reagents. They found that the following concentrations gave an optimum yield for hydroxylation of anisole(34):

1.2×10^{-4} M catechol
6.0×10^{-5} M ferric perchlorate
5.0×10^{-3} M H_2O_2
2.1×10^{-3} M anisole
pH 4.0 in 0.005 M acetate buffer

I similarly varied the concentrations of the reagent in reactions with pyridine. The results indicated that the concentrations Connors and Albert chose also gave an optimum yield for hydroxylation of pyridine and its derivatives. Variation of reagent concentrations with yield will be shown in Section III.

2. Hydroxylation Time Course Studies

Reaction mixtures were prepared in 25-ml volumetric flasks by adding 1.0 ml of 3×10^{-3} M aqueous catechol, 1.0 ml of 1.5×10^{-3} M ferric perchlorate, and 20.0 ml of substrate solution. The solutions were brought to volume with pH 4.0 acetate buffer. Their temperatures were adjusted to 27.5° or 80° by placing them in a water

bath.

Reactions were initiated by adding 0.1 ml of 3% hydrogen peroxide solution to the 25-ml volumetric flask. The reaction flask was placed in the water bath for indicated time periods; a 20.0 ml aliquot of the reaction mixture was pipetted into 5.0 ml of borate buffer. The temperature of this final mixture was equilibrated to 25.0^{o} before fluorescence readings were taken. Fluorescence was measured at the optimum excitation and emission wavelengths. Plots of fluorescence against time were subsequently prepared.

3. Proposed Analytical Method

To a 25-ml volumetric flask are added 1.0 ml of 3×10^{-3} M aqueous catechol, 1.0 ml of 1.5×10^{-3} M ferric perchlorate, and 20.0 ml of the heterocyclic compound such that its concentration in the diluted solution will be in the range 10^{-6} to 10^{-4} M. The solution is diluted to the mark with pH 4.0 acetate buffer. The pH of this reaction solution should be in the range of 3.9 to 4.1.

The reaction vessel is placed in a 80^{o} water bath

and reaction is initiated by adding 0.1 ml of 3% hydrogen peroxide solution. After 10 minutes at 80°, a 20.0 ml aliquot is pipetted into 5.0 ml of pH 12.8 borate buffer (0.4 M sodium borate + 0.2 M sodium hydroxide). This solution is then equilibrated to 25° before fluorescence is measured. Fluorescence intensity is read at the optimum excitation and emission wavelengths. Fluorescence of a reagent blank carried through the same procedure is also noted. A standard curve is prepared by subjecting known concentrations of the same heterocyclic compound to the procedure.

4. pH Dependence of Fluorescence Before and After Hydroxylation

10^{-4} M quinoline was dissolved in media of various pH. The samples were equilibrated to 25° before fluorescence was recorded. Fluorescence of the various media was also recorded. A plot of fluorescence of quinoline vs. pH was obtained. 10^{-4} M quinoline was subjected to the analytical procedure described on p. 22. After 10 minutes at 80°, 20.0 ml aliquots were pipetted into 5.0 ml of buffer of varying pH ranging from pH 3.0 to 12.5. These solutions were equilibrated to 25° before fluo-

rescence was measured. Fluorescence of a reagent blank carried through the same procedure was also noted.

5. Estimation of Product Yield and Distribution

Each of the three 4×10^{-4} M hydroxypyridine isomers were brought to pH 12.8 with borate buffer. The fluorescence of each solution was recorded. 4×10^{-4} M pyridine was subjected to the analytical procedure described on p. 22. The fluorescence of the product corrected for the blank was noted.

6. Interference Studies

To a 25-ml volumetric flask were added 1.0 ml of 3×10^{-3} M aqueous catechol, 1.0 ml of 1.5×10^{-3} M ferric perchlorate, and 20.0 ml of the mixture containing the sample and one interfering compound such that each of their concentrations in the diluted solution was 10^{-5} M. The procedure was carried out as described for the proposed analytical method. Fluorescence of a blank containing only the reagent and sample carried through the same procedure was also noted.

III. RESULTS

A. Hydroxylation of Pyridine with the Hamilton System

1. Variation of Hydrogen Peroxide Concentration

The initial concentrations of hydrogen peroxide were varied. Other initial reactant concentrations were: 6.0×10^{-5} M Fe(III), 1.2×10^{-4} M catechol, 1.98×10^{-4} M pyridine. The reaction flask was placed in a 80° water-bath for 10 minutes, this reaction time being chosen was based on previous work done by Connors and Albert. Reaction was stopped by cooling in an ice-bath. A 20.0 ml aliquot of the reactants was added to 5.0 ml of pH 12.8 borate buffer (0.4 M sodium borate and 0.2 M sodium hydroxide). These flasks were then equilibrated to 25° before fluorescence measurements were taken. Fluorescence intensities were recorded at λ_{ex} 300 nm and λ_{em} 350 nm. Fig. 2 shows that fluorescence intensity was greatest when the ratio $[H_2O_2]$ / [pyridine] was 25 or higher.

2. Dependence of Yield on Catechol and Fe(III) Concentrations

Hamilton (17,18) observed a maximum rate for the loss of peroxide at a catechol concentration of 2.5×10^{-4} M. His initial reactant concentrations were: 3.94×10^{-5} M Fe(III), 1×10^{-2} M anisole, 1.75×10^{-3} M H_2O_2, pH 4.3 in 0.005 M acetate buffer. Based on the data from Fig. 2, the concentration of catechol was varied. Other initial reactant conditions include: 5×10^{-3} M H_2O_2, 1.98×10^{-4} M pyridine, 6.0×10^{-5} M Fe(III), pH 4.0 in 0.005 M acetate buffer. The reaction flasks were placed in a 80° water-bath for 10 minutes, and reactions were stopped by subjecting them to an ice-bath. A 20.0 ml aliquot of the reactants was pipetted into 5.0 ml of borate buffer and fluorescence was noted as before. Fluorescence seems to be highest at a catechol concentration of 1.2×10^{-4} M as shown in Fig. 3. This corresponds to a [Fe(III)] / [catechol] ratio of 0.5. This is identical with what Connors and Albert (1,2) found for aromatics. To determine whether the yield was related to the [Fe(III)] / [catechol] ratio, catechol concentration was kept constant at its optimum (1.2×10^{-4} M) while Fe(III) concentration was varied. The data depicted in Fig. 4 establish that the ratio of catalysts, namely the [Fe(III)] / [catechol] ratio, affects the overall yield. The data in Table IV demonstrate that an optimum catechol concentration does occur at a

value of about 1.2×10^{-4} M.

Fig. 2. Change of fluorescence with initial hydrogen peroxide concentration at 80°: 1.98×10^{-4} M pyridine, 6.0×10^{-5} M ferric perchlorate, 1.2×10^{-4} M catechol, pH 4.0, 10 minutes reaction time. Fluorescence measured at λ_{ex} 300 nm and λ_{em} 350 nm.

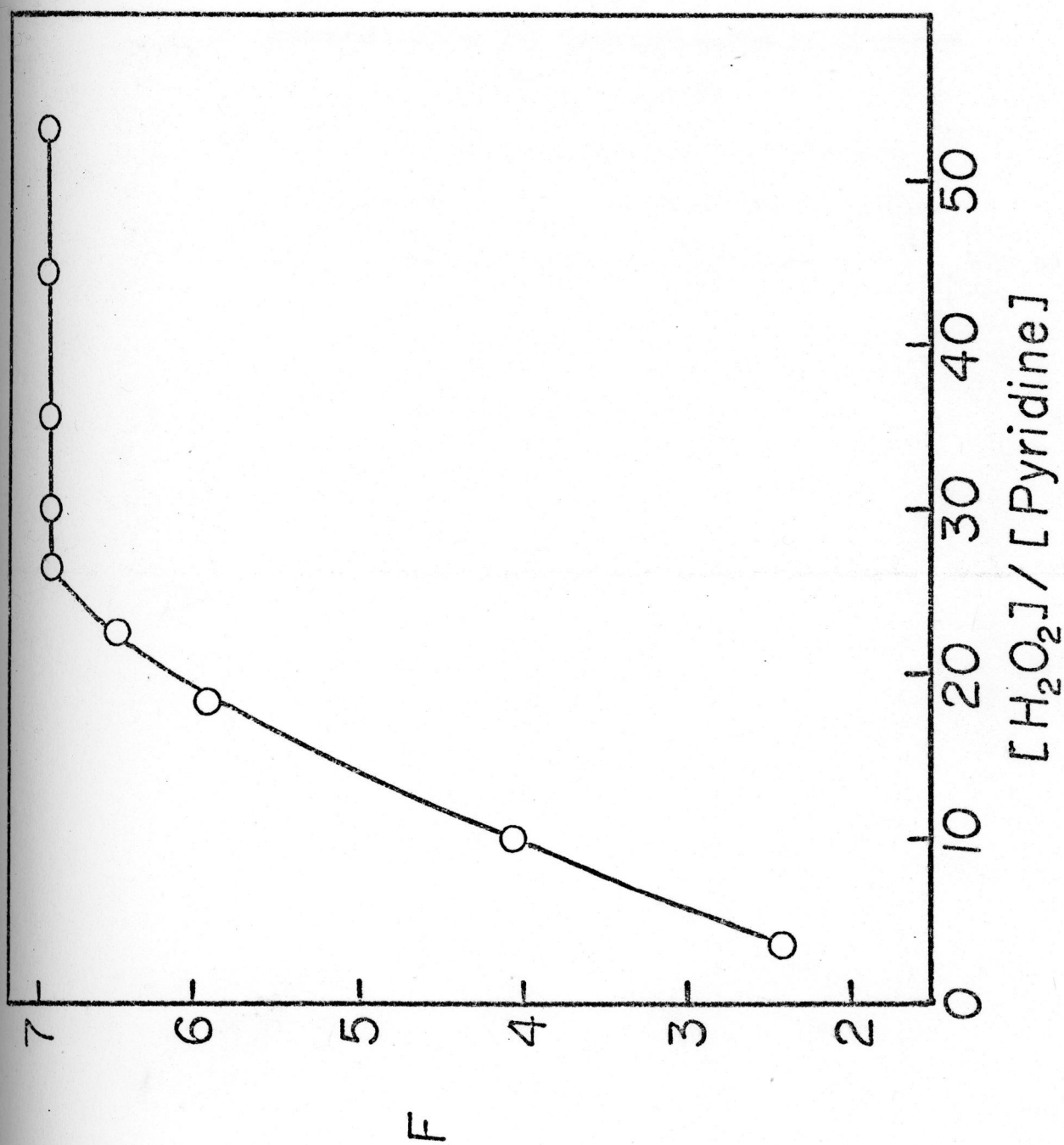

F
7
6
5
4
3
2
0
10
20
30
40
50
[H2O2] / [Pyridine]

Fig. 3. Change of fluorescence with initial catechol concentration for hydroxylation of pyridine at 80^{o}: 1.98×10^{-4} M pyridine, 6.0×10^{-5} M ferric perchlorate, 5×10^{-3} M hydrogen peroxide, pH 4.0, 10 minutes reaction time. Fluorescence measured at λ_{ex} 300 nm and λ_{em} 350 nm.

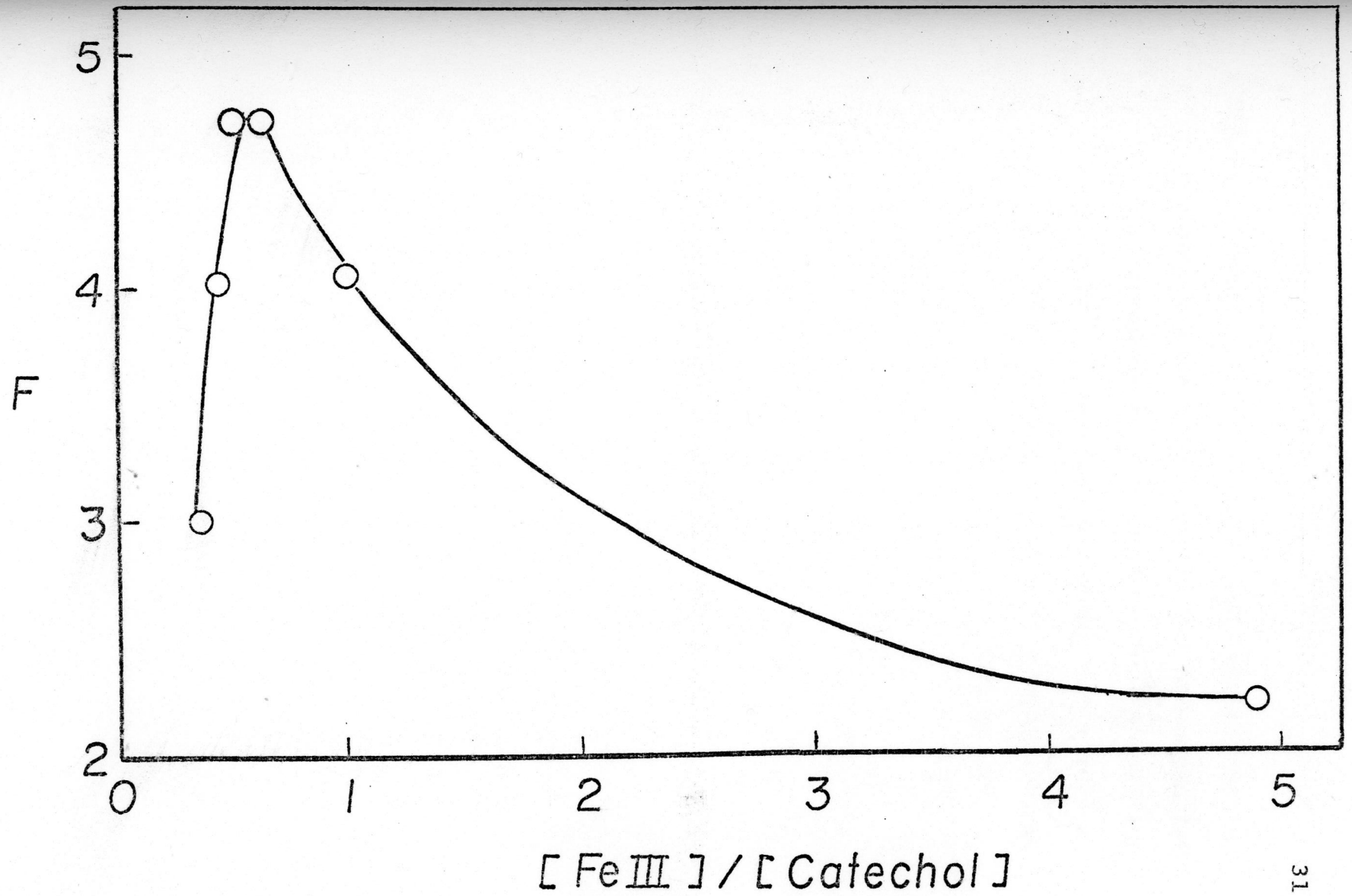

5
4
3
2
F
0
1
2
3
4
5
[Fe III] / [Catechol]

Fig. 4. Change of fluorescence with initial ferric perchlorate concentration for hydroxylation of pyridine at 80°: 1.98×10^{-4} M pyridine, 5×10^{-3} M hydrogen peroxide, 1.2×10^{-4} M catechol, pH 4.0, 10 minutes reaction time. Fluorescence measured at λ_{ex} 300 nm and λ_{em} 350 nm.

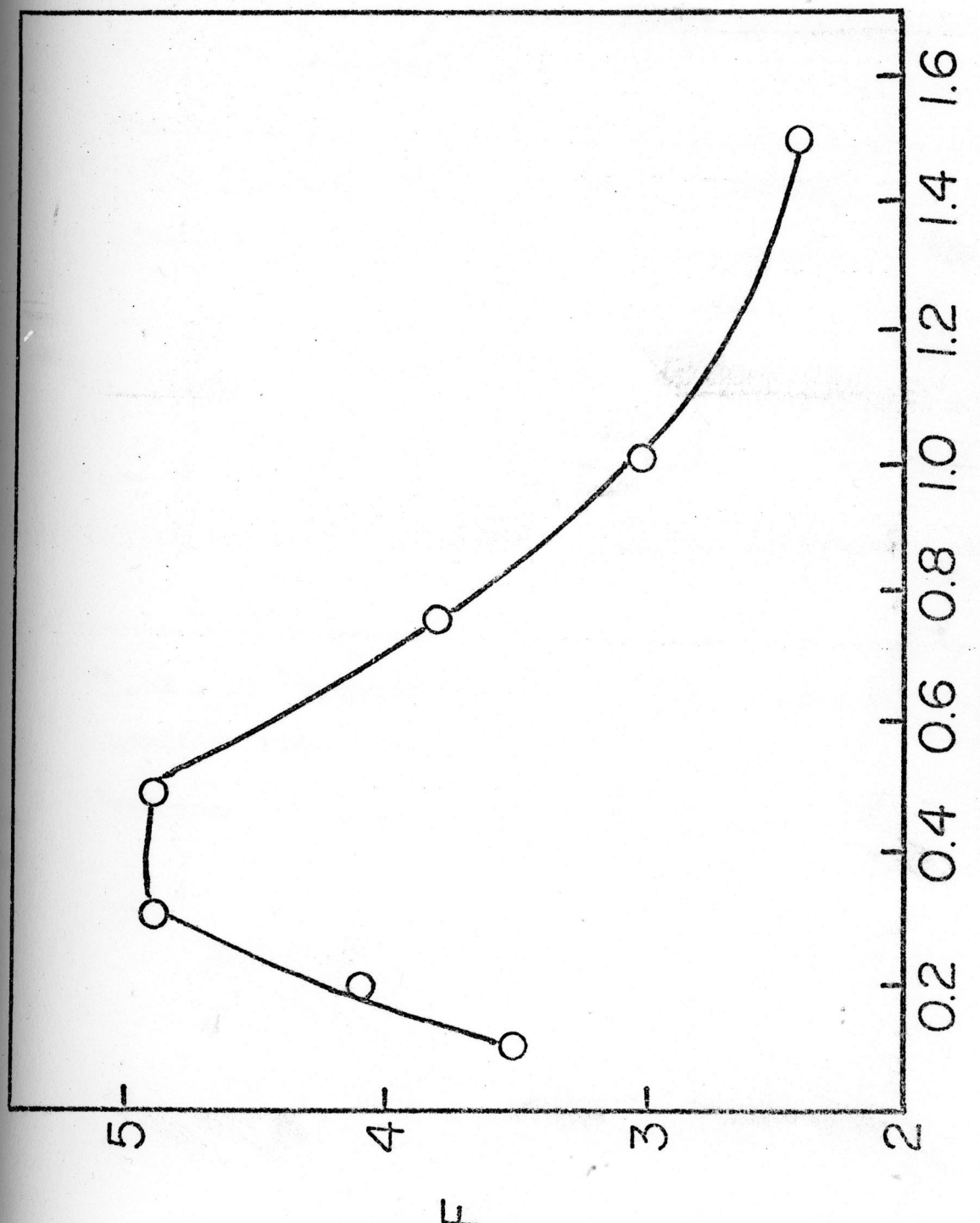

5
4
3
2
F
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
[FeIII]/[Catechol]

Table IV. Effect of [Catechol] on Yield at Constant [Fe(III)] / [Catechol] for the Hydroxylation of Pyridine at 80°.[a]

10^4 x [Catechol] M^{-1}	[Fe(III)] / [Catehol]	F[b]
0.6	0.50	3.3
1.2	0.50	4.8
2.4	0.50	2.5

[a] 1.98×10^{-4} M pyridine; 5×10^{-3} M H_2O_2; pH 4.0; reaction time 10 min.

[b] Fluorescence values corrected for blank.

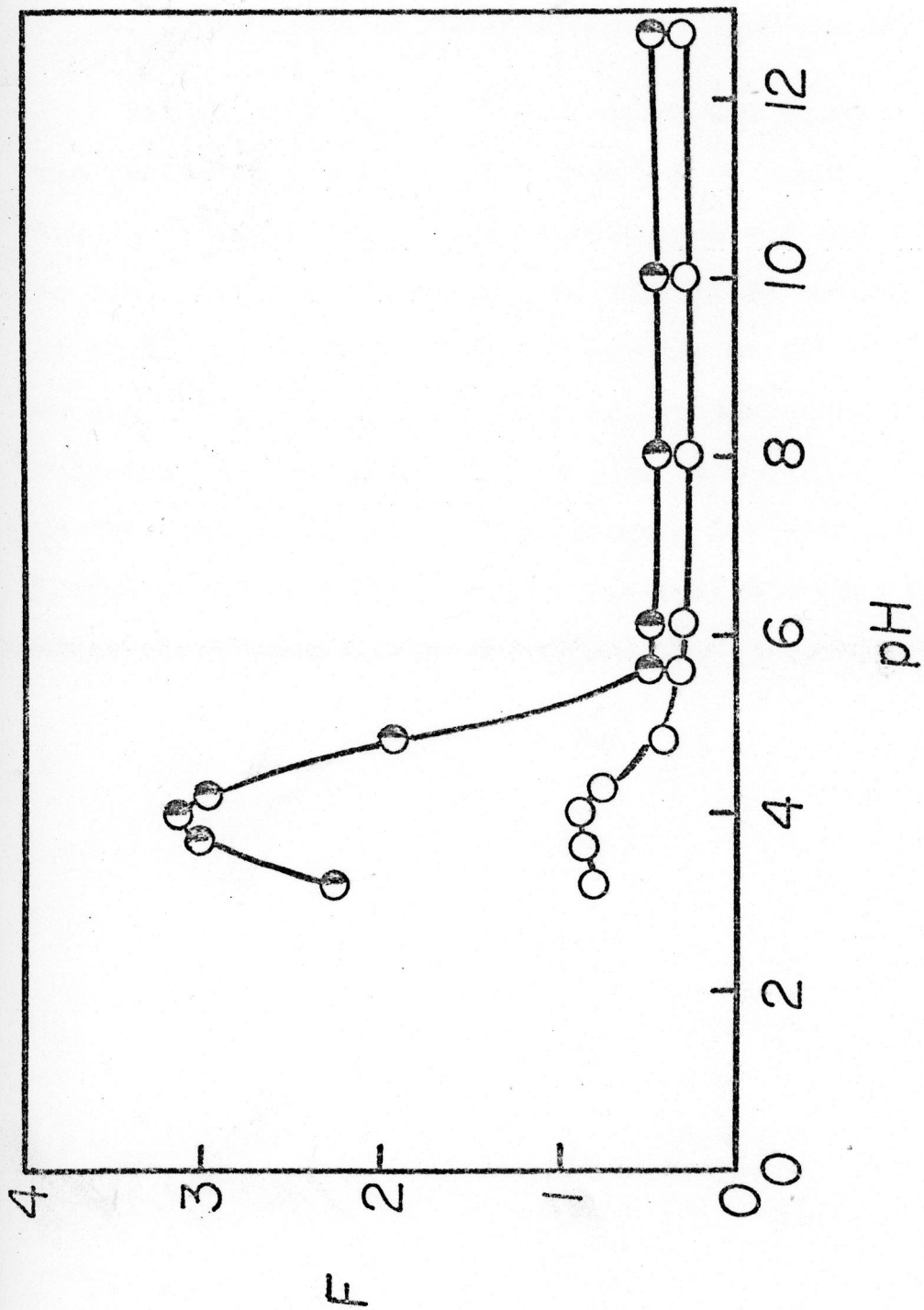
3. Dependence of Yield on pH

Hamilton (17,18) indicated that the rate of loss of peroxide for the hydroxylation of anisole was independent of the pH from 3.5 to 4.2. Fig. 5 shows that fluorescence was highest at pH 4.0 and the hydroxy-pyridine fluoresces strongly in the anion form.

Fig. 5: Dependence of fluorescence on reaction pH for treatment of pyridine with the Hamilton system: 1.98×10^{-4} M pyridine, 1.2×10^{-4} M catechol, 6.0×10^{-5} M ferric perchlorate, 5×10^{-3} M H_2O_2, reaction time 10 min. at 80°, products brought to pH 12.8 with borate buffer before fluorescence measurements were taken.

◑ Fluorescence of pyridine after hydroxylation. Products were pipetted into a borate buffer of pH 12.8 before fluorescence measurements were recorded.

○ Fluorescence of pyridine after hydroxylation without bringing final pH to 12.8.


4
3
2
1
0
F
0
2
4
6
8
10
12
pH

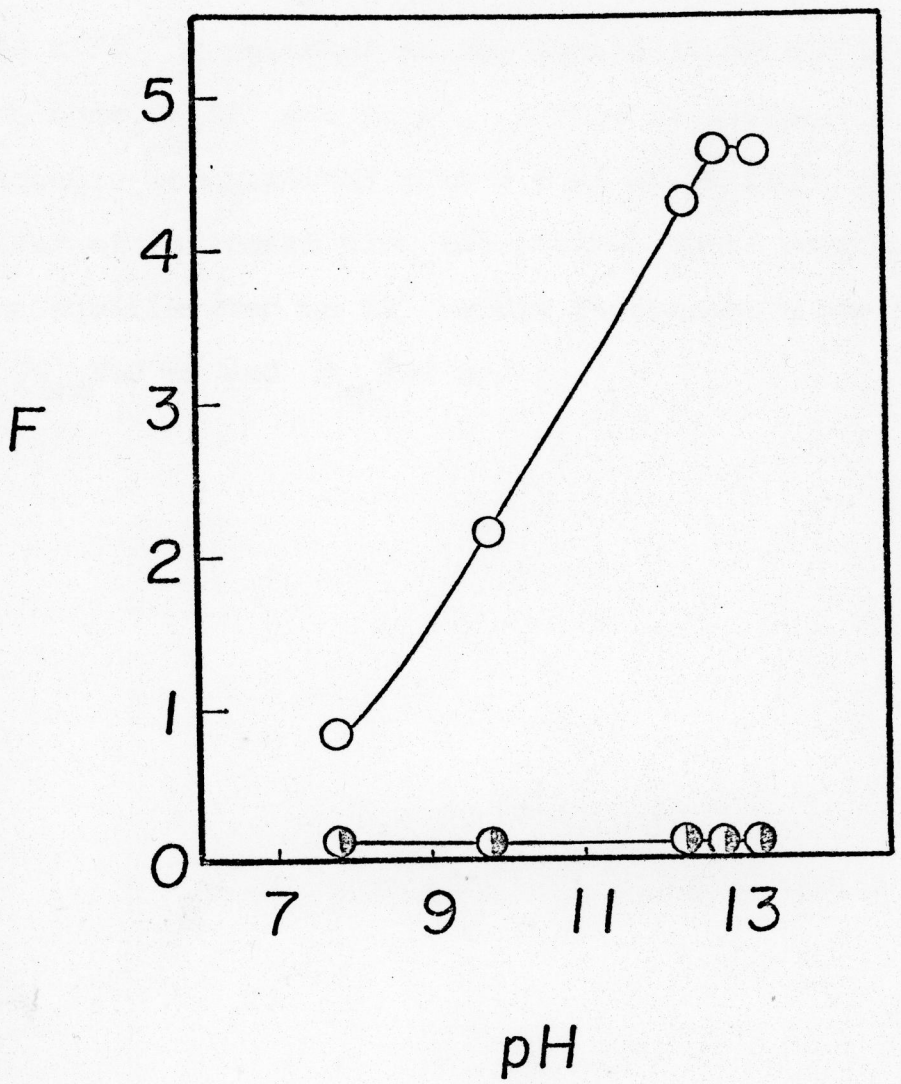
4. Dependence of Fluorescence of Products on pH

250 ml of 1.98×10^{-4} M pyridine was subjected to the following reagents: 1.2×10^{-4} M catechol, 6.0×10^{-5} M Fe(III), acetate buffer pH 4.0 and 0.005 M in concentration. Reaction was started by adding 1.0 ml of 3% H_2O_2. After heating the mixture at 80° for 10 minutes, 20.0 ml aliquots were each pipetted into various buffers. The reagent mixture with no pyridine was subjected to the same conditions. Fluorescence intensities of the products and reagent blanks at various pH's were recorded after equilibrating them to 25°.

Fig. 6: Dependence of fluorescence of products on pH for treatment of pyridine with the Hamilton reagents; 1.98×10^{-4} M pyridine, 1.2×10^{-4} M catechol, 6.0×10^{-5} M Fe(III), 5×10^{-3} M H_2O_2, reaction time 10 min. at 80^{o} in acetate buffer of pH 4.0. Products were brought to various pH. Fluorescence measurements of these products were taken after equilibrating them to 25^{o}. The reagent mixture with no pyridine was subjected to the same conditions.

○ Fluorescence of products corrected for buffer blanks.

◑ Fluorescence of reagents corrected for buffer blanks.


F
5
4
3
2
1
0
7
9
11
13
pH

B. Hydroxylation Time Course Studies

Using the concentrations of reagents that gave an optimum yield, namely 1.2×10^{-4} M catechol, 6.0×10^{-5} M Fe(III), 5×10^{-3} M H_2O_2, time studies using 1.98×10^{-4} M pyridine as the sample in pH 4.0 buffer were done at 80° and 27.5°. A 20.0 ml aliquot of the reactants was pipetted into 5.0 ml of pH 12.8 borate buffer at indicated time intervals. These samples were then equilibrated to 25° before fluorescence was measured at λ_{ex} 300 nm and λ_{em} 350 nm.

Fig. 7: Change of fluorescence with time for hydroxylation of pyridine at 80^{o}: 1.98×10^{-4} M pyridine, 6.0×10^{-5} M ferric perchlorate, 1.2×10^{-4} M catechol, pH 4.0.

○ $[H_2O_2]$ / [pyridine] = 35.4

◑ $[H_2O_2]$ / [pyridine] = 17.7

● $[H_2O_2]$ / [pyridine] = 3.54

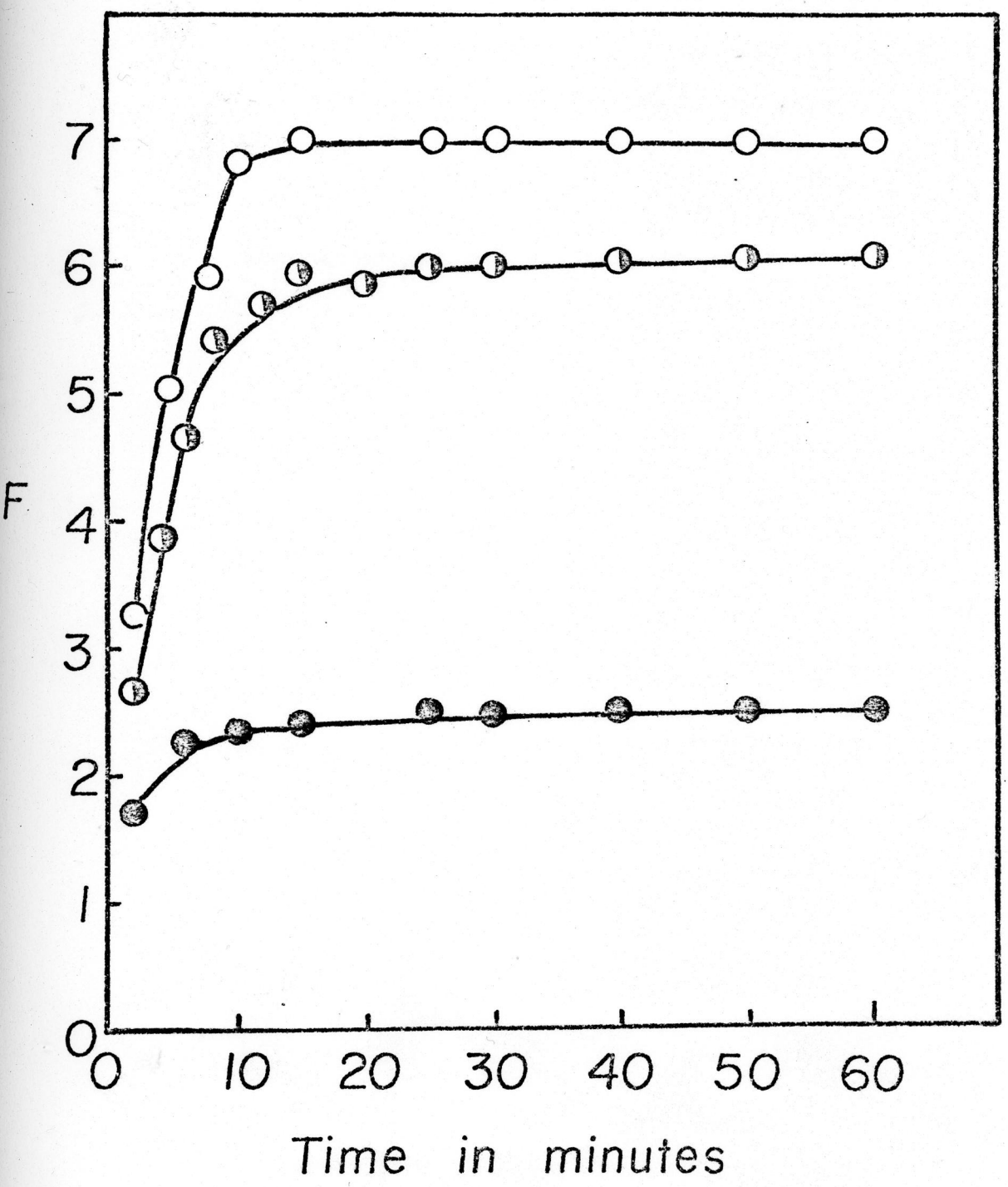
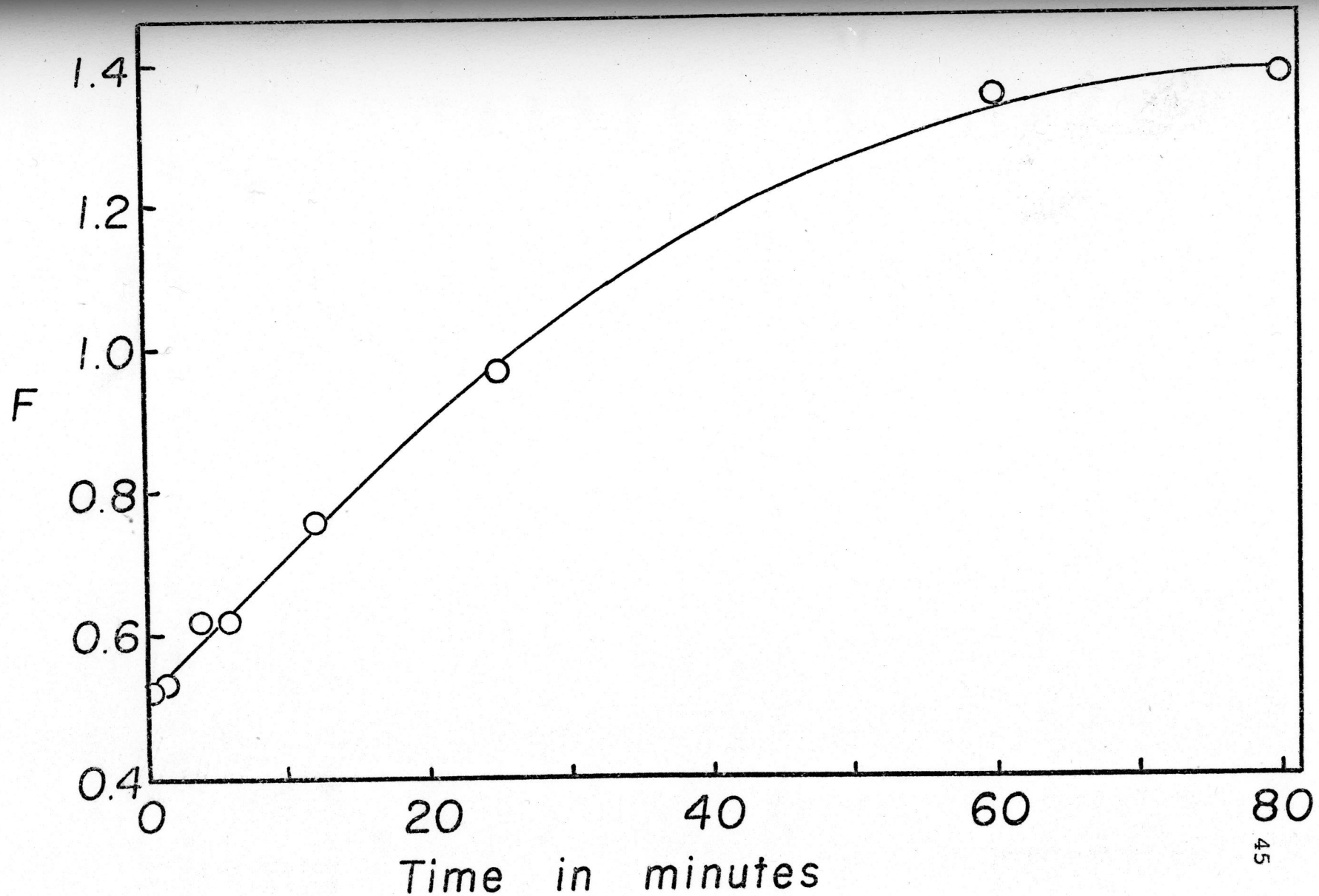

F
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
Time in minutes

Fig. 8: Change of fluorescence with time for hydroxylation of pyridine at 27.5°: 1.98×10^{-4} M pyridine, 6.0×10^{-5} M ferric perchlorate, 1.2×10^{-4} M catechol, 5×10^{-3} M hydrogen peroxide, pH 3.9. Fluorescence intensities were measured at λ_{ex} 300 nm and λ_{em} 350 nm.


1.4
1.2
1.0
0.8
0.6
0.4
F
0
20
40
60
80
Time in minutes

Figures 7 and 8 report the time course for the hydroxylation of pyridine under the described conditions. The data show that fluorescence does not seem to change after 10 minutes when hydroxylation was carried out at 80°.

C. pH Dependence of Fluorescence Before and After Hydroxylation

10^{-4} M quinoline in various pH media were equilibrated to 25° before fluorescence was measured. The fluorescence of the buffer blanks was noted. Fig. 9 shows the fluorescence of quinoline as a function of pH. A sigmoid curve is obtained, indicating that the acid and base forms of quinoline have different quantum yields. The pK_a of quinoline from the literature (32) is 4.90.

10^{-4} M quinoline was subjected to the hydroxylation reagent: 1.2×10^{-4} M catechol, 6.0×10^{-5} M ferric perchlorate, 5×10^{-3} M H_2O_2, pH 4.0. After 10 minutes at 80°, a 20.0 ml aliquot was pipetted into 5.0 ml of buffer of varying pH ranging from pH 3.0 to 12.5. These solutions were then equilibrated to 25° before fluorescence was measured. The fluorescence of a reagent blank carried through the same procedure was also noted. Fluo-

rescence was high at low and high pH as shown in Fig. 10.

Fig. 9. Fluorescence intensity of quinoline solutions vs. pH. pH buffers were prepared according to Bates (29-31). Concentration of quinoline was 8.46×10^{-4} M. Fluorescence of quinoline (F) = Fluorescence of quinoline in buffer - Fluorescence of buffer blank. Fluorescence was measured at λ_{ex} 320 nm and λ_{em} 380 nm.

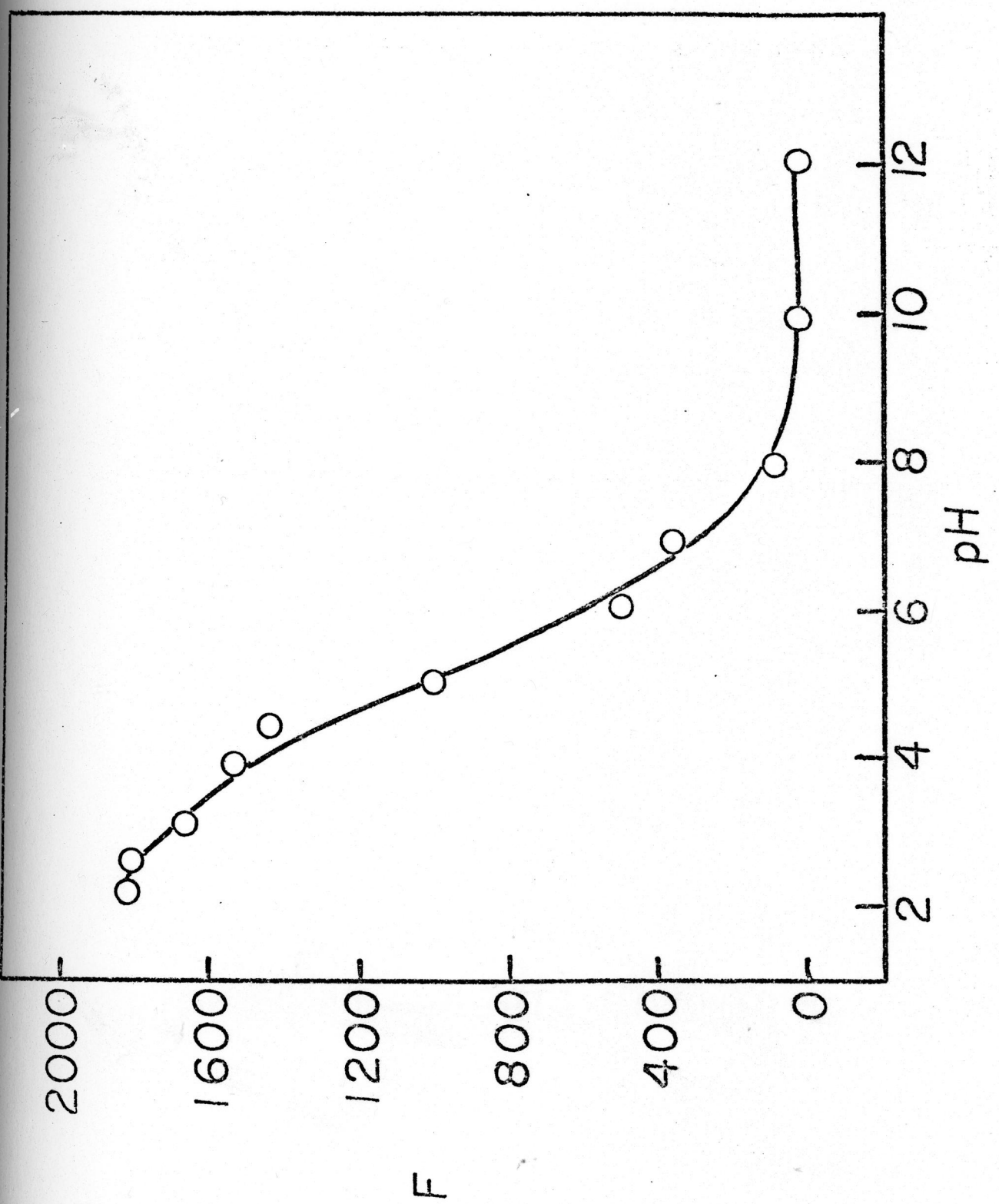
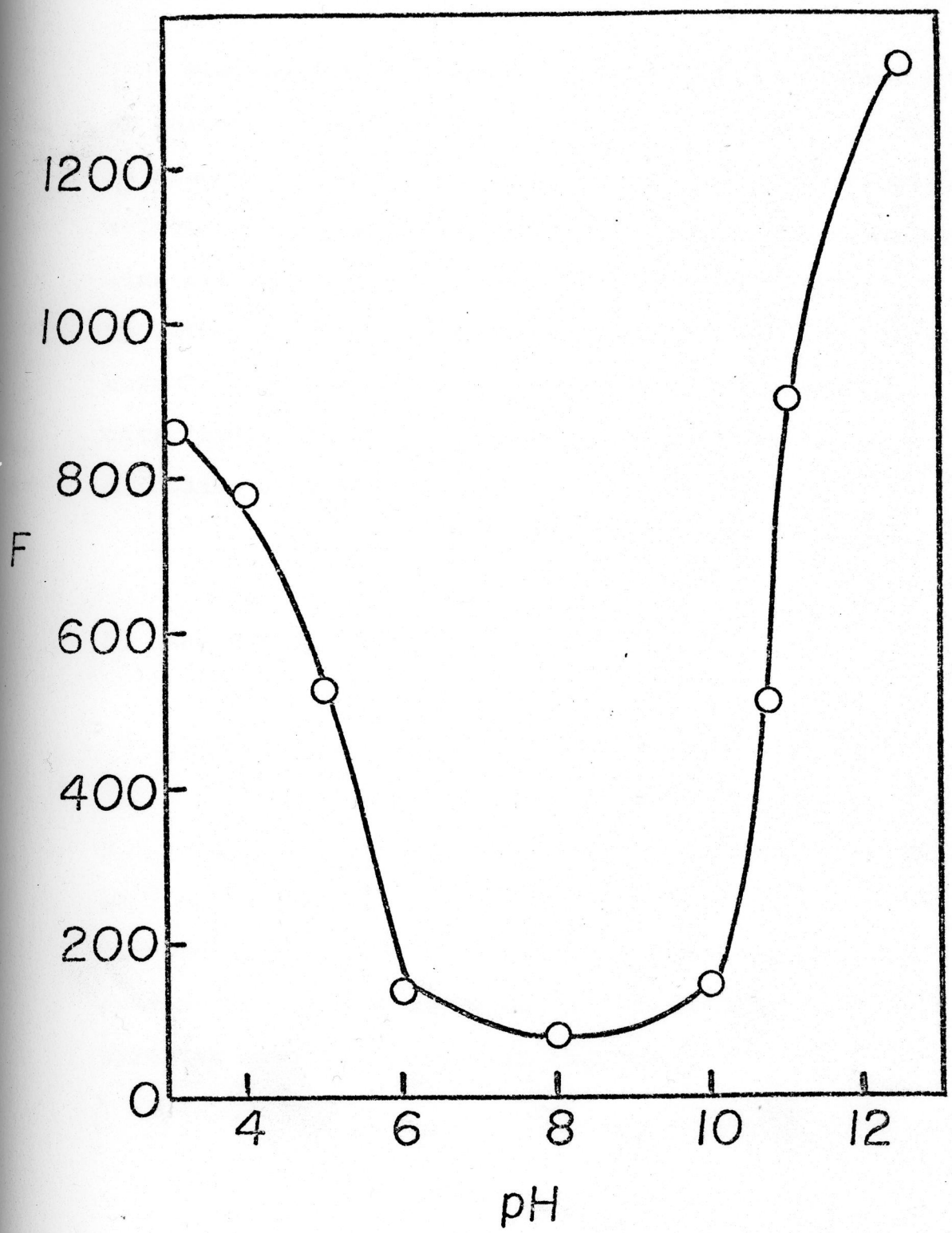

2000
1600
1200
800
400
0
F
2
4
6
8
10
12
pH

Fig. 10. Fluorescence of quinoline after hydroxylation is plotted against pH. Concentration of quinoline was 8.46×10^{-4} M. pH buffers were prepared according to Bates. Hydroxylation reagents are: 1.2×10^{-4} M catechol, 6.0×10^{-5} M Fe(III), 5×10^{-3} M H_2O_2, pH 4.0, reaction time 10 min. Fluorescence was measured at λ_{ex} 320 nm and λ_{em} 380 nm.


1200
1000
800
F
600
400
200
0
4
6
8
10
12
pH

Figures 9 and 10 show the dependence of fluorescence of quinoline on pH before and after hydroxylation. It is observed that fluorescence of quinoline before hydroxylation at low pH is higher than fluorescence of quinoline after hydroxylation at all pH studied. It is therefore apparent that it is more sensitive to determine quinoline directly in acid medium than to measure it after hydroxylation. Quinaldine behaves similarly to quinoline in this respect.

D. Analytical Results

Using the conditions determined above, namely 1.2×10^{-4} M catechol, 6.0×10^{-5} M Fe(III), 5×10^{-3} M H_2O_2, in pH 4.0 buffer, reaction time 10 minutes at 80^{o}, 10^{-4} to 10^{-5} M solutions of pyridine and pyridine derivatives were treated. One of the typical plots of a concentration-response curve for the hydroxylation of pyridine samples is shown in Fig. 11.

Fig. 11. Concentration-response curve for the hydroxylation of pyridine at 80°; 6.0×10^{-5} M ferric perchlorate, 1.2×10^{-4} M catechol, 5×10^{-3} M hydrogen peroxide, pH 4.0, 10 minutes reaction time; fluorimetric finish in borate buffer pH 12.8. Fluorescence intensities were measured at λ_{ex} 300 nm and λ_{em} 350 nm.

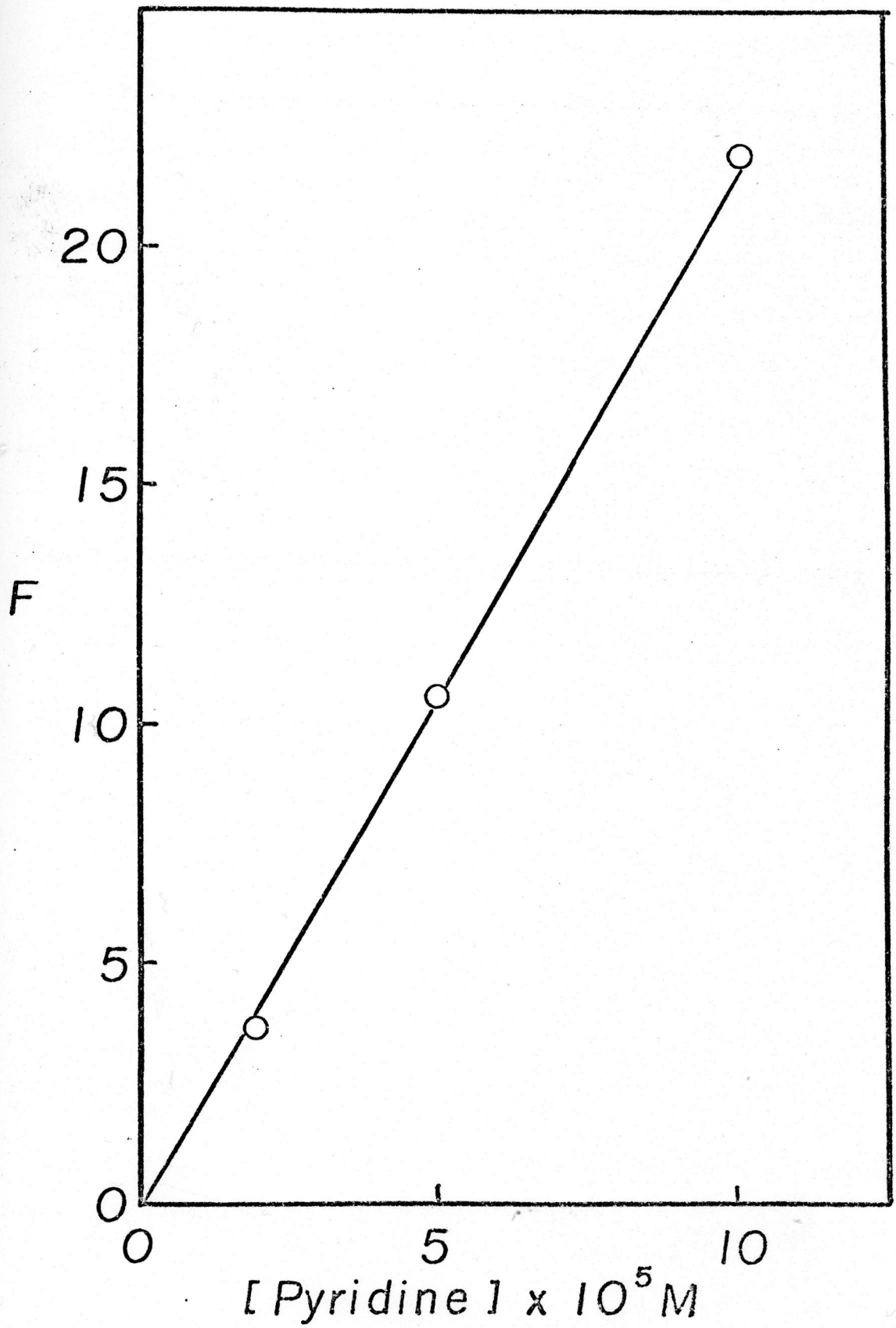

20
15
F
10
5
0
0
5
10
[Pyridine] x 10^5 M

Table V lists the simple heterocyclic compounds that have been successfully treated by the Hamilton reagent. Linear working curves of fluorescence against concentration were observed for the compounds in the concentration levels indicated. Fluorescence intensities were higher after hydroxylation for these compounds. The increases in analytical sensitivity for these compounds are listed in Table XI.

Table V. Heterocyclic Compounds Determined by the Hamilton Hydroxylation Procedure at 80°.

Sample	Analytical Wavelengths (nm) λ_{ex}	λ_{em}	Conc. Levels Determined
Pyridine	300	350	10^{-6} - 10^{-4} M
2-Picoline	300	350	10^{-6} - 10^{-4} M
4-Dimethyl-aminopyridine	340	410	10^{-5} - 10^{-4} M
2,6-Pyridine-dicarboxylic acid	290	375	10^{-5} - 10^{-4} M

Table VI lists the heterocyclic compounds that have been treated by the Hamilton system with results indicating that fluorescence intensities of these compounds were higher before hydroxylation than after hydroxylation. Fluorescence was measured at the optimum excitation and emission wavelengths listed.

Table VI. Heterocyclic Compunds Unsuitable for Analysis by this Technique.

Pyridine Compounds	λ_{ex} (nm)	λ_{em}
Quinoline	320	380
Quinaldine	320	380
3-Quinoline carbonitrile	333	380
Isoquinoline	318	333
4-Quinoline carboxaldehyde	315	390
2-Pyridinealdoxime	270	333
2-Aminopyridine	260	353
Pyridoxine·HCl	315	380
2,3-Pyridine dicarboxylic acid	300	390
2-chloro-6-methoxypyridine	290	375

E. Estimation of Product Yield and Distribution

Solutions of 4×10^{-4} M o-, m-, and p- hydroxypyridine were prepared. Their final pH's were adjusted to 12.8 with borate buffer. The fluorescence intensities of these solutions corrected for the buffer blank are recorded in Table VII. The hydroxylation procedure described on p. 22 was applied to 4×10^{-4} M pyridine, and the fluorescence of the product corrected for the reagent blank was measured, giving a fluorescence intensity of 30.8 units with the same instrument settings (λ_{ex} 300 nm, λ_{em} 350 nm, and sensitivity 10X).

Table VII. Fluorescence Intensities of 4×10^{-4} M Hydroxypyridine Isomers in pH 12.8 Borate Buffer.

Hydroxypyridine Isomers	Fluorescence Intensities*
o-	57.4
m-	90.5
p-	8.1

*The fluorescence intensities were corrected for buffer blanks, sensitivity setting on Perkin-Elmer MPF-4 fluorescence spectrophotometer was 10X. Fluorescence measurements were all made at λ_{ex} 300 nm and λ_{em} 350 nm.

Connors and Albert (2) reported that pyridine does not produce m-hydroxypyridine in significant yield on treatment with the hydroxylating agent. Since the present analytical reagents and reaction conditions were identical to those of Connors and Albert, the products formed should be identical with what they found. Fig. 6 shows that the fluorescence of products formed on hydroxylation increases with pH. Moreover, the fluorescence intensities remained the same from pH 12.5 to 13.0. Fluorescence spectra of individual hydroxypyridine isomers indicate that the anion forms have higher quantum yields than the protonated forms. Since the pK_a values of o- and p- hydroxypyridines are 11.6 and 11.1 respectively while pK_a of m-hydroxypyridine is 8.7 (33); it is expected that the fluorescence of products would not change significantly from pH 10 to 11 if m-hydroxypyridine is the major product. Increase in fluorescence is observed from pH 10 to 11 as shown in Fig. 6. My results therefore support the fact that m-hydroxypyridine is not formed significantly. Calculations of apparent percentage yield (Table VIII) were made assuming that o- and p- hydroxypyridines are the products formed on hydroxylation of pyridine. An example of the calculation is shown below: assuming 90% of all products formed is the o- isomer, the fluorescence

intensity due to the o- isomer will be 57.4 x 0.9 $\simeq$ 51.7. Then, 10% fraction of the product will be the p- isomer; it will give a fluorescence intensity of 8.08 x 0.1 $\simeq$ 0.81. Hence, if the yield of hydroxylation is 100%, the fluorescence intensity on hydroxylation of 4×10^{-4} M pyridine will be 52.51 units. A fluorescence intensity of 30.8 units was obtained. The apparent yield is given by 30.8/52.51 x 100% which is 58.7%. The other percentage yields shown in Table VIII were calculated similarly.

Table VIII. Calculations of Percentage Yield on Hydroxylation of Pyridine Based on Data in Table VII.

Fraction of *o*- isomer	Fraction of *p*- isomer	% Yield
1.0	0.0	53.6
0.9	0.1	58.7
0.8	0.2	64.8
0.7	0.3	72.3
0.6	0.4	81.7
0.5	0.5	94.1

F. Interference Studies

For applicable compounds, fluorescence gives greater sensitivity and selectivity than absorbance measurements. Attempts were made to see if pyridine and 2-picoline could be analyzed in the presence of structurally similar compounds. Mixtures of pyridine and one interfering sample were subjected to the procedure described on p. 22. The results of interference studies are listed in Tables IX and X. Pyridine, benzene, 2-picoline, 3-picoline, and 4-picoline were all nonfluorescent before hydroxylation. After hydroxylation, pyridine, and 2-picoline became fluorescent while benzene, 3-picoline, and 4-picoline remained nonfluorescent. However, the results listed in Tables IX and X indicate that 3- and 4- picolines interfere significantly with the determination of pyridine, while benzene interferes to a small extent. The analysis of 2-picoline was also interfered with by the presence of 3- or 4- picoline.

Table IX. Mixtures of pyridine and one interfering sample were subjected to hydroxylation at 80^{o}; 6.0×10^{-5} M ferric perchlorate, 1.2×10^{-4} M catechol, 5×10^{-3} M H_2O_2, pH 4.0, 10 minutes reaction time; fluorimetric finish in borate buffer pH 12.8. Concentration of pyridine in the reaction flask was 9.9×10^{-5} M; concentration of the interfering sample in the same flask is listed in the table. Percent interference is calculated by the following formula

$$\frac{F_{(s + i)} - F_s}{F_s} \times 100\ \%$$

where $F_{(s + i)}$ = fluorescence of sample + interfering compound after hydroxylation

F_s = fluorescence of sample after hydroxylation

Table IX. Interference in Mixtures of Similar Compounds in the Determination of 10^{-4} M Pyridine

Interferent	Percent Interference	Conc. of Interfering Substance
3-Picoline	13	1.6×10^{-5} M
4-Picoline	15	1.6×10^{-5} M
Benzene	14	2.3×10^{-4} M

Table X: Mixtures of 2-picoline and one interfering sample were subjected to hydroxylation at 80^{o}; 6.0×10^{-5} M ferric perchlorate, 1.2×10^{-4} M catechol, 5×10^{-3} M hydrogen peroxide, pH 4.0, 10 minutes reaction time; fluorimetric finish in borate buffer pH 12.8. Concentration of 2-picoline in the reaction flask was 7.9×10^{-5} M; concentration of the interfering sample in the same flask is listed in the table. Percent interference is calculated by the formula in Table IX.

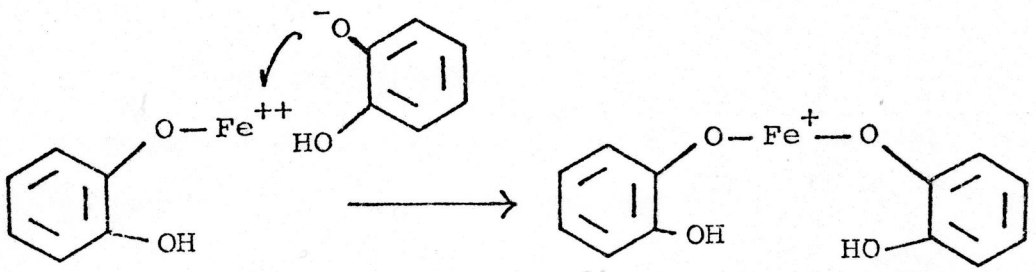
Table X. Interference by Isomers in the Fluorimetric Determination of 8×10^{-5} M 2-Picoline after Hydroxylation.

Interferent	Percent Interference	Conc. of Interfering Substance
3-Picoline	15	1.6×10^{-5} M
4-Picoline	8	1.6×10^{-5} M

IV. DISCUSSION

A. The Hamilton Hydroxylation System

Fig. 2 shows the extent of hydroxylation of pyridine with the Hamilton reagent as a function of the initial ratio of peroxide to pyridine. Fluorescence was highest at a $[H_2O_2]/[\text{pyridine}]$ ratio of 25 or higher. It is not obvious why such a high ratio is necessary. Figures 3 and 4 show that the ratio $[\text{Fe(III)}]/[\text{catechol}]$ affects the yield. An optimum yield was obtained when this ratio was 0.5 and the catechol concentration was 1.2×10^{-4} M. This ratio dependence suggests that if complex I is formed from Eq. 2, it might combine with another catechol molecule to form complex IV as shown in Eq. 11.



Eq.11

Complex II

Complex IV

A maximum fluorescence occurs around 1.2×10^{-4} M catechol, possibly because high catechol concentration would compete with the pyridine sample for the hydroxylating agent. Fig. 5 demonstrates that an optimum pH for the hydroxylation is 4.0. This is identical with what Connors and Albert found (34). The fluorescence of the hydroxypyridine products was much enhanced when subjected to the borate buffer of pH 12.8 as shown in Fig. 6. It appeared that the anion form of the hydroxypyridines has a high quantum yield. Independent experiments showed that the anion form of the o-, m-, and p- hydroxypyridines has a high quantum yield.

Connors and Albert (34) reported that pyridine gives no color in the Hamilton hydroxylation-4-aminoantipyrine colorimetric method. Separate experiments showed that o- and p- hydroxypyridines give no color on treatment with 4-aminoantipyrine whereas m-hydroxypyridine does give a color. These results suggest that pyridine does not produce m-hydroxypyridine in significant yield on treatment with the hydroxylating agent, although the o- and p- isomers may be produced.

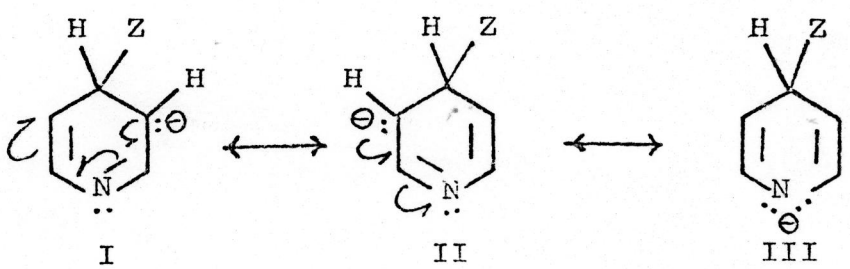
My results show that the fluorescence of pyridine is enhanced after hydroxylation. o-, m-, and p-hydroxypyridines are more fluorescent than pyridine,

as shown by the fluorescence spectra of the individual hydroxypyridine isomers. Moreover, more enhancement was observed when the hydroxypyridine products were subjected to high pH. The individual hydroxypyridine isomers also have higher quantum yields compared to their nonionic forms. The fluorescence of products as a function of pH suggested that m-hydroxypyridine is not formed predominantly. The fluorescence spectrum of pyridine after hydroxylation is similar to those of o- and m- hydroxypyridines, but not p-hydroxypyridine. Both the o- and m- isomers have an excitation maximum of 300 nm and an emission maximum of 350 nm, while the p- isomer does not show any distinct excitation or emission maxima. Since the analytical reagents and reaction conditions were identical to those of Connors and Albert (1,2), the products formed should also be identical with theirs. Pyridine does not produce m-hydroxypyridine in significant yield. Based on these results, it is therefore highly probable that the reaction with the reagent produces o-hydroxypyridine.

Assuming that the product formed on hydroxylation of pyridine is o-hydroxypyridine, the percentage yield is 53.7 as shown in Table VIII. To test whether o-hydroxypyridine was the only product formed, 2,6-disubstituted pyridines were subjected to the Hamilton

reagent. Fluorescence enhancement was obtained when 2,6-pyridine dicarboxylic acid was subjected to the reagent conditions. Thus it appears that some p-hydroxypyridine could be formed. If so, it should not be possible to hydroxylate a 2,4,6-trisubstituted pyridine. The fluorescence spectrum of 2,4,6-trimethyl-pyridine remained the same after it was treated with the reagents. Hence, if o- and p- isomers are the products, the yield would be 54% or greater as shown in Table VIII.

The pyridine ring resembles a benzene ring that contains strongly electron-withdrawing groups (35). As a result, pyridine undergoes electrophilic substitution much less readily than benzene. Even if it occurs, substitution would occur predominantly at the m- position for stability reasons. On the other hand, the reactivity of pyridine toward nucleophilic substitution is very large compared to benzene. Nucleophilic attack at the p- position yields a carbanion that is a resonance hybrid of structures I, II, and III.



I II III

Structure III is especially stable because nitrogen can accomodate the negative charge most readily. Nucleophilic attack at the o- position gives similar structures. It is therefore expected that nucleophilic substitution of the pyridine ring will occur most rapidly at the o- and p- positions. The electronegativity of nitrogen in pyridine makes the ring unreactive toward electrophilic substitution and yet highly reactive toward nucleophilic substitution. The foregoing discussion suggests that the mechanism of Hamilton reagents on pyridine is non-electrophilic. Complex IV from Eq. 11 might also be one of the intermediates in the reaction. Further experiments would be required to elucidate the mechanism.

B. Analysis by Hydroxylation

1. Proposed Method

The proposed analytical method for the determination of aqueous solutions of pyridine compounds can be found in the Experimental Section on page 22. A 10 minute reaction time was chosen since fluorescence intensity does not seem to change after 10 minutes.

2. Range of Applicability

The analytical method is applicable to several of the pyridine compounds tested in the range 10^{-6} - 10^{-4} M.

Table XI shows the gain in analytical sensitivity after hydroxylation of the various pyridine compounds. Gain is defined by the following expression:

$$\frac{F_{(\text{sample after hydroxylation})} - F_{(\text{reagent})}}{F_{(\text{sample in water})} - F_{(\text{water})}}$$

where

$F_{(\text{sample after hydroxylation})}$ = fluorescence intensity of sample after hydroxylation

$F_{(\text{reagent})}$ = fluorescence intensity of reagent blank treated under the same procedure

$F_{(\text{sample in water})}$ = fluorescence intensity of aqueous solution of sample

$F_{(\text{water})}$ = fluorescence intensity of water

There is no pH dependence on $F_{(\text{sample in water})}$ for the samples listed in Table XI.

Table XI. Increase in Analytical Sensitivity for Pyridine Compounds Subjected to Hydroxylation.

Sample Compound	Conc. of Sample (M)	Gain
Pyridine	5×10^{-5}	160
2-Picoline	8×10^{-5}	50
4-Dimethyl-aminopyridine	2×10^{-4}	30
2,6-Pyridine-dicarboxylic acid	7×10^{-5}	20

Table XII compares the lowest concentration determined and the estimated detectable limit by the present method with the estimated detectable limit by absorbance measurements calculated from ε_{max}. ε_{max} values were taken from Organic Electronic Spectral Data (36). Estimated detectable limit (E.D.L.) by absorbance is defined as the concentration of the compound to give an absorbance of 0.1 in a 1 cm cell. Estimated detectable limit by the present method is defined as the concentration of the sample such that $F_{(sample)} - F_{(reagent)} =$ 1 unit on sensitivity setting of 30X of the Perkin Elmer MPF-4 Fluorescence Spectrophotometer, and:

$F_{(sample)}$ = fluorescence intensity of sample after hydroxylation;

$F_{(reagent)}$ = fluorescence intensity of reagent blank treated through the same procedure.

Table XII. Comparison of the Lowest Concentration Determined (L.C.D.), and the Estimated Detectable Limit (E.D.L.) by the Present Method with the Estimated Detectable Limit by Absorbance Measurements.

Sample	L.C.D. (Present Method)	E.D.L. (Present Method)	E.D.L. (Absorbance)
Pyridine	3×10^{-6} M	3×10^{-6} M	4×10^{-5} M
2-Picoline	3×10^{-6} M	3×10^{-6} M	6×10^{-5} M
4-Dimethyl-aminopyridine	2×10^{-5} M	1×10^{-5} M	5×10^{-5} M
2,6-Pyridine dicarboxylic acid	7×10^{-6} M	4×10^{-6} M	2×10^{-5} M

3. The Analytical Finish

The fluorimetric finish offers the advantage of being selective. Catechol, acting as a catalyst, does not interfere with the determination of the sample after hydroxylation. The borate buffer acts both to quench the hydroxylation reaction and to serve as a medium for fluorimetric determination, simplifying the analytical procedure. Fluorescence intensity of the various samples after hydroxylation remains the same for more than 24 hours.

4. Mixtures and Interferences

Albert (2) indicated that due to the non-specificity of the hydroxylating agent, it would be difficult to determine mixtures of aromatics.

My resllts show that the analytical method as described on page 22 allows the determination of 10^{-4} M pyridine in the presence of 2×10^{-4} M benzene with relatively small interference. When 3- or 4- picoline was subjected to the hydroxylating reagent, low fluorescence intensities were observed; while 2-picoline and pyridine both gave high fluorescence intensities after

hydroxylation. It was expected that the percent interference would be small when pyridine or 2-picoline was analyzed in the presence of 3- or 4- picoline. It is not obviou why significant interferences were observed as shown in Tables IX and X. The proposed analytical method therefore seem to be appropriate for the analysis of individual pyridine compounds; further interference studies were not pursued.

V. SUMMARY

The Hamilton system, consisting of an aqueous solution of ferric perchlorate, catechol, and hydrogen peroxide afforded a means of introducing a hydroxy group into aromatic compounds rapidly and in high yield when compared with other hydroxylating agents. Consequently, the application of this system to the quantitative determination of some heterocyclic compounds was investigated.

This work was designed to provide experimental data to test the potential of the Hamilton system as an analytical reaction for the quantitative determination of pyridine and some of its derivatives. For these compounds, the proposed analytical method is more sensitive than analysis by absorption spectroscopy. Second, the fluorimetric finish is simpler than the colorimetric finish, since adding a stabilizer or a colorimetric developing reagent becomes unnecessary. Third, this analytical method is useful when pyridine must be determined in the presence of benzene.

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