Investigations Into The Improvement Of Methodologies In Clinical Chemistry.

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INVESTIGATIONS INTO THE IMPROVEMENT OF
METHODOLOGIES IN CLINICAL CHEMISTRY.

LEHIGH UNIVERSITY, PH.D., 1978

INVESTIGATIONS INTO THE IMPROVEMENT OF METHODOLOGIES IN CLINICAL CHEMISTRY

by

Dennis M. Todd

A Dissertation
Presented to the Graduate Committee
of Lehigh University
in Candidacy for the Degree of
Doctor of Philosophy
in
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Lehigh University
1978

Approved and recommended for acceptance as a dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

September 21, 1978

Date

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A very special thanks is due his sister, Shelly, for the beautiful memories and love.

DEDICATION

To Linda and Damian

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ABSTRACT

Two studies have been conducted directed toward the improvement of existing clinical chemistry methodologies.

Chemical methods available for the measurement of blood triglyceride levels involve the isolation of the triglycerides followed by hydrolysis to free glycerol and subsequent oxidation of the liberated glycerol by periodate. Numerous methods exist for the quantitation of liberated glycerol and, thus, the original triglyceride levels. The method described here involves the use of *tris*-(2,2'-bipyridine)-iron(II) as a colorimetric reagent for unused periodate. *Tris*-(2,2'-bipyridine)-iron(II) exhibits a rather intense charge transfer absorption band at 522 nm. The oxidation of *tris*-(2,2'-bipyridine)-iron(II) by periodate in acidic solutions followed spectrophotometrically by observing the decrease in absorbance at 522 nm. Aqueous solutions containing glycerol were subjected to oxidation by excess periodate and the unused periodate was measured using *tris*-(2,2'-bipyridine)-iron(II). Glycerol in solutions as dilute as 2.5 ng/ml was easily detectable with a reproducibility of about 2%. The kind of acid used and its concentration were important factors. Interferences observed in acetic acid media were

found to be due to the precipitation of an iron(III)-periodate complex. Extension of the method to the standard triglyceride analysis was unsuccessful. The solvents used for the extraction of triglycerides from blood affected the dissociation of the *tris*-(2,2'-bipyridine)-iron(II) reagent.

The possibility of using fluorescamine in a fluorometric analysis for urinary δ-aminolevulinic acid was also studied. Fluorescamine will react with δ-aminolevulinic acid and generate a fluorophor emitting at 490 nm (390 nm excitation). The extension of this technique to a urine analysis presented several problems. A method was investigated by which one could differentiate the fluorophor generated by the fluorescamine-δ-aminolevulinic acid reaction. It was found that for successful use of fluorescamine in the analysis of urinary δ-aminolevulinic acid the method of separating the δ-aminolevulinic acid from urine was of profound importance.

I. Introduction

The data produced by clinical chemistry laboratories are used for the diagnosis and treatment of disease. As the field of clinical chemistry has developed, there has been a corresponding increase in the number of requested tests. Although a number of methods may exist for the determination of a chemical constituent in a biological sample, there is always room for improvement in the quality, sensitivity, and specificity of the individual tests. Whether the improvement comes through the use of more sensitive analytical techniques or a reduction in the number of manipulations, or simplification of such manipulations, the results should be the same and that is an increase in the accuracy of the result. The need for simplicity can not be overstated. With the advent of automation coupled with an ever increasing work load, the need for fast and accurate backup procedures is a must.

This dissertation consists of two parts. Both are concerned with the improvement of existing methodologies. Section III involves a study aimed at improving the standard chemical method for triglyceride analysis. In this study the use of *tris*-(2,2'-bipyridine)-iron(II) as a colorimetric reagent for the measurement of unused periodate in the standard chemical triglyceride analysis has been investigated.

Section IV involves the development of a method for the quantitation of urinary δ-aminolevulinic acid by fluorometry. Although the use of analytical luminescence techniques has not enjoyed the popularity obtained by other analytical techniques it none-the-less offers the clinician a highly sensitive tool with which to assay for trace amounts of biologically important compounds. The theoretical aspects of luminescence will not be discussed and the reader may consult other texts on the fundamentals of luminescence (36,37,38).

Each section contains its own introductory material; hence the rather short formal introduction.

II. General Experimental

A. Ultra-violet Visible Absorption Spectra

All absorption spectra were recorded on a Beckman Model DK-2A ratio recording spectrophotometer using quartz-window absorption cells of 1.00 cm optical path-length. All absorption spectra, unless otherwise stated, were taken at room temperature.

B. Luminescence Spectra

All luminescence measurements were made using a Farrand Model 10424 spectrofluorometer. It was fitted with a General Electric UA-2 250 watt low pressure mercury lamp as the excitation source and an RCA 1P-28 photomultiplier tube as the detector. Emission spectra were recorded on an Esterline Angus XY350 Recorder. Excitation monochromator slit widths were 2.0 mm and 0.5 mm, respectively. Emission monochromator slit widths were 1.0 mm and 0.5 mm, respectively, making the spectral bandpass of the excitation and emission monochromators were *ca*. 5 nm.

All luminescence spectra shown are uncorrected for variation in the response of the photomultiplier tube and source intensity with wavelength. 10x10x48 mm quartz cells were used for all luminescence measurements.

C. Mass Spectra

All mass spectral measurements were made using a

Finnigan Automated 4000 GC/MS system used in the solid probe analysis configuration. The data were collected and normalized by the accompanying Data General Computer System.

D. Materials

All chemicals used were of reagent grade quality. Distilled water was used in all experiments. δ-aminolevulinic acid was obtained from Sigma Chemical Co., St. Louis, Mo. Tris-2,2'-bipyridine-iron(II) was obtained from G. F. Smith Chemical Co., Columbus, Ohio. Fluorescamine was obtained as a gift from Hoffman-LaRoche, Nutly, N.J.

E. Ion Exchange Chromatography

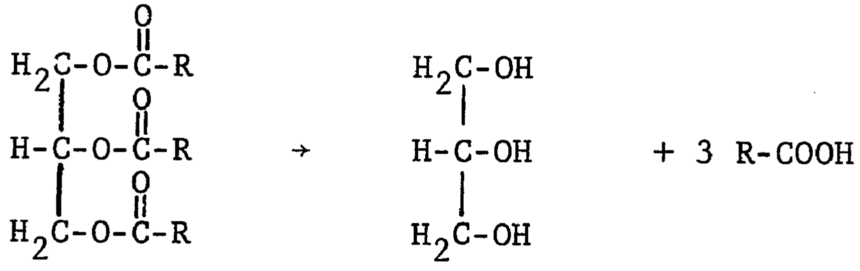
Dowex 50W-X8, 200-400 mesh, cation exchange resin was used for all chromatographic procedures. The resin consists of sulphonic acid residues attached to cross-linked polystyrene beads and binds various types of cations during separation via cation attachment to the $-SO_3^-$ group.

Experimental details will be provided in appendix A.

III. An Improved Triglyceride Analysis Involving Tris-(2,2'-bipyridine)-iron(II)

A. Introduction

Triglycerides are fatty acid esters of glycerol.



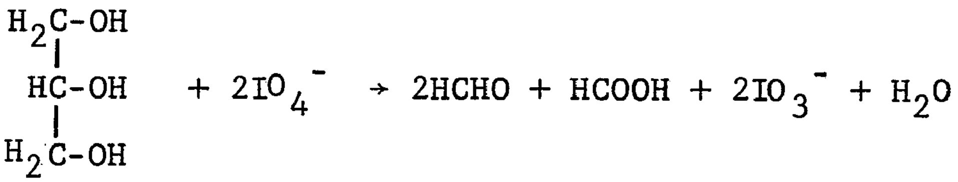
They make up the most abundant fraction of neutral glycerides in the body. They are stored mainly in adipose tissue and are intimately linked with carbohydrate metabolism. Various hyperlipoproteinemias are associated with increased levels of triglycerides and it is therefore necessary to be able to measure those levels in blood. In addition, the triglyceride levels, along with the levels of other blood lipids, can be correlated with tendencies towards heart disease.

The various metal ion chelates of 1,10-phenanthroline, 2,2'-bipyridine, and related imine compounds are extremely valuable as reversible redox indicators for titrations involving strong oxidizing agents such as cerium (IV). Prior to this use, the ligands themselves were widely used as colorimetric reagents in the spectrophotometric detection of iron because of the intense red color of the iron (II) ligand complex.

Malaprade, in 1928, found that periodic acid and its salts were capable of cleaving quantitatively the carbon-carbon bond of 1,2 diols (1). Later it was established that for the cleavage to take place the hydroxyl groups had to be on adjacent carbon atoms. Thus, periodate became established as a useful analytical reagent. Its use was later extended to the oxidation of hydroxycarbonyl and dicarbonyl compounds and 1,2-amino alcohols (2).

B. Periodate Oxidations of Glycerol

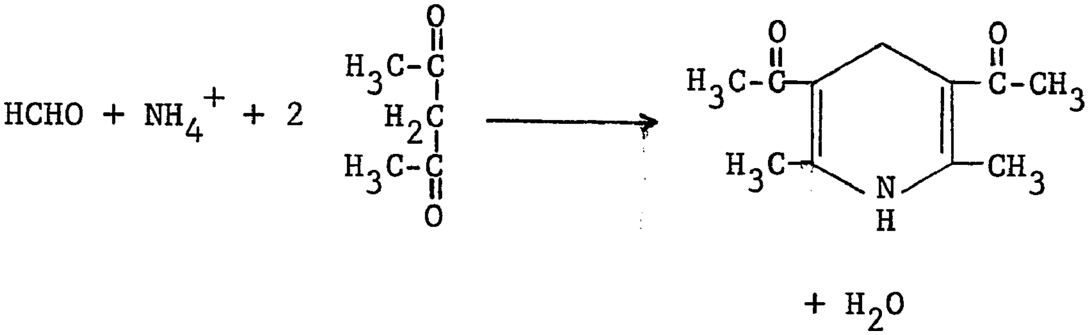
The oxidation of glycerol is typically "Malapradian":



$$\begin{matrix} H_2C\text{-}OH \\ | \\ HC\text{-}OH \\ | \\ H_2C\text{-}OH \end{matrix} + 2IO_4^- \rightarrow 2HCHO + HCOOH + 2IO_3^- + H_2O$$

The oxidation rate is not very fast and therefore this method does not lend itself to direct titration with standard periodate solutions. Consequently an excess of periodate is added and the reaction allowed to proceed to completion.

A number of methods have been used to quantitate the glycerol that was present before oxidation. Titration of the formic acid produced with a standard base serves as the basis for the determination of glycerol in the presence of ethylene glycol (3,4). It has also been used for the determination of glycerol in the presence of a variety of organic compounds so long as they are not oxidized by periodate or, if they are, do not produce formic acid (4). The most commonly used methods involve a colorimetric reaction with the formaldehyde produced. In the Hantzsch condensation reaction (5), a yellow colored diacetyl lutidine is produced from the reaction of formaldehyde with ammonium ion and acetyl acetone:



The absorption peak of the diacetyl lutidine is at 415 nm. Alternatively, its fluorescence at 510 nm with excitation by 412 nm light can be measured. The method of Van Handel and Zilversmit (6) uses an acidic solution of chromotripic acid to form a pink colored derivative. The absorption peaks of the colored derivative is at 564 nm. One can also measure the excess periodate with a standard solution of thiosulfate (7).

Recently enzymatic methods have come into routine use in clinical laboratories. The one for triglycerides involves the use of three enzymes for the measurement of glycerol: glycerol kinase, pyruvate kinase, and lactate dehydrogenase (20):

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol phosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenol pyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}$$

The last reaction is monitored by the decrease in absorbance at 340 nm due to the loss of NADH.

The work presented in this section is an attempt to develop an improved method for the determination of blood triglyceride levels. Tris-(2,2'-bipyridine)-iron(II) was used as a colorimetric reagent for unused periodate.

Willard and Greathouse (8) first described a method for the determination of manganese (II) by oxidation with periodic acid or its salts:

$$2Mn^{2+} + 5IO_4^- + 3H_2O \rightarrow 2MnO_4^- + 5IO_3^-$$

Manganese oxidations by periodate in the presence of various metals are extensively reviewed by G. F. Smith (4). Sensie and Silva (9) proposed a method for the detection of periodate using tris-(2,2'-bipyridine)-manganese(II). Very little supporting data were presented, but this was the first mention of the possible oxidation of a metal-(2,2'-bipyridine) complex by periodate.

C. Tris-(2,2'-bipyridine)-iron (II)

Tris-(2,2'-bipyridine)-iron (II) was first described in 1888 by Blau. It is an intensely red colored compound showing a number of absorption maxima. Figure

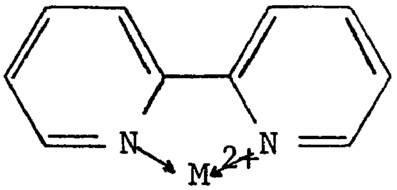
1 shows the uv-visible absorption spectrum of tris-(2,2'-bipyridine)-iron (II) in aqueous solution with its characteristic charge transfer band at 522 nm. At pH 5.0 the formation of the complex is rapid and can be represented by the following stepwise formation scheme:

$$\mathrm{Fe}^{2+} + \text{2,2'-bipyridine} \xrightleftharpoons{k_1} \text{Fe(2,2'-bipyridine)}^{2+}$$

$$\text{Fe(2,2'-bipyridine)}^{2+} + \text{2,2'-bipyridine} \xrightleftharpoons{k_2} \text{Fe(2,2'-bipyridine)}_2^{2+}$$

$$\text{Fe(2,2-bipyridine)}_2^{2+} + \text{2,2'-bipyridine} \xrightleftharpoons{k_3} \text{Fe(2,2'-bipyridine)}_3^{2+}$$

2,2'-bipyridine functions as a typical bidentate ligand molecule through the nitrogen atoms forming five membered rings.

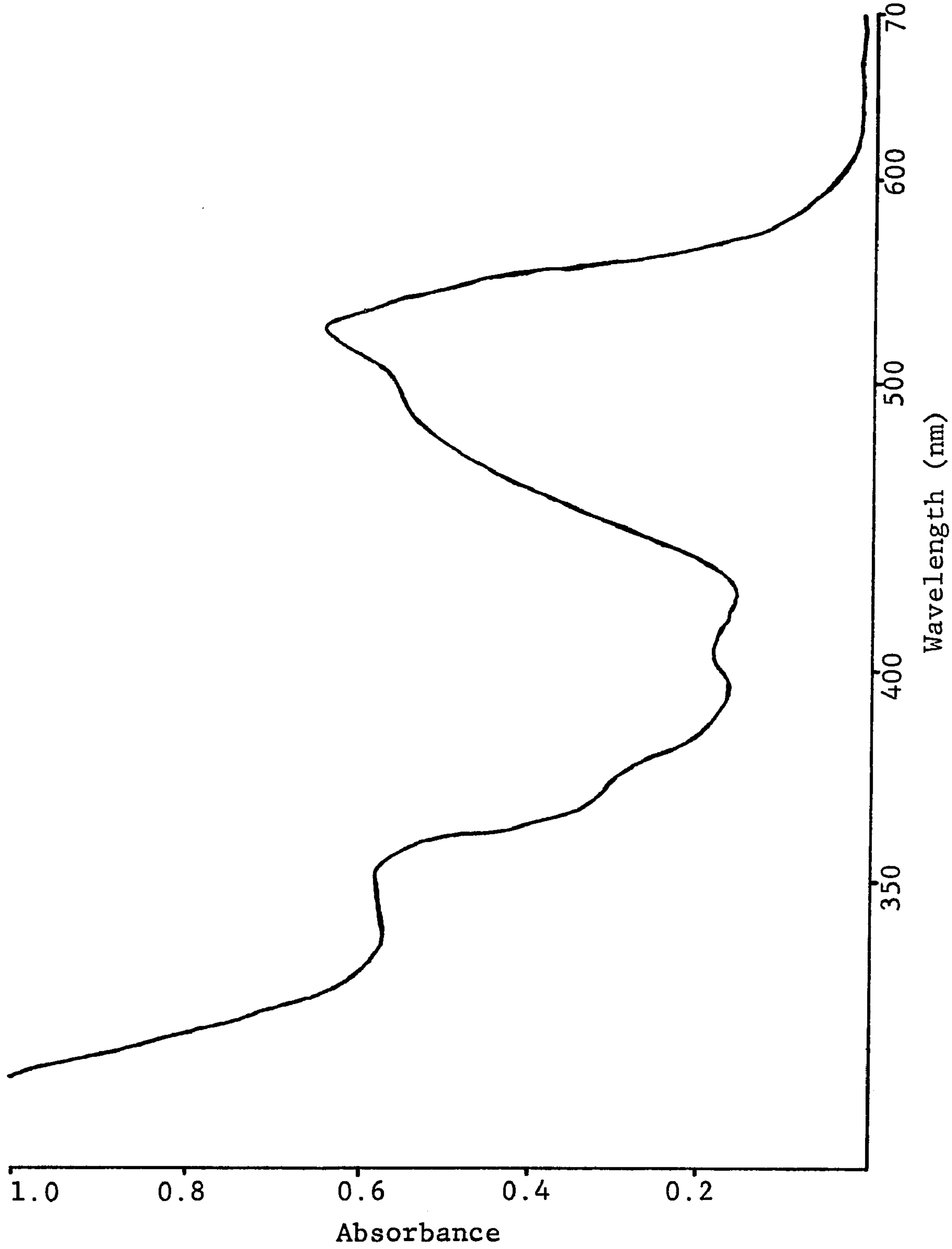


mono-(2,2'-bipyridine)-metal (II)

Figure 1. Visible absorption spectrum of *tris*-(2,2'-bipyridine)-iron(II) in aqueous solution.

12.5 μmol $Fe(bipy)_3^{2+}$, 2 mmol acetic acid, total volume = 50 ml, 3:10 dilution, b = 1.00 cm, heating 2 minutes at 100°C.

Figure 1



Oxidation of the tris-(2,2'-bipyridine)-iron (II) to tris-(2,2'-bipyridine)-iron (III) is easily obtained using cerium (IV) and other strong oxidizing agents.

$$\mathrm{Fe(2,2'\text{-}bipyridine)_3^{2+} + Ce\ (IV) \rightarrow Fe(2,2'\text{-}bipyridine)_3^{3+} + Ce\ (III)}$$

The tris-(2,2'-bipyridine)-iron (III) gives a pale blue color and is easily converted back to tris-(2,2'-bipyridine)-iron (II) by the addition of a mild reducing agent, e.g. sodium sulfite or hydrogen peroxide.

Schilt (10) measured the formal oxidation-reduction potentials for the iron (II)-iron (III) bipyridine complex couple in sulfuric acid; the results are given in Table 1. It has been noted previously that the formal potentials are very dependent on the acid concentration as well as the kind of acid used (11). The overall formation constant for tris-(2,2'-bipyridine)-iron (II) has been evaluated through potentiometric measurements and reported as log $\beta_3 = 16.4$.

TABLE 1

Formal Oxidation-reduction Potentials for tris-(2,2'-bipyridine)-iron(II) in H_2SO_4 solutions at 25°C (10).

Formal Concentration of H_2SO_4 :	0.01 F	0.05	0.11	0.50
Formal Potential :	1.069 V.	--	1.026	--

Concentration:	1.0	2.0	4.0	6.0
Potential :	1.00	1.00	0.95	0.88

Concentration:	10	12
Potential :	0.8	--

D. Oxidation of *tris*-(2,2'-bipyridine)-iron(II) with Periodate.

The estimated standard reduction potential for paraperiodic acid-iodate couple (12):

$$H_5IO_6 + H^+ + 2e^- \quad = \quad IO_3^- + 3H_2O$$

is given as $E^o \sim +1.6$ V. vs. NHE. No data have been given describing the exact experimental conditions but a consensus among a number of authors seems to agree with the above value (3,21). The ionization equilibria of paraperiodic acid (12).

$$H_5IO_6 \rightleftarrows H^+ + H_4IO_6^- \qquad K_{a_1} = 1 \times 10^{-3}$$

$$H_4IO_6^- \rightleftarrows IO_4^- + 2H_2O \qquad K = 43$$

$$H_4IO_6^- \rightleftarrows H^+ + H_3IO_6^{3-} \qquad K_{a_2} = 3 \times 10^{-7}$$

suggest that the principal species present in acidic aqueous solutions are periodate (IO_4^-), and paraperiodic acid (H_5IO_6). At pH $\geq$ 2 the amount of $H_4IO_6^-$ becomes significant.

Figure 2 shows the typical absorption spectrum of a solution of *tris*-(2,2'-bipyridine)-iron(II) completely oxidized to *tris*-(2,2'-bipyridine)iron(III) by an excess of cerium (IV). The addition of cerium (IV) gave complete and instantaneous color change at room temperature to the characteristic pale-blue color of *tris*-(2,2'-bipyridine)-iron(III).

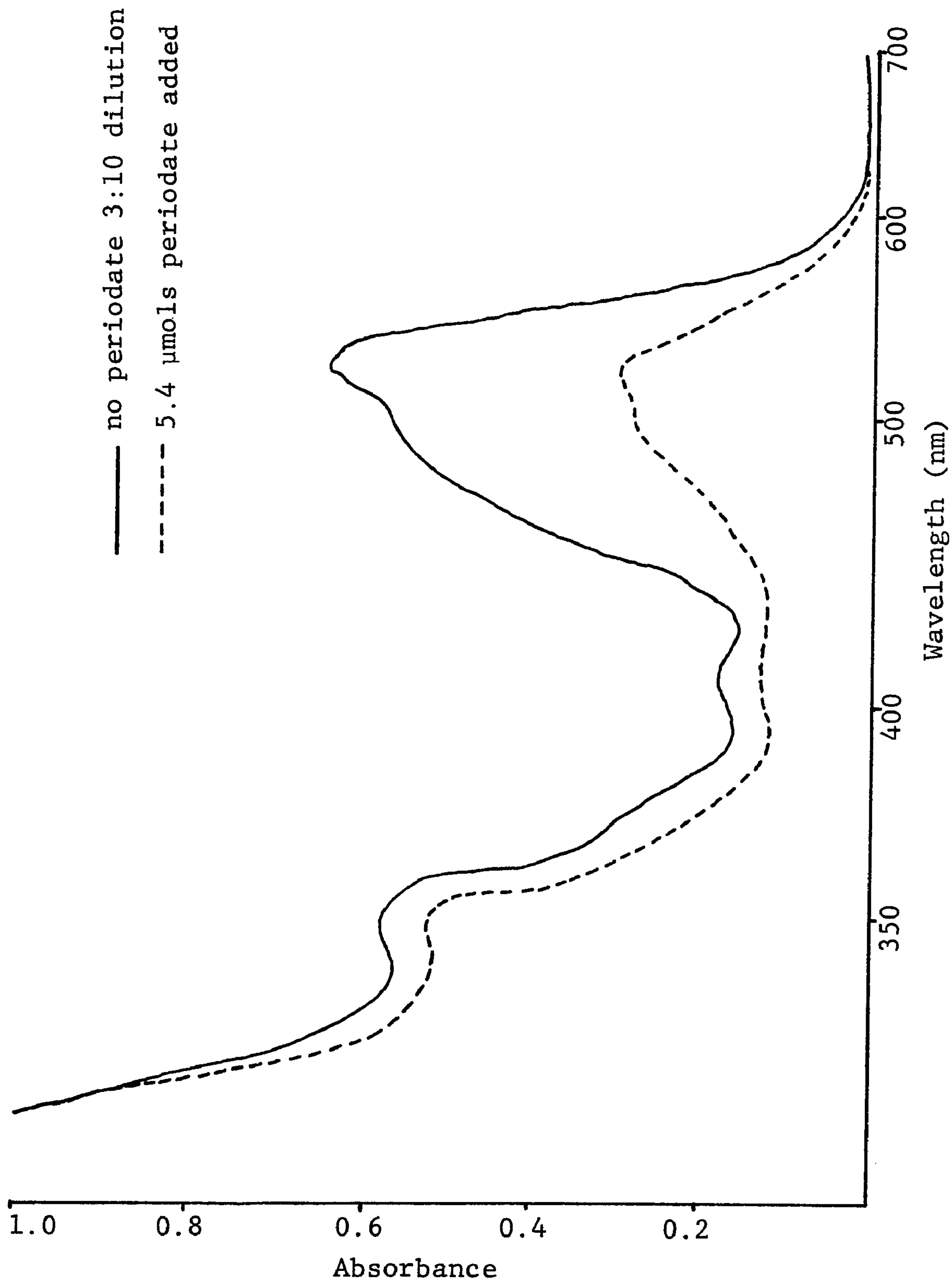
The oxidation of *tris*-(2,2'-bipyridine)-iron(II) by periodate was not instantaneous, but upon prolonged standing the pale-blue color of *tris*-(2,2'-bipyridine)-iron(III) did appear. It was necessary to place the *tris*-(2,2'-bipyridine-iron(II)-periodate solutions in a 100°C water bath for five minutes to get loss of the red color. After heating, there was no blue color discernable.

It is evident from Figure 2 that the addition of periodate causes a decrease in the original absorbance of *tris*-(2,2'-bipyridine)-iron(II). The absorption maximum at 610 nm in Figure 3 is attributable to the *tris*-(2,2'-bipyridine)-iron(III) species. In those solutions where periodate was used as the oxidizing agent and where heating at 100°C for five minutes took place, there was no visible evidence of the *tris*-(2,2'-bipyridine)-iron(III) species, i.e., no blue color. In addition, the absorption spectra of those solutions did not show a maximum at 610 nm. As stated above, if the periodate-*tris*-(2,2'-bipyridine)-iron(II)

Figure 2. Visible absorption spectrum of tris-(2,2'-bipyridine)-iron(II) in aqueous solution after oxidation with periodate.

12.5 μmol $Fe(bipy)_3^{2+}$, 2mmol acetic acid, 5.4 μmol IO_4^-, total volume = 50 ml, 3:10 dilution, b = 1.00 cm, heating 2 minutes at 100°C.

Figure 2


no periodate 3:10 dilution
5.4 μmols periodate added
Wavelength (nm)
350
400
500
600
700
Absorbance
1.0
0.8
0.6
0.4
0.2

solution was allowed to stand at room temperature for about 2 hours, the characteristic pale-blue color appears and the absorption spectrum shown in Figure 4 resembles that shown in Figure 3. Figure 4 represents a tris-(2,2'-bipyridine)-iron(II) solution to which a large excess of periodate was added and the mixture was allowed to stand at room temperature for 2 hours. The oxidation was not complete as evidenced by the absorption maximum at 522 nm but none-the-less, the blue color due to the presence of tris-(2,2'-bipyridine)-iron(III) was clearly visible. Also in Figure 4 the 610 nm absorption maximum of tris-(2,2'-bipyridine)-iron-(III) is present.

It is clearly evident that periodate will oxidize tris-(2,2'-bipyridine)-iron(II) to tris-(2,2'-bipyridine)-iron(III). The lack of an absorption maximum at 610 nm and no visible blue coloration in those solutions heated for 5 minutes at 100°C seems to be contradictory. This apparent contradiction can be explained if one considers dissociation of the tris-(2,2'-bipyridine)-iron(III). This is not unreasonable because those solutions of tris-(2,2'-bipyridine)-iron(II) oxidized to tris-(2,2'-bipyridine)-iron(III) by cerium (IV) and those oxidized by periodate at room temperature had rapid loss of the blue color.

Figure 3. Visible absorption spectrum of tris-(2,2'-bipyridine)-iron(II) in aqueous solution after oxidation with Ce (IV).

10.84 µmol $Fe(bipy)_3^{2+}$, 2 mmol acetic acid, excess Ce (IV), total volume = 50 ml, b = 1.00 cm.

Figure 3

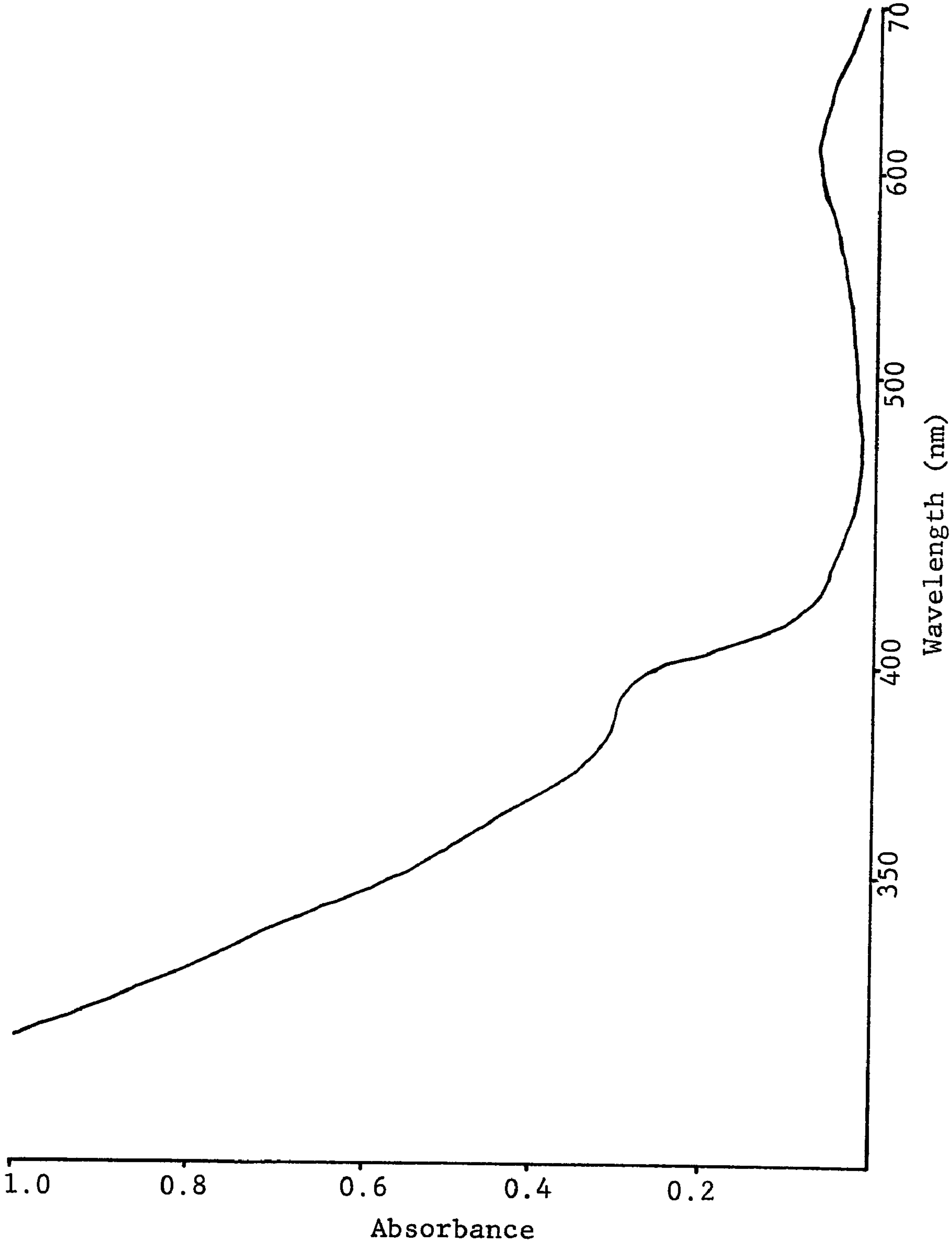
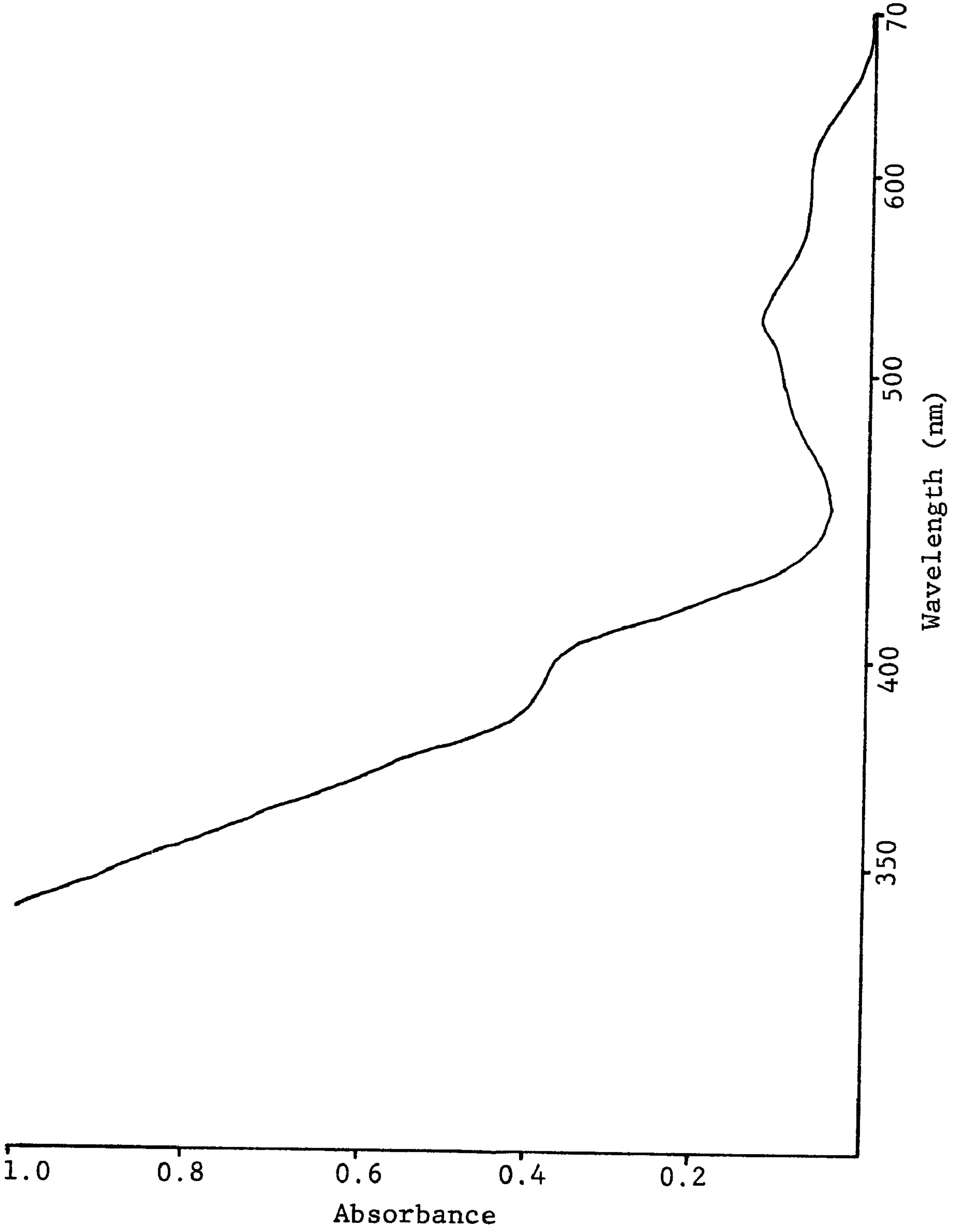


Figure 4. Visible absorption spectrum of <u>tris</u>-(2,2'-bipyridine)-iron(II) in aqueous solution after oxidation with periodate.

10.84 μmol $Fe(bipy)_3^{2+}$, 2 mmol acetic acid, excess periodate, total volume = 50 ml, b = 1.00 cm, no heating and spectrum recorded 2 hours after adding periodate.

Figure 4



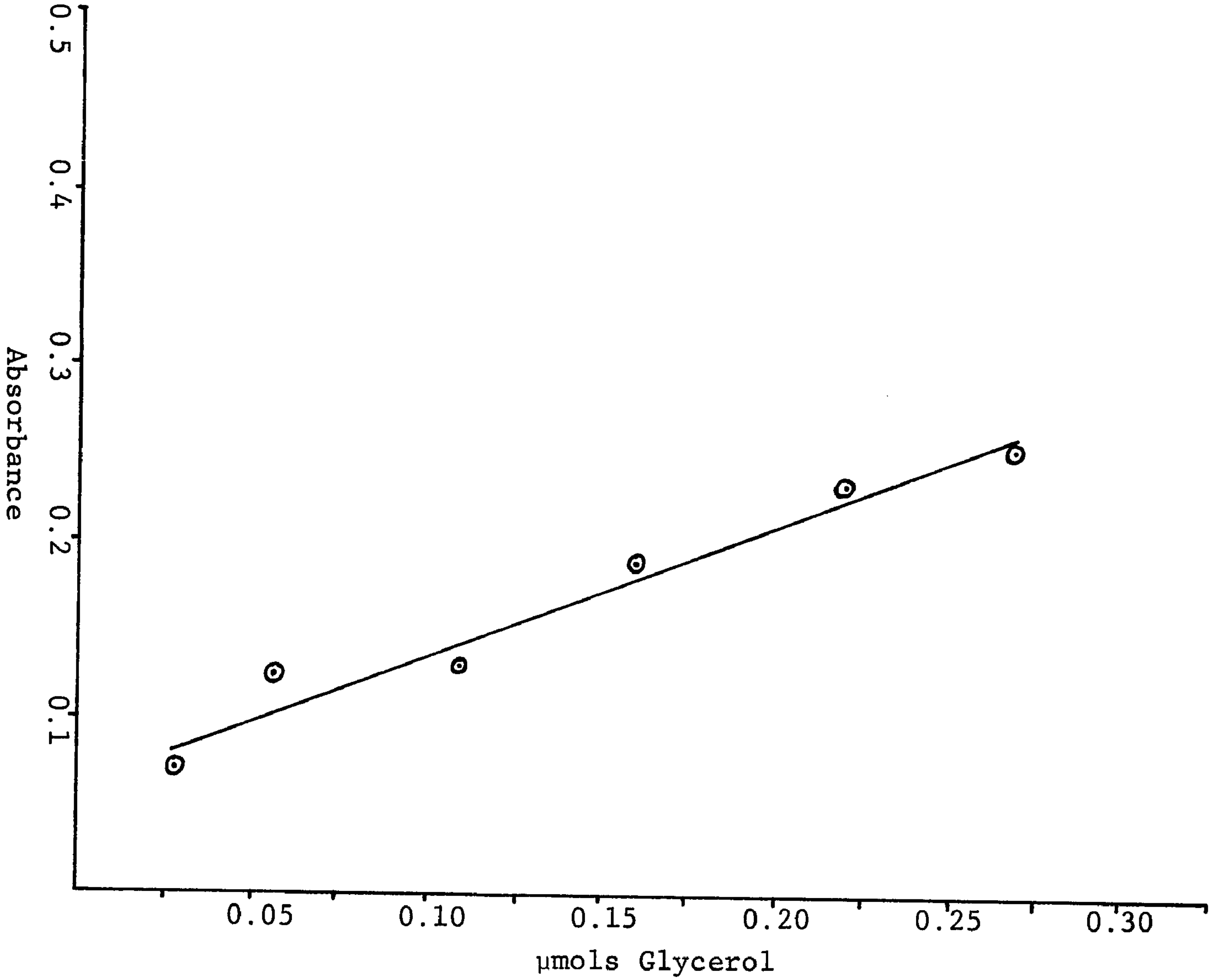
As previously stated, the intent of this work is to develop an improved method for triglyceride analysis. After extraction of the triglycerides from the blood the glycerol must be liberated. The most common method in use today is hydrolysis by a strong base such as sodium or potassium hydroxide. The rate of reaction of periodic acid with organic compounds is most rapid near pH 4 (3). Because of this it is necessary to neutralize any excess base left after hydrolysis and before oxidation takes place. In order to combine the neutralization and oxidation steps most procedures make use of a mixed periodate-acid reagent. Acetic acid is the most commonly used acid. Initial experiments, therefore, used an acetic acid-periodate mixture. For reasons to be discussed later phosphoric acid was substituted for acetic acid.

Figure 5 shows a typical calibration curve obtained from the determination of glycerol by periodate oxidation using *tris*-(2,2'-bipyridine)-iron(II) as a colorimetric reagent to determine the unused periodate. The oxidation of glycerol is complete within 5 minutes under these conditions, *i.e.* pH ∿ 4.0, but it is necessary to heat the solution after addition of the *tris*-(2,2'-bipyridine)-iron(II) to effect its oxidation by periodate in any reasonable length of time. As little as 2.5 mg glycerol per ml of solutionwas easily detectable and

Figure 5. Representative calibration curve for glycerol analysis.

6.25 μmol $Fe(bipy)_3^{2+}$, 1.83 μmol IO_4^-, 0.1 mmol H_3PO_4, total volume = 50 ml, b = 1.00 cm. 5 minutes allowed for oxidation of glycerol by IO_4^-. Solution heated 2 minutes at 100°C after addition of $Fe(bipy)_3^{2+}$.

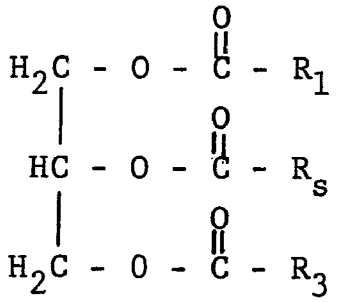
Figure 5


0.5
0.4
0.3
0.2
0.1
Absorbance
0.05
0.10
0.15
0.20
0.25
0.30
µmols Glycerol

the reproducibility of the method was about 2% with a relative standard deviation of 5.9%.

Giegel, Ham, and Clema (19) introduced a method which accomplishes the separation of triglycerides through the partition of serum lipids between a water/isopropanol phase and a nonane solvent phase. The non-polar triglycerides are extracted into the nonane. The extraction into nonane is highly specific for triblycerides with very little (≤1%) extraction of interfering substances such as phospholipids. The hydrolysis is accomplished through the use of an isopropanol/sodium hydroxide reagent followed by the standard periodate oxidation of liberated glycerol. Liberated glycerol is then estimated by the Hantzch condensation methodology.

The above procedure was used substituting *tris*-(2,2'-bipyridine)-iron(II) for the Hantzsch reagents. It was found that after extraction and hydrolysis of triolein:



where R_1, R_2, and R_3 are oleic acid residues esterified to glycerol, there was inhibition of the periodate oxidation of tris-(2,2'-bipyridine)-iron(II). Triolein is the triglyceride commonly used as a standard for the triglyceride analysis. The various constituents of the final reaction mixture were then considered to ascertain their part in the apparent inhibition.

Table 2 outlines the results of these experiments. Considering Table 2 it becomes readily apparent that the cause seems to be the isopropanol used in the hydrolyzing reagent.

Initial experiments using the acetic acid-periodate reagent gave two interesting observations. The expected reaction

$$2\ \mathrm{Fe(2,2'\text{-}bipyridine)}_3^{2+} + \mathrm{IO_4^-} + 2\mathrm{H^+} \rightarrow 2\mathrm{Fe(2,2'\text{-}bipyridine)}_3^{3+} + \mathrm{IO_3^-} + \mathrm{H_2O}$$

should give a 2:1 (tris-(2,2'-bipyridine)-iron(II): periodate) reaction stoichiometry. When one considers the observed stoichiometry of the acetic acid-periodate solutions, as indicated by Figure 6, one sees an apparent 1:1 (tris-(2,2'-bipyridine)-iron(II):periodate) reaction. Secondly with prolonged standing, without heating and with heating at 100° for 5 minutes, there

TABLE 2

Each reaction solution contains 16.26 µmols *tris*-(2,2'-bipyridine)-iron(II), 8.95 µmols IO_4^-, 0.5 mmols acid in additon to the following. Total volume = 50 ml*:

Additional Reagent	Result**
0.5 ml hydrolyzing reagent (0.1 M NaOH in isopropanol)	Negative
0.5 ml isopropanol	Negative
0.5 ml H_2O	Positive
0.5 ml concentrated (18 M) H_2SO_4	Positive
0.5 ml hydrolyzing reagent and 3 drops 18 M H_2SO_4	Negative
0.5 ml 0.1 M NaOH	Positive
Blank (no IO_4^- added)	

* Heated 2 minutes at 100°C.

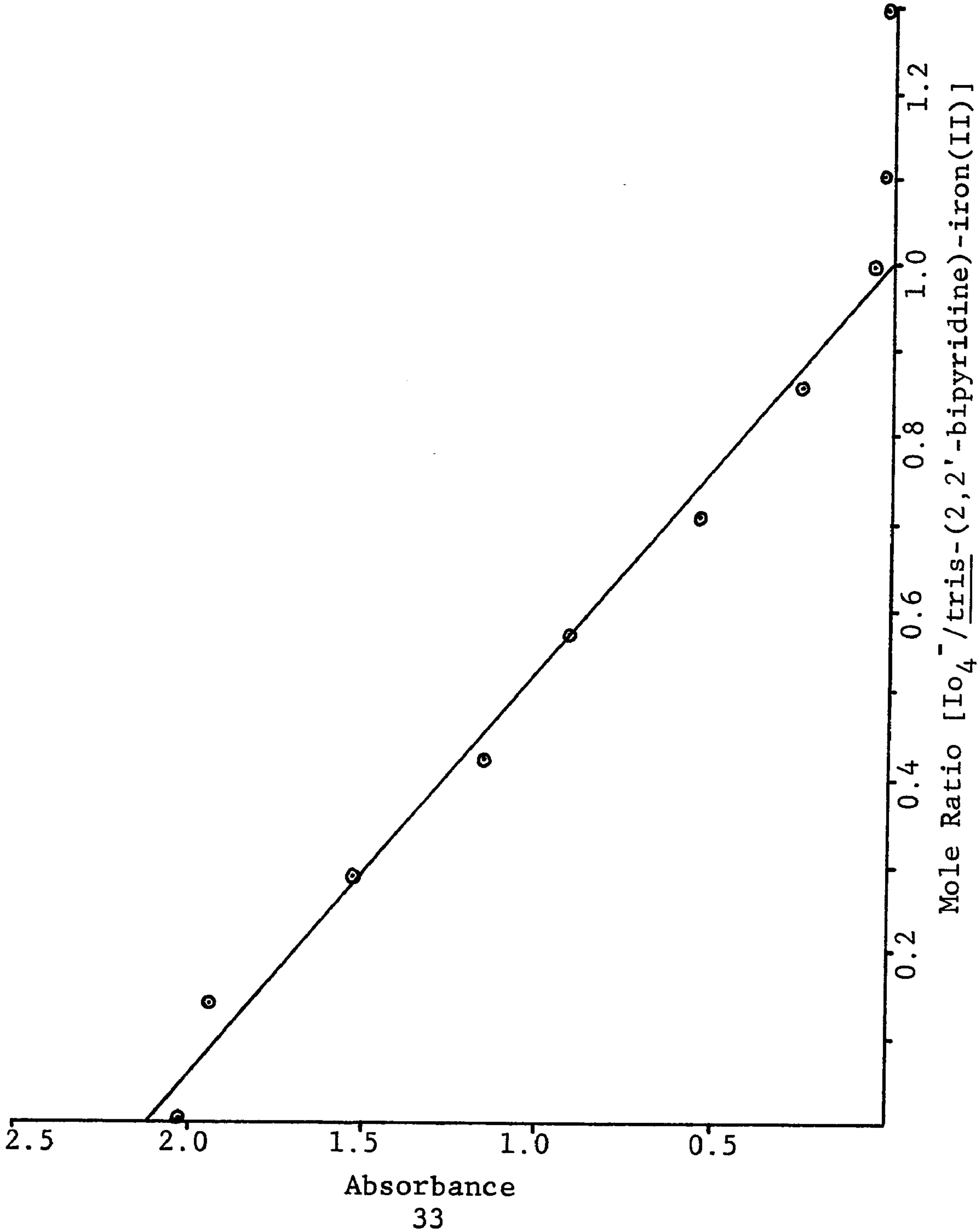
**Negative = no loss of color compared to blank

Positive = loss of color compared to blank.

Figure 6. Oxidation of tris-(2,2'-bipyridine)-iron(II) by periodate in acetic acid.

12.5 μmol $Fe(bipy)_3^{2+}$, 2 mmol acetic acid, total volume = 50 ml, b = 1.00 cm, heated 2 minutes at 100°C.

Figure 6


Mole Ratio [IO_4^-/tris-(2,2'-bipyridine)-iron(II)]
0.2
0.4
0.6
0.8
1.0
1.2
Absorbance
2.5
2.0
1.5
1.0
0.5

was precipitate formation.

The following work was conducted to ascertain why there seemed to be an apparent 1:1 (tris-(2,2'-bipyridine)-iron(II):periodate) stoichiometry in acetic acid, what was the precipitate formed and its effect on the periodate oxidation of tris-(2,2'-bipyridine)-iron(II), and what is the effect of isopropanol on that oxidation.

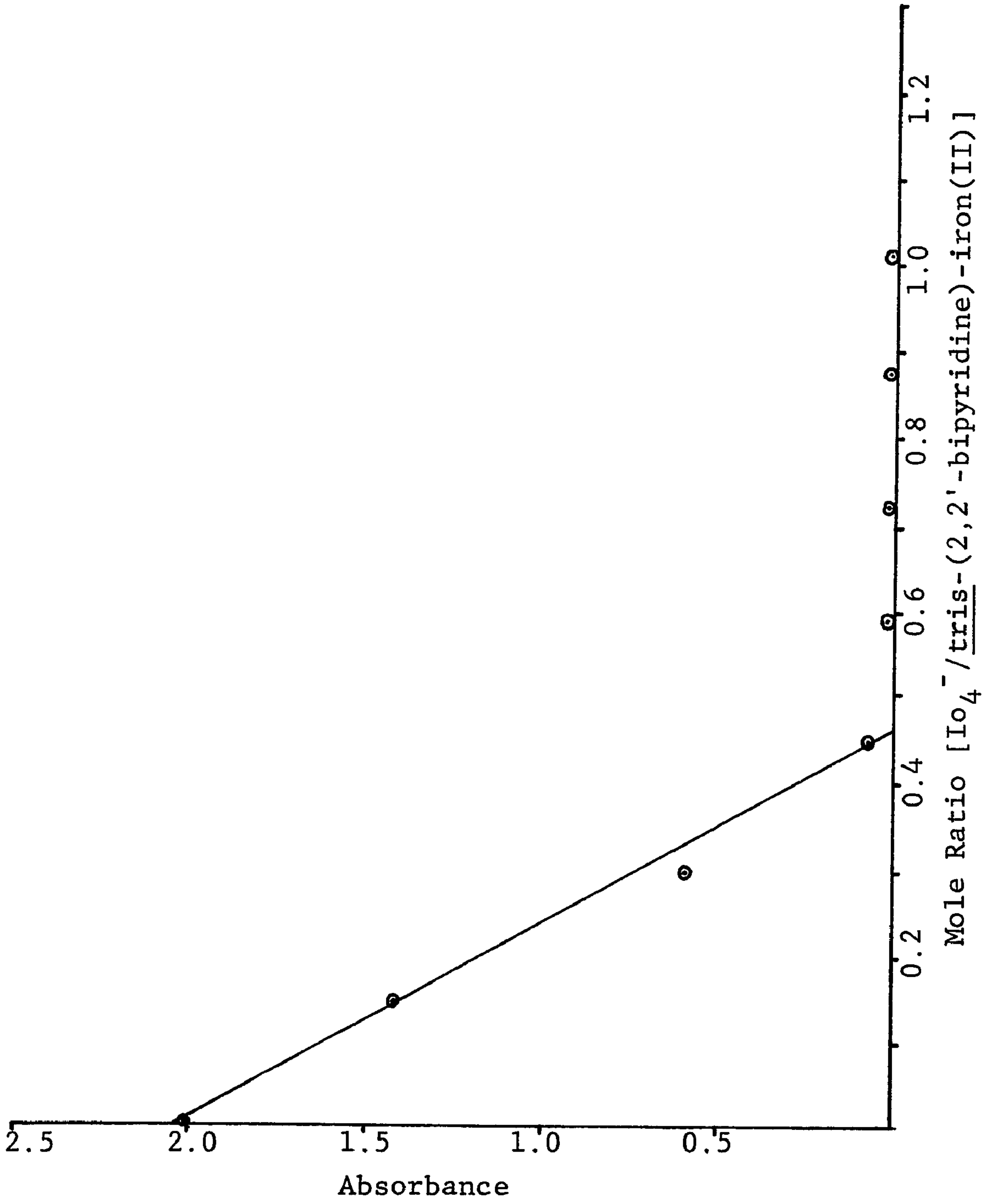
E. Effect of Kind of Acid and Its Concentration

Acetic, phosphoric, and sulfuric acids were considered. With the use of acetic acid there was formation of a yellow-brown precipitate. Sulfuric acid media showed similar precipitate formation, but visibly less when compared to the acetic acid solution and only in those sulfuric acid solutions where there was an excess of periodate in comparison to tris-(2,2'-bipyridine)-iron(II) concentration. Precipitate formation occurred rapidly in those solutions (acetic or sulfuric acid media) heated for 5 minutes at 100^{o}C. At no time did the solutions containing phosphoric acid show evidence of the yellow-brown precipitate. Figures 7 and 8 show results for the reaction between periodate and tris-(2,2'-bipyridine)-iron(II) in, respectively, sulfuric and phosphoric acid media. It is clearly evident that in both cases the expected 2:1 (tris-(2,2'-bipyridine)-iron(II):periodate) reaction stoichiometry is approxi-

Figure 7. Oxidation of tris-(2,2'-bipyridine)-iron(II) by periodate in sulfuric acid.

12.5 μmol $Fe(bipy)_3^{2+}$, 0.05 mmol H_2SO_4, total volume = 50 ml, b = 1.00 cm, heated 2 minutes at 100°C.

Figure 7

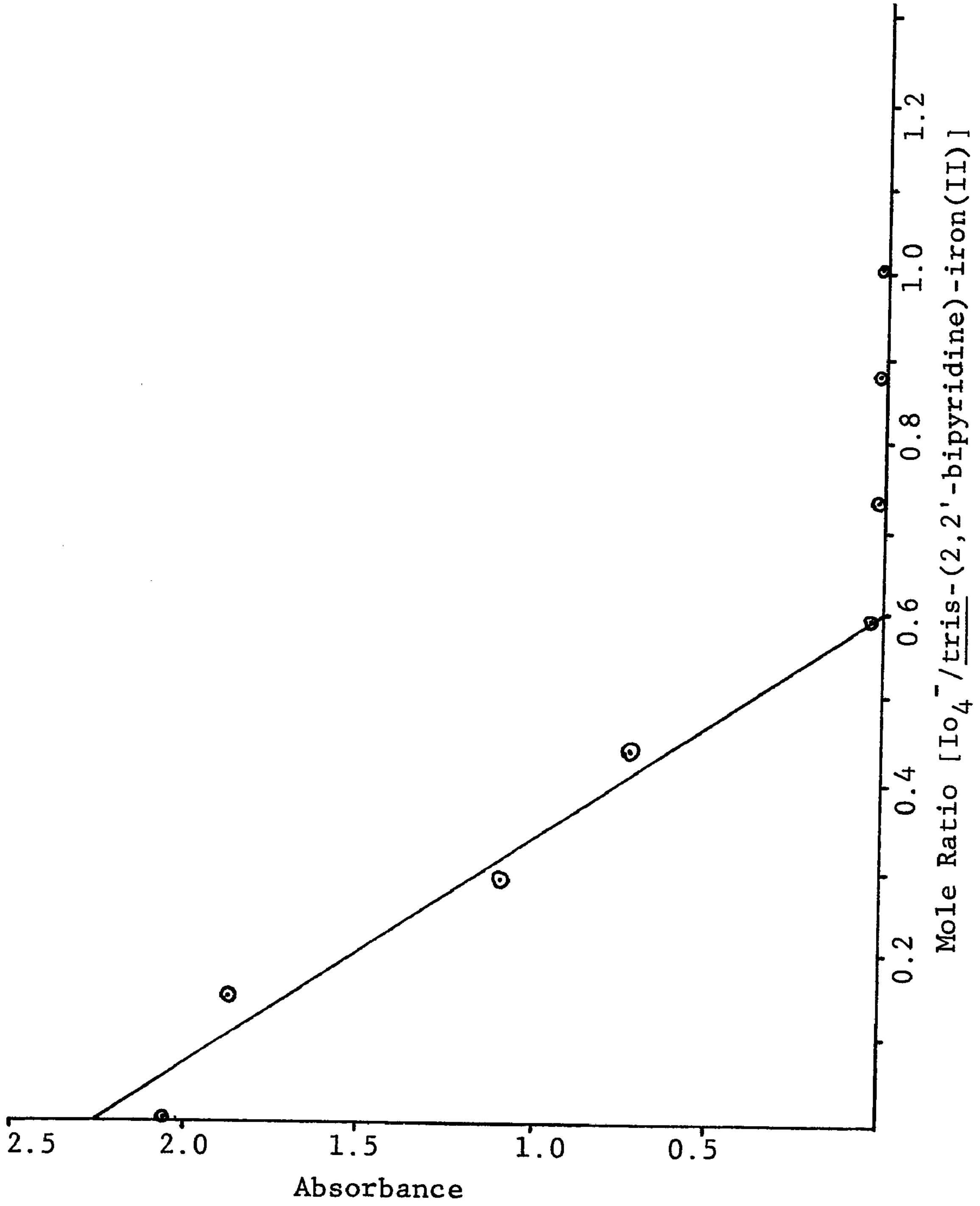


Mole Ratio [Io$_4^-$/tris-(2,2'-bipyridine)-iron(II)]
0.2
0.4
0.6
0.8
1.0
1.2
Absorbance
2.5
2.0
1.5
1.0
0.5

Figure 8. Oxidation of tris-(2,2'-bipyridine)-iron(II) by periodate in phosphoric acid.

12.5 μmol $Fe(bipy)_3^{2+}$, 0.1 mmol H_3PO_4, total volume = 50 ml, b = 1.00 cm, heated 2 minutes at 100°C.

Figure 8


Mole Ratio [Io$_4^-$/tris-(2,2'-bipyridine)-iron(II)]
0.2
0.4
0.6
0.8
1.0
1.2
Absorbance
2.5
2.0
1.5
1.0
0.5

mated. In all cases the solutions were filtered prior to absorbance measurements in order to remove any precipitate formed. The absorption spectrum of those solutions containing sulfuric acid showed evidence of light scattering which indicates incomplete removal of the precipitate and the actual stoichiometry is perhaps less than 2:1. The spectra for the acetic acid media showed similar evidence of light scattering which again would indicate that the actual stoichiometry is less than the observed 1:1 (tris-(2,2'-bipyridine)-iron(II):periodate). There was no evidence of light scattering from the phosphoric acid solutions.

Another important factor concerning the acid used is its concentration. The iron-2,2'-bipyridine system is similar to the iron-1,10-phenanthroline in that the dissociation of the iron (III) complex in both systems shows an acid-dependent term. They differ in that the iron(II)-2,2'-bipyridine complex also shows an acid-dependent term whereas the iron(II)-1,10-phenanthroline complex does not (11).

Table 3 shows the effect of varying the phosphoric acid concentration on the dissociation of tris-(2,2'-bipyridine)-iron(II). Results determined by absorbance measurements were similar for acetic and for sulfuric acid solutions. The data in Table 3 show that the optimum amount of phosphoric acid present ranged between

TABLE 3

Reaction mixture contains 6.25 moles $Fe(bipy)_3^{2+}$ in the presence of 1.83 μmoles IO_4^- (Total volume = 50 ml) λ_{abs} = 522 nm

	mmols H_3PO_4	Absorbance
1.	0.00	0.745
2.	0.05	0.607
3.	0.07	0.598
4.	0.09	0.603
5.	0.10	0.609
6.	0.12	0.573
8.	0.15	0.566
9	0.50	0.380
10.	1.00	0.178
11.	2.00	0.069
12.	3.00	0.046

0.05 to 0.1 mmols per 50 ml of reaction mixture or between 1×10^{-3} $\underline{M}$ and 2×10^{-3} $\underline{M}$. In this range of concentrations the decrease in absorbance at 522 nm was kept to about 10% with an additional loss of 10% in absorbance due to heating.

The data show that the kind of acid chosen is important in preventing formation of the precipitate while the concentration is important with respect to dissociation of *tris*-(2,2'-bipyridine)-iron(II). It should also be noted that in those solutions where heating took place there was no evidence of *tris*-(2,2'-bipyridine)-iron(III) either by absorbance measurements at 610 nm or by the appearance of a blue color. The combination of heating for 5 minutes at 100°C and the phosphoric acid concentration used (2×10^{-3} $\underline{M}$) caused dissociation of *tris*-(2,2'-bipyridine)-iron(III). Support for this assumption comes from considering the conditions outlined earlier in conjection with Figure 4. Hydrochloric acid was not considered because periodate oxidizes chloride to chlorine (8). Nitric acid was not considered for reasons which will be discussed later.

F. Precipitate Investigation

Because this proposed triglyceride analysis involves measuring the unused periodate after glycerol oxidation it is imperative that there be no extraneous reactions involving periodate. Precipitate formation as described in the previous experiments could constitute such an interference. It therefore becomes necessary to characterize the precipitate composition.

The ultraviolet absorption spectrum of the possible complexes of iron (II) with 2,2'-bipyridine and of the various forms of 2,2'-bipyridine itself have been fairly well characterized (13, 14, 15). The absorption maxima with their corresponding molar absorptivities reported by previous investigators are listed in Table 4. Figure 9 is a typical absorption spectrum (ultraviolet region) of a reaction solution containing acetic acid, tris-(2,2'-bipyridine)-iron(II), and periodate. The mixture was filtered prior to making the absorption measurements; the precipitate was collected and dried under vacuum. The absorption spectrum of tris-(2,2'-bipyridine)-iron-(III) in the ultraviolet region is shown in Figure 10.

Comparisons of the spectrum in Figure 9 with Table 4 reveals the following. The peak at 298 nm corresponds to the reported λ_{max} for tris- and mono-(2,2'-bipyrdine)-iron(II). It also corresponds to the reported λ_{max} for the protonated-2,2'-bipyridine molecule. The spectrum of tris-(2,2'-bipyridine)-iron(III) indicates the presence of a band at 203 nm with a shoulder appearing at 313 nm (Figure 10). Both the visible and ultraviolet

TABLE 4

Westheimer and Beufey (13)

H(2,2'-bipyridine)$^+$	λ_{max} = 302 nm	$\varepsilon = 14.7 \times 10^3$ $\text{mol}^{-1}\text{l}\ \text{cm}^{-1}$
	λ_{max} = 241 nm	$\varepsilon = 7.24 \times 10^3$
HH(2,2'-bipyridine)$^{++}$	λ_{max} = 290 nm	$\varepsilon = 15.2 \times 10^3$

Hazra and Lahiri (14)

2,2'-bipyridine	λ_{max} = 281 nm	ε = 12,835
	λ_{max} = 233	ε = 9726
H(2,2'-bipyridine)$^+$	λ_{max} = 301 nm	ε = 15,180
	λ_{max} = 241 nm	ε = 7230

Baxendal and George (15)

2,2'-bipyridine	λ_{max} = 280 nm	$\varepsilon = 1.34 \times 10^4$
	λ_{max} = 231 nm	$\varepsilon = 1.05 \times 10^4$
H(2,2'-bipyridine)$^+$	λ_{max} = 298 nm	$\varepsilon = 1.36 \times 10^4$
	λ_{max} = 240 nm	$\varepsilon = 5.9 \times 10^3$
Mono-(2,2'-bipyridine)-iron(II)	λ_{max} = 298 nm	$\varepsilon = 1.61 \times 10^4$
	λ_{max} = 248 nm	$\varepsilon = 1.03 \times 10^4$
tris-(2,2'-bipyridine)-iron(II)	λ_{max} = 298 nm	$\varepsilon = 2.38 \times 10^4$
	λ_{max} = 248 nm	$\varepsilon = 9.65 \times 10^3$

Figure 9. Ultraviolet absorbance spectrum of *tris*-(2,2'-bipyridine)-iron(II) after oxidation by periodate.

12.5 μmol $Fe(bipy)_3^{2+}$, 25.9 μmol IO_4^-, 2 mmol acetic acid, total volume = 50 ml, b = 1.00 cm, 1:20 dilution, heated 2 minutes at 100°C.

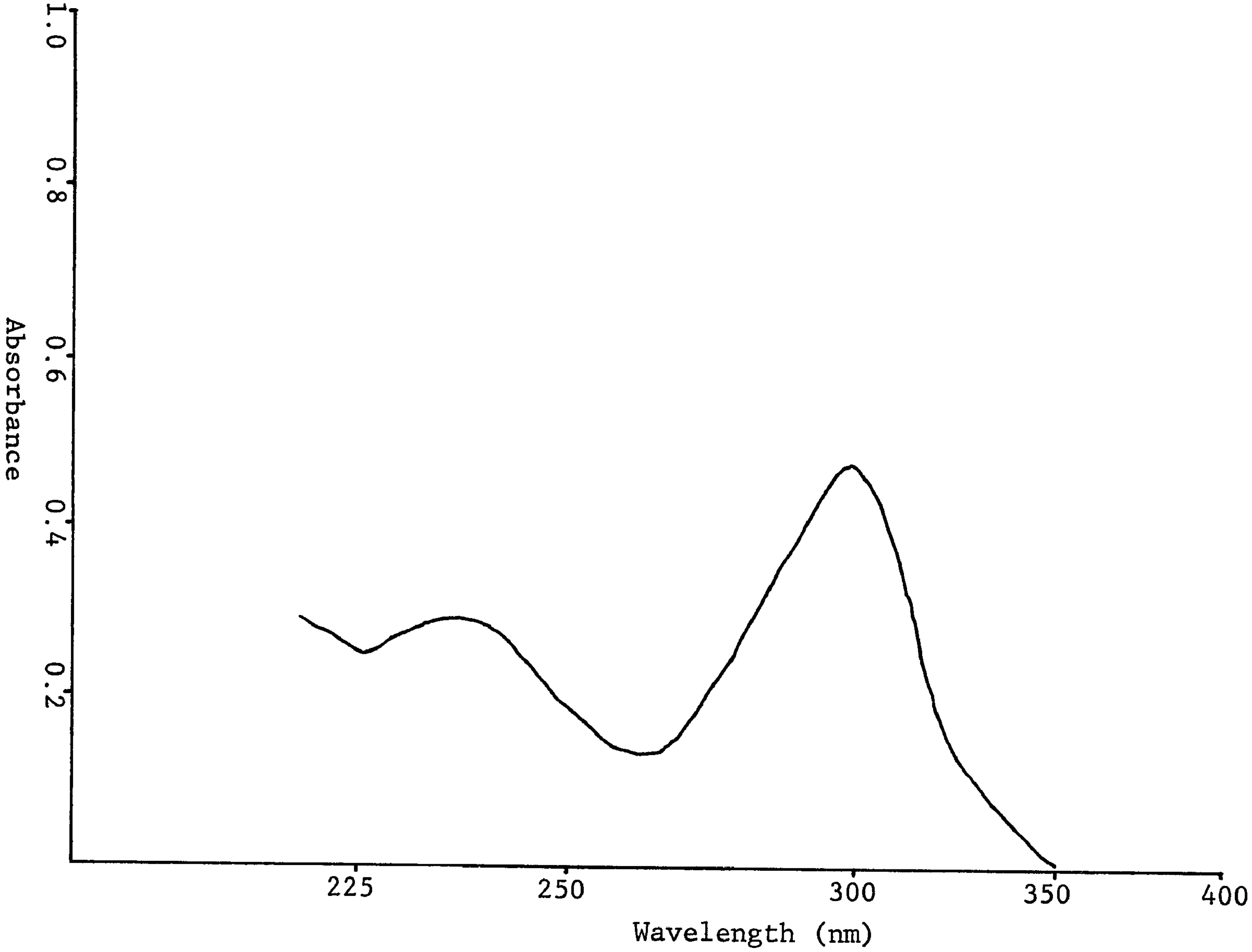
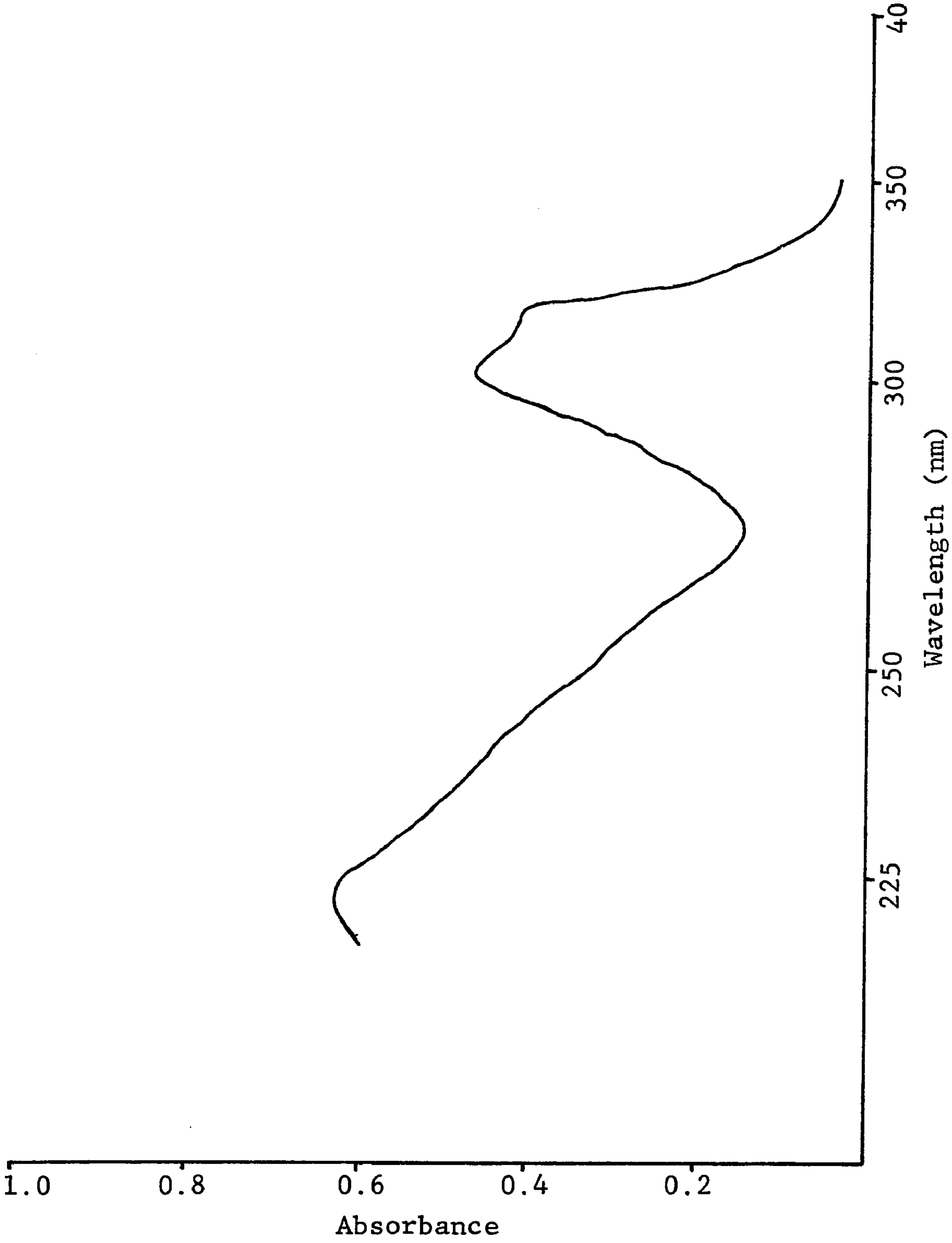


Figure 9

Figure 10. Ultraviolet absorption spectrum of *tris*-(2,2'-bipyridine)-iron(II) after oxidation with Ce (IV).

10.84 μmol $Fe(bipy)_3^{2+}$, 2 mmol acetic acid, excess Ce (IV), total volume = 50 ml, 1:20 dilution, b = 1.00 cm, no heating.

Figure 10



absorption spectra of the reaction mixture do not indicate the presence of tris-(2,2'-bipyridine)-iron(III). Because the reaction mixture contains an excess of periodate, it can be assumed that the band at 298 nm corresponds either to protonated 2,2'-bipyridine or to mono-(2,2'-bipyridine)-iron(II). Periodate shows a λ_{max} at 225 nm and therefore does not constitute an interference at 298 nm (22). The band appearing at 237 nm lies between the reported λ_{max} for protonated-2,2'-bipyridine (240 nm) and unprotonated-2,2'-bipyridine (231 nm). Further, since there appears to be no band at 248 nm, it can be assumed that there is no appreciable quantity of mono-(2,2'-bipyridine)-iron(II) present. The possibility of a mixture of the unprotonated and protonated-2,2'-bipyridine molecules is therefore indicated.

On the basis of the absorption spectra of the reaction solutions (those containing acetic acid, tris-(2,2'-bipyridine)-iron(II), and periodate where precipitate formation occurred) and published absorption bands it is not unreasonable to assume there is a mixture of protonated and unprotonated-2,2'-bipyridine because the pK_a for protonated-2,2'-bipyridine is 4.44 (16). There seems to be very little if any mono- or tris-(2,2'-bipyridine)-iron(II) or tris-(2,2'-bipyridine)-iron(III) present. This does not support the assumption that the oxidation of tris-(2,2'-bipyridine)-iron(II) by periodate has occurred but neither does it rule out this possibility. A series of qualitative tests was undertaken to confirm whether or not oxidation had occurred. The tests were conducted on aliquots of the reaction solution from Figure 9. The results are shown in Table 5.

The test results can be summarized as follows. The addition of zinc powder (a fairly strong reducing agent) should have converted any iron (III) present to iron (II) with reformation of tris-(2,2'-bipyridine)-iron(II). Because the test was negative it can be assumed there was little or no iron (III) present in the aliquot. This was further confirmed by the absence of precipitate formation with the addition of ammonium hydroxide. The acidic potassium thiocyanate gave a slightly positive reaction. This is not contradictory because the thiocyanate reaction is much more sensitive and specific for iron (III). The addition of ferrous salts gave an instantaneous and strongly positive reaction (appearance of red color). This indicates two things: first, it confirms the presence of 2,2'-bipyridine and, secondly, that any iron (III) reduced by the zinc would have reformed tris-(2,2'-bipyridine)-iron(II). Tests for the presnce of iodine showed none present while the presence of iodate and/or periodate was confirmed.

TABLE 5

Species tested for	Reagent	Result
1. Fe(III)	Zn powder	Negative
2. Fe(III)	NH_4OH	Negative
3. Fe(III)	Acidic KSCN	Positive (slightly)
4. 2,2'-bipyridine	Ferrous Ammonium Sulfate	Positive
5. I_2	Starch	Negative
6. IO_3^- and IO_4^-	Starch; I^-; H^+	Positive

The dried precipitate was subjected to the tests shown in Table 6. The precipitate was yellow-brown in color and insoluble in water. It dissolved in concentrated (18 M) sulfuric acid and in concentrated (15 M) phosphoric acid but was insoluble in dilute (0.2 M) nitric acid. Attempts to extract the precipitate into benzene and into ethyl ether failed. Qualitative tests indicated the presence of large quantities of iron (III) along with large quantities of iodate and/or periodate.

The precipitate was subjected to solid probe mass spectral analysis. Figure 11 is the mass spectrum of 2,2'-bipyridine. Figure 12 and 14 represent the mass spectra obtained from the precipitate. Figure 13 represents the ion chromatogram of the precipitate temperature programmed for a 10°C rise per minute from 50 to 300°C. In all cases the fragmentation pattern was recorded from 40 to 600 m/e. The ion chromatogram (Figure 13) shows two distinct peaks. The mass spectrum obtained at points along the first peak (Figure 12) are characteristic of 2,2'-bipyridine with a parent peak at 156.1 m/e. The mass spectrum obtained at points along the second ion chromatogram peak (Figure 14) reveal a similar fragmentation pattern to Figure 12 but now three new peaks appear at 55.8, 126.9, and 253.8 m/e. These peaks correpond respectively to iron, iodide, and diiodine, I_2.

TABLE 6

Precipitate Qualitative Analysis

Species Tested for	Reagent	Result
Fe(III)	Acid KSCN	Strong Positive
IO_3^- and/or IO_4^-	Starch/I^-	Strong Positive

Figure 11. Mass spectrum of 2,2'-bipyridine.

Figure 11

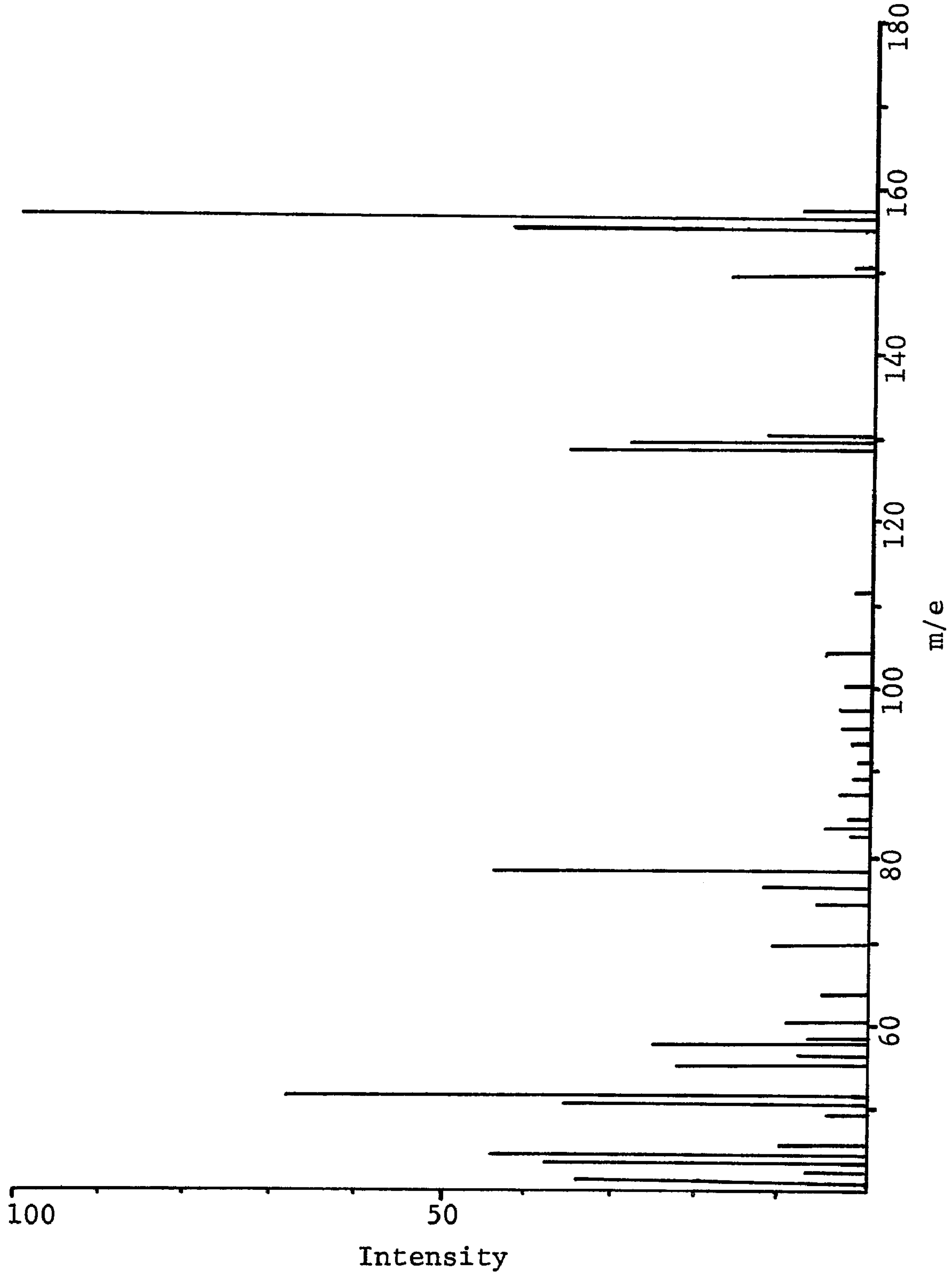


Figure 12. Mass spectrum of unknown precipitate from first ion chromatogram peak.

Figure 12

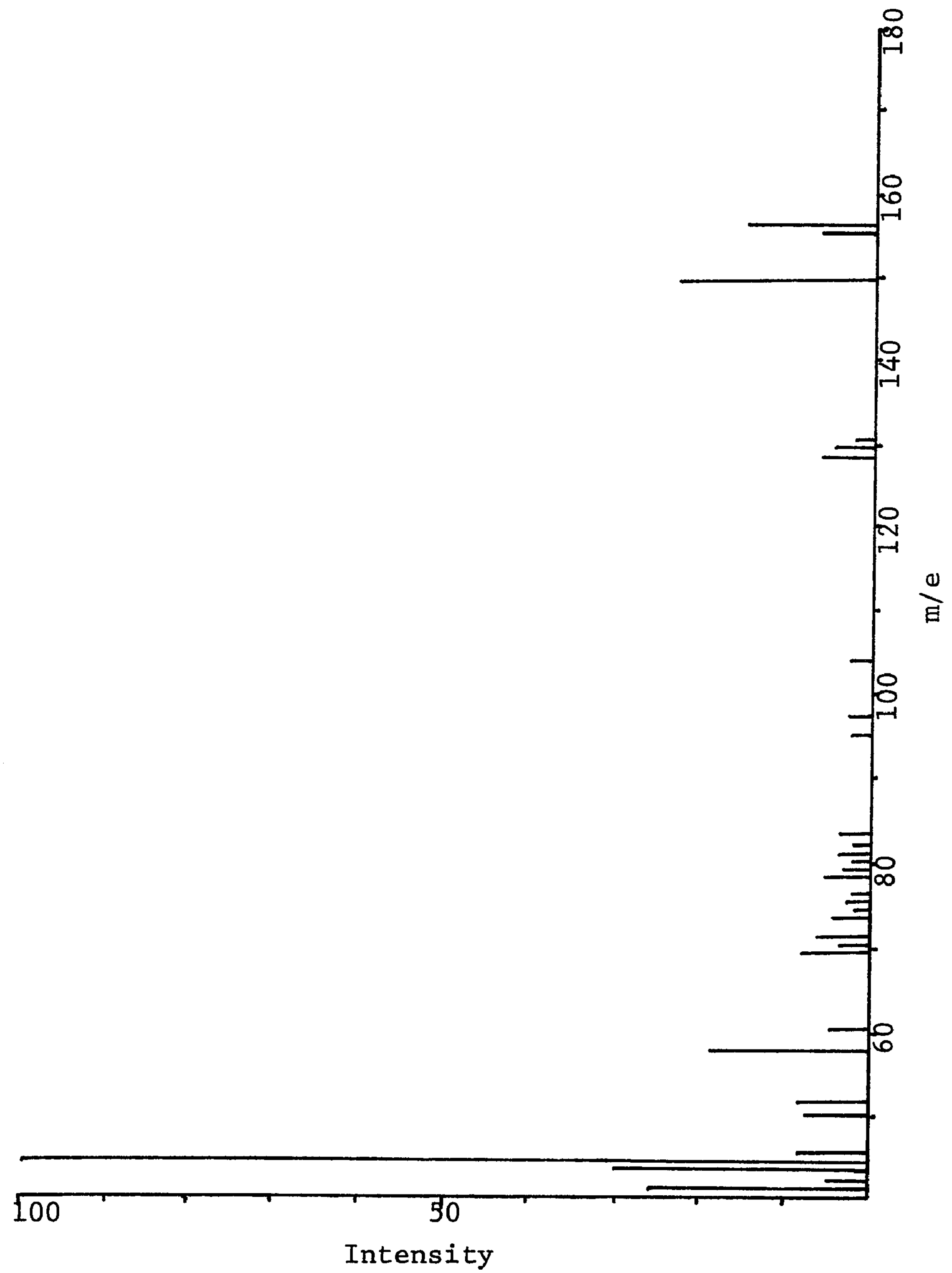


Figure 13. Mass spectral reconstructed ion chromatogram of unknown precipitate.

10°C/min. from 50-300°C.

Figure 13

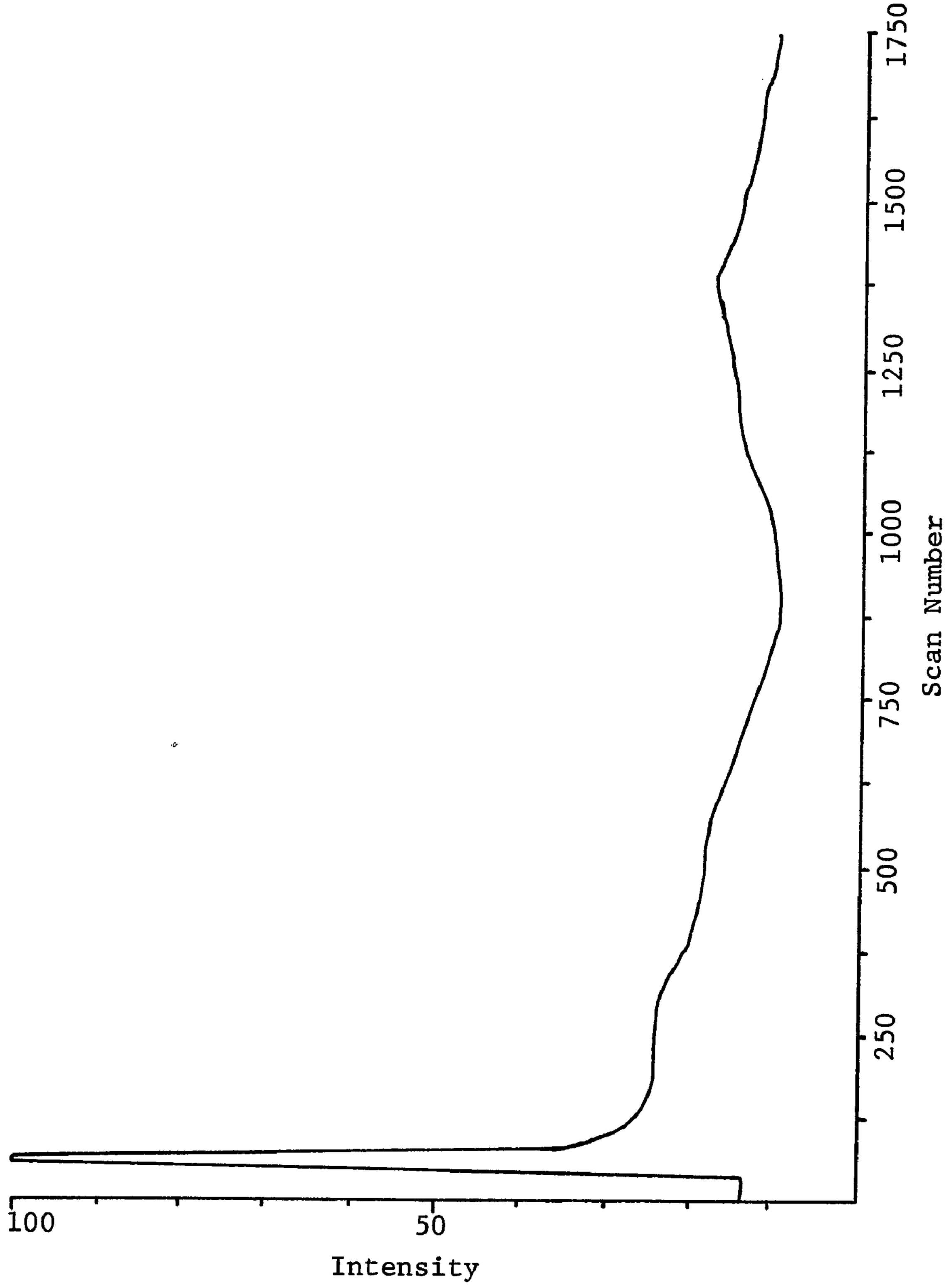
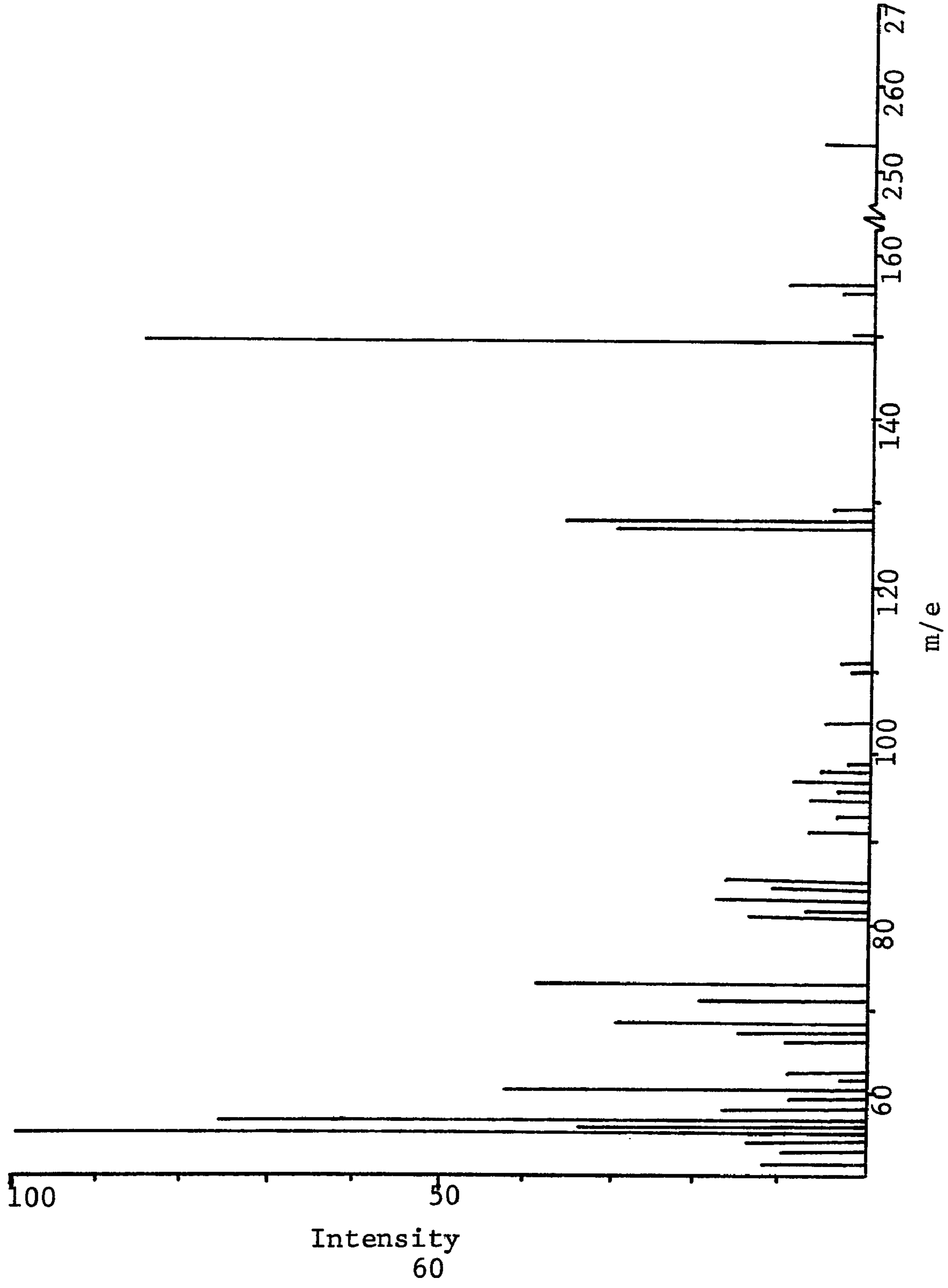


Figure 14. Mass spectrum of unknown precipitate from second ion chromatogram peak.

Figure 14



From the data presented so far and the fact that Willard and Greathouse (8) were concerned with the formation of an iron-periodate complex in their oxidations of manganese (II) using periodate as the oxidizing agent it is logical to assume the precipitate studied here is an iron(III)-periodate complex.

Although very little physical data are presented, Campbell and Nyman (17) offer evidence for the existence of two periodato complexes of iron (III). The 4:3 (iron:iodine) complex, having the formula $H_3 Fe_4I_3O_{24}H_{12}$, is formed in acid solutions; is yellow-ochre in color; forms a brownish-glassy compound on drying and is insoluble in dilute (0.02 M) nitric acid. The 1:2 (iron:iodine) complex, having the formula $Fe(H_2IO_6)_2(OH)_2$, is formed in alkaline solutions; yellow-green in color; forms a pale green glassy compound on drying; insoluble in dilute sodium hydroxide solutions and is converted to the 4:3 complex by the addition of acid.

At this point it becomes possible to propose a mechanism to answer the questions: Why the apparent 1:1 (tris-(2,2'-bipyridine)-iron(II):periodate) stoichiometry in acetic acid? Does the precipitate formed pose a problem in the use of tris-(2,2'-bipyridine)-iron(II) as a colorimetric reagent for unused periodate in the standard chemical triglyceride analysis?

The following mechanism could apply to those reactions run in acetic acid.

(1) $Fe(bipy)_3^{2+} + 3H^+ \rightleftarrows Fe^{2+} + 3H(bipy)^+$ *

(2) $4Fe(bipy)_3^{2+} + 2IO_4^- + 4H^+ \rightarrow 4Fe(bipy)_3^{3+} + 2IO_3^-$
$+ 2H_2O$

(3) $4Fe(bipy)_3^{3+} + 12H^+ \rightleftarrows 4Fe^{3+} + 12H(bipy)^+$

(4) $H(bipy)^+ \rightleftarrows H^+ + bipy$

(5) $4Fe^{3+} + 3IO_4^- + 12H_2O \rightarrow \underline{(Fe_4I_3O_{24}H_{12})}^{3-} + 12H^+$

Combining (2), (3), and (5)

(6) $4Fe(bipy)_3^{2+} + 5IO_4^- + 4H^+ + 10H_2O \rightarrow$
$12H(bipy)^+ + 2IO_3^- + \underline{(Fe_4I_3O_{24}H_{12})}^{3-}$

The following mechanism could apply to those reactions run in sulfuric and phosphoric acid.

(7) $FE(bipy)_3^{2+} + 3H^+ \rightleftarrows Fe^{2+} + 3H(bipy)^+$

(8) $4Fe(bipy)_3^{2+} + 2IO_4^- + 4H^+ \rightarrow 4Fe(bipy)_3^{3+} + 2IO_3^-$
$+ 2H_2O$

* Solution pH 4.0.

(9) $4Fe(bipy)_3^{3+} + 12H^+ \rightleftarrows 4Fe^{3+} + 12H(bipy)^+$

(10) $4Fe^{3+} + 4H_2PO_4^- \rightarrow 4Fe(H_2PO_4)^{2+}$

Combining (8), (9), and (10)

(11) $4Fe(bipy)_3^{2+} + 2IO_4^- + 12H^+ + 4H_2PO_4^- \rightarrow$

$4Fe(H_2PO_4)^{2+} + 2IO_3^- + 2H_2O$

For sulfuric acid, SO_4^{2-} can be substituted for $H_2PO_4^-$.

Equation 6 yields a mole ratio of 1.25 [periodate/ _tris_-(2,2'-bipyridine)-iron(II)] while Equation 11 yields a mole ratio of 0.5. These values are consistent with the observed mole ratios for those experiments shown in Figures 6, 7, and 8. Table 7 gives available literature values for the stability constants of several iron (III)-ligand complexes.

Several conclusions can be drawn from the proposed mechanisms and observations previously discussed. It can be assumed that the relative strength of the iron (III)-periodate complex is greater than the iron(III)-acetate complex because of the observed 1:1 reaction stoichiometry. In those solutions containing sulfuric acid there was visibly less precipitate when compared to those solutions containing acetic acid. At the same time, the observed reaction stoichiometry in sulfuric

TABLE 7

Stability Constants (18)

NO_3^-

$$K_1 = 10^{1.00}$$

$$\beta = 10 = \frac{[Fe(NO_3)^{2+}][H^+]}{[Fe^{3+}][HNO_3]}$$

SO_4^{2-}

$K_1 = 10^{2.03}$ $K_2 = 10^{0.97}$ $\beta(Fe^{3+}HL^-)10^{1.78}$

$$K_1 = 10^{2.03} = \frac{[Fe(SO_4)^+]}{[Fe^{3+}][SO_4^{2-}]}$$

$$K_2 = 10^{0.97} = \frac{[Fe(SO_4)_2^-]}{[Fe(SO_4)^+][SO_4^{2-}]}$$

$$\beta = 10^{1.78} = \frac{[Fe(HSO_4)^{2+}]}{[Fe^{3+}][HSO_4^-]}$$

Acetate

$$K_1 = 10^{3.2}$$

$$K_1 = 10^{3.2} = \frac{[Fe(Ac)^{2+}]}{[Fe^{3+}][Ac^-]}$$

PO_4^{3-}

$(Fe^{3+}H_2L^-)10^{3.45}$ $(Fe^{3+}HL^{2-})10^{8.36}$

$$\beta = 10^{3.45} = \frac{[Fe(H_2PO_4)^{2+}]}{[Fe^{3+}][H_2PO_4^-]} \quad \beta = 10^{8.36} = \frac{[Fe(HPO_4)^+]}{[Fe^{3+}][HOP_4^{2-}]}$$

acid approximated the expected 2:1 (*tris*-(2,2'-bipyridine)-iron(II):periodate) stoichiometry. This could indicate that in solutions containing a ligand which forms a relatively stable complex with iron (III), *e.g.* sulfate, the precipitate formed could be an iron (III)-iodate complex rather than an iron(III)-periodate complex. By similar reasoning one can assume that the relative stability of the phosphate-iron(III) complex is greater than the stability of either the iron(III)-iodate complex or the iron(III)-periodate complex.

Consequently, in order for *tris*-(2,2'-bipyridine)-iron(II) to be used successfully as a colorimetric reagent for unused periodate in a triglyceride analysis, the anion of the acid used must be capable of forming a more stable complex with iron (III) than those formed with iron (III) and periodate or iodate. Alternatively, one can choose an imine which forms a more stable complex with iron (III) than 2,2'-bipyridine does.

G. Solvent Effects

In those triglyceride methodologies involving liquid-liquid extraction of the triglycerides from blood, the hydrolyzing agent must be soluble in the extracting solvent. In the case of the method proposed by Giegel, Ham, and Clema (19) isopropanol is used as the solvent for the hydrolyzing agent, sodium hydroxide. This method involves the extraction of the non-polar triglycerides into nonane. Isopropanol is used as the solvent for the sodium hydroxide because of its solubility in nonane; it is very soluble in nonane and infinitely soluble in water. Thus when the hydrolysis is complete and water is added the polar glycerol partitions into the isopropanol-water phase and the liberated glycerol is then oxidized by periodate.

As indicated earlier the above method was used in the *tris*-(2,2'-bipyridine)-iron(II) system and there appeared to be inhibition of the oxidation of *tris*-(2,2'-bipyridine)-iron(II). Again, earlier data indicated that isopropanol was responsible for the apparent inhibition. Methanol and ethanol have also been used as solvents for hydrolyzing agents in other chemical triglyceride analysis. Experiments were carried out to test if the inhibition of *tris*-(2,2'-bipyridine)-iron(II) oxidation by periodate was unique to isopropanol. The results are given in Table 8. The results showed that the alcohols studied gave results similar to isopropanol, *i.e.* apparent inhibition of the oxidation of *tris*-(2,2'-bipyridine)-iron(II) by periodate. The reaction solutions described in Table 8 contained 5.5% alcohol.

TABLE 8

16.26 μmols tris-(2,2'-bipyridine)-iron(II), 8.95 μmols IO_4^-, 1.5 mmol acid, and .5 ml of following (total volume = 9.0 ml)*:

	Reagent	Result
1.	methanol	Negative
2.	ethanol	Negative
3.	m-propanol	Negative
4.	isopropanol	Negative
5.	isopropanol added after 10 minutes	Positive
6.	water	Positive
7.	blank (no IO_4^-)	

* heated 2 minutes in 100°C water bath.

** negative = no loss of absorbance compared to blank
positive = loss of absorbance compared to blank

Figure 15 illustrates the results obtained from a set of experiments undertaken to determine the effect of varying amounts of isopropanol on an acidic solution of *tris*-(2,2'-bipyridine)-iron(II). Each reaction contained 6.25 μmols *tris*-(2,2'-bipyridine)-iron(II), 0.1 mmols H_3PO_4, and some designated amount of isopropanol. The total volume was 50 ml. Similar experiments were carried out under the same conditions as above but this time with 1.83 μmoles periodate added. The results of these experiments were the same as those shown in Figure 15. The addition of potassium thiocyanate to the blank (containing no periodate) was negative for iron (III). The addition of potassium thiocyanate to solutions containing periodate were positive, indicating the presence of iron (III) and showing that some oxidation has occurred.

As indicated by the results shown in Figure 15 at the 1% level which corresponds to 0.5 ml isopropanol in a total volume of 50 ml, there was essentially none of the original 6.25 μmols *tris*-(2,2'-bipyridine)-iron(II) left. Because the reaction mixture contained no periodate it can be inferred that the decrease in absorbance at 522 nm was due to the dissociation of *tris*-(2,2'-bipyridine)-iron(II). The blank gave an absorbance of 0.854 which is consistent with the

Figure 15. Effect of solvent concentration on the absorbance of <u>tris</u>-(2,2'-bipyridine)-iron(II) at 522 nm.

625 μmols $Fe(bipy)_3^{2+}$, 0.1 mmol H_3PO_4, isopropanol, total volume = 50 ml, b = 1.00 cm, heated for 5 minutes at 100°C.

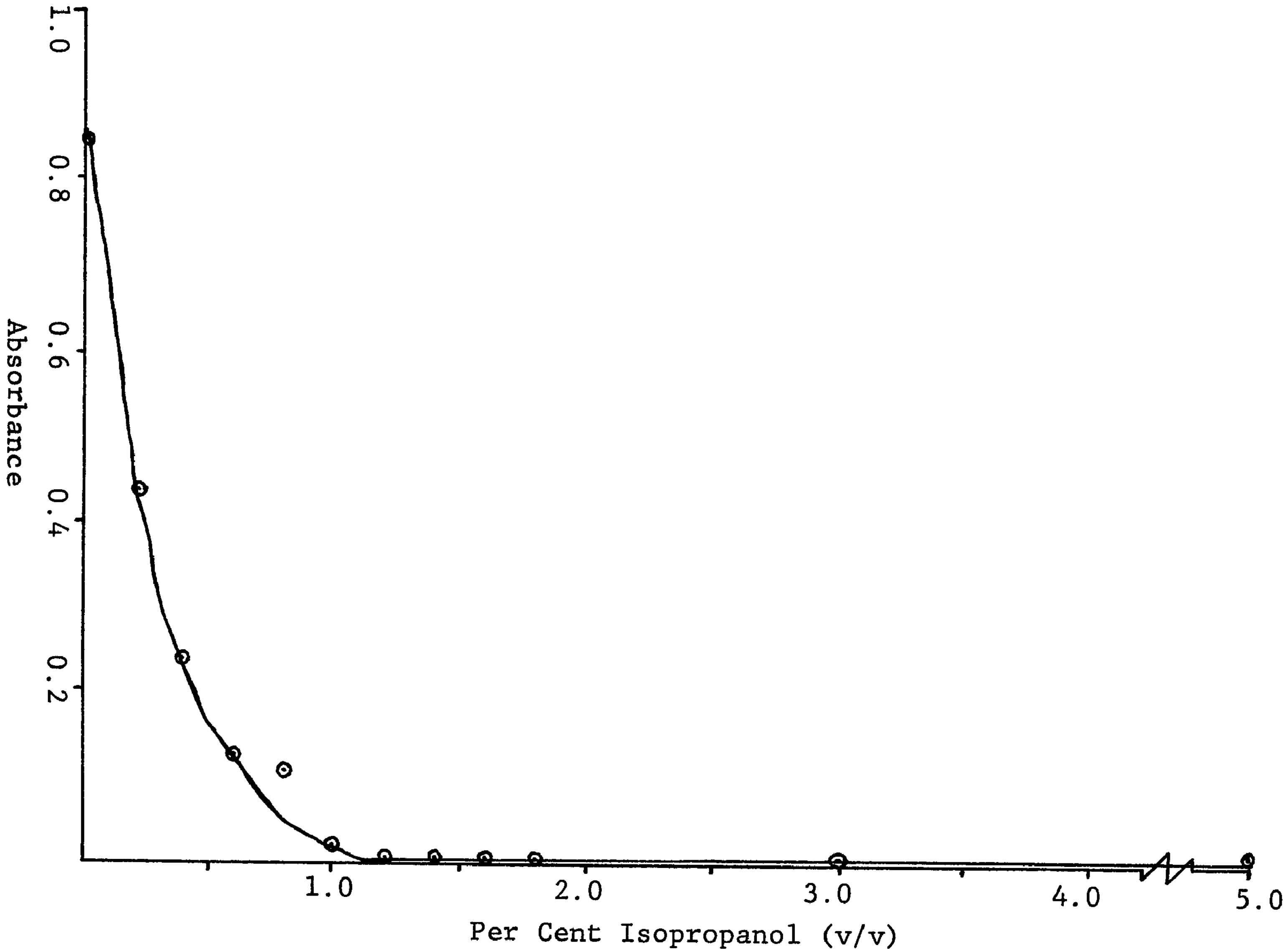

Absorbance
1.0
0.8
0.6
0.4
0.2
1.0
2.0
3.0
4.0
5.0
Per Cent Isopropanol (v/v)

Figure 15

expected value taking into account the decrease in absorbance due to the presence of the acid (21% decrease for 0.1 mmols H_3PO_4/50 ml). It was found that the dissociation of *tris*-(2,2'-bipyridine)-iron(II) in isopropanol solution required a minimum of 4 minutes at 100°C to reach equilibrium. The assumption that the isopropanol inhibited the oxidation of *tris*-(2,2'-bipyridine)-iron(II) by periodate was based on initial experimentation where the reaction solutions were heated at 100°C for only 2 minutes.

The major effect of alcohols, in particular isopropanol, on *tris*-(2,2'-bipyridine)-iron(II) seems to be that of enhancing the complex dissociation in acidic solutions. Periodate itself is unaffected because the presence of iron (III) was confirmed in those solutions containing isopropanol, *tris*-(2,2'-bipyridine)-iron(II), acid, and periodate which were heated 5 minutes at 100°C.

Summary

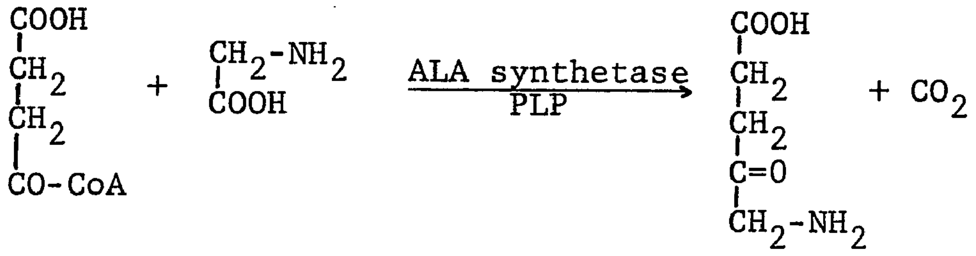
Although the original intent of this work has not been realized, *i.e.* to develop an improved chemical triglyceride analysis, a number of interesting observations have been made.

Tris-(2,2'-bipyridine)-iron(II) can be used as a colorimetric reagent for unused periodate in the standard glycerol analysis in aqueous solutions. This in itself is significant because most methods in use today (non-enzymatic) involve the use of some rather drastic reaction conditions or else unstable reagents. Tris-(2,2'-bipyridine)-iron(II) is very stable and can be used under rather mild reaction conditions. Extending its use to a triglyceride analysis involving liquid-liquid extractions did not work. Its failure in this respect does suggest that other imine metal chelates without the acid dependent dissociation characteristics of tris-(2,2'-bipyridine)-iron(II) might be more successful. In particular the manganese complex of 2,2'-bipyridine could be a likely candidate.

IV. Fluorescence Determination of δ-Aminolevulinic Acid

Introduction

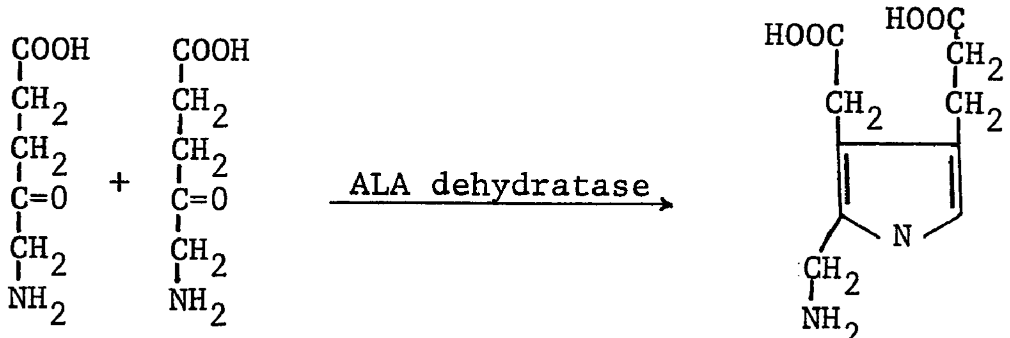
δ-aminolevulic acid (ALA) is synthesized in the liver and marrow of the long bones. Evidence suggests that it is formed through the condensation of glycine and succinyl CoA by pyridoxal-phosphate (PLP) and ALA synthetase.



$$HOOC-CH_2-CH_2-CO-CoA + CH_2(NH_2)-COOH \xrightarrow[\text{PLP}]{\text{ALA synthetase}} HOOC-CH_2-CH_2-C(=O)-CH_2-NH_2 + CO_2$$

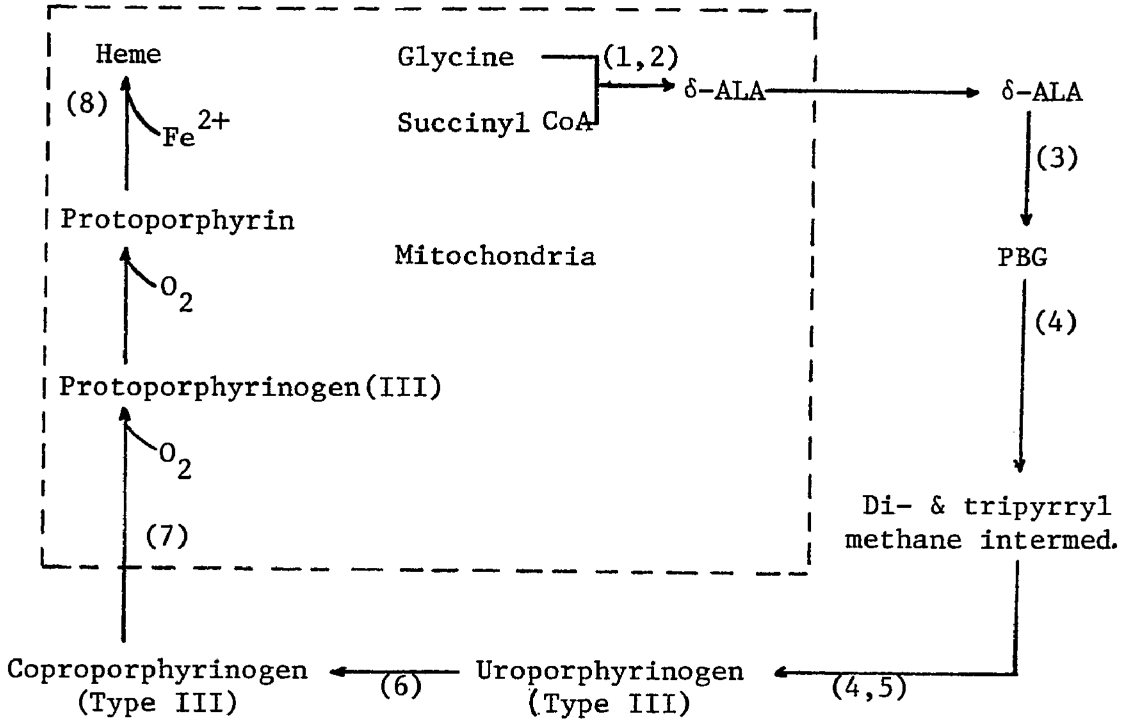
δ-aminolevulinic acid

This is the first step in the synthesis of heme and appears to be the rate limiting step. ALA acts as a negative feedback inhibitor of ALA synthetase. Two molecules of ALA combine through the action of ALA dehydrase (or PBG synthetase) to form porphobilinogen (PBG) with the release of two molecules of water:



PBG

The overall scheme for heme synthesis is given below (23).



(1) Pyridoxal Phosphate

(2) δ-amino Levulinic Acid Synthetase

(3) Aminolevulinic Acid Dehydratase

(4) Uroporphyrinogen I Synthetase

(5) Uroporphyrinogen III Cosynthetase

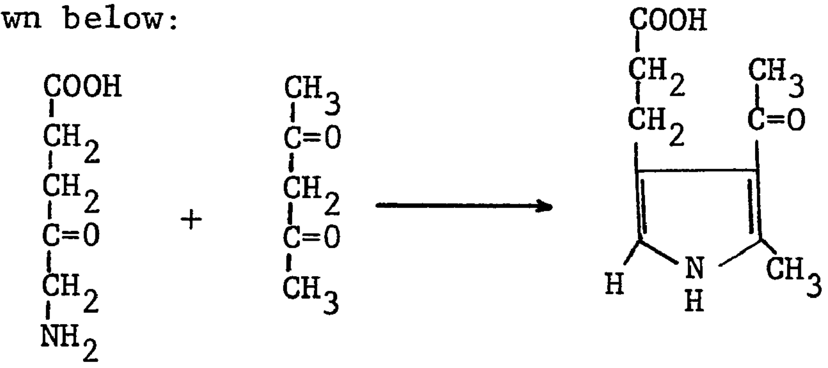
(6) Uroporphyrinogen Decarboxylase

(7) Coproporphyrinogen Oxidase

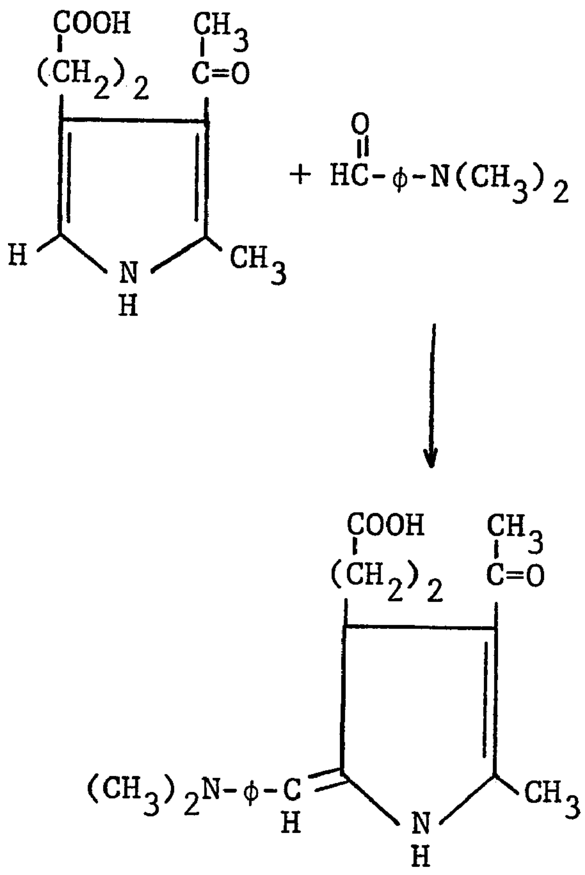
(8) Fe(II)-protoporphyrin Chelatase

ALA became diagnostically important when it was found that increased levels could be correlated with occupational lead exposure and subclinical lead poisoning in children. This is due to the fact that lead inhibits ALA dehydratase. The inhibition is believed to occur through the coordination of lead with the sulfhydral groups in the enzyme. Normal ALA levels in urine are 1.5-7.5 mg per 24 hours and 0.1-0.6 mg per 100 ml of random fresh urine. In cases of lead poisoning, ALA may reach levels as high as 60 mg per 24 hours.

The most widely used method for the determination of ALA is that of Mauzerall and Granick (24). The assay involves the passage of urine, at pH 4 to 7, through a Dowex 2-X8 anion exchange resin to retain urinary PBG which constitutes an interference. The effluent is then passed through a Dowex 50 W-X8 cation exchange column which retains the ALA. Elution of the retained ALA is accomplished by passing 0.5 M sodium acetate through the column. The collected eluate is then heated with acetylacetone at pH 4 to 6 to form a pyrrole which is shown below:



The pyrrole is subsequently heated with Erlich's reagent to form a colored complex; the absorbance is measured at 533 nm.



Erlich's reagent, p-dimethylaminobenzaldehyde, forms the colored complex with pyrroles having free alpha hydrogens. Problems arise because there are a number of compounds in urine which react with Erlich's reagent which have not been characterized. Aminoketones and glucosamine will also form condensation products with acetylacetone and therefore interfere. The fact that only 10-20% of the color formed in normal urines is actually from ALA has prevented wider use of this method for the mass screening of high risk populations. Indeed, the more recent editions of standard clinical chemistry texts, i.e. Tietz and Henry (39,40), no longer include the analysis.

Samuels and Fisher (25) have proposed a semi-quantitative method for measuring urinary ALA levels by thin-layer electrophoresis followed by thin-layer chromatography. The spots are developed using the standard Erlich's reagent. The technique tends not to be as sensitive as the method of Mauzerall and Granick but is much more rapid. The total running time is about 30 minutes as compared to 2 hours.

Lamola, Joselow, and Yamane (26) proposed a fluorometric technique for the detection of lead poisoning. The technique involves the measurement of zinc protoporphyrin (ZPP) in whole blood. ZPP emits fluorescence radiation at 625 nm when excited by 397 nm light. The method has several problems associated with it. The levels of ZPP do not rise immediately, but rather it is a delayed phenomenon. Thus a patient may actually have lead intoxication and not show a positive test. The actual correlation of ZPP with lead intoxication is secondary. ZPP is predominantly associated with erythrocytes and it is not until the lead intoxication has led to an anemic state that it can be associated with ZPP. Therefore iron-deficiency anemias in general lead to false positives by this particular method.

The most sensitive method in use today for the screening of high risk populations is the determination of δ-amminolevulinic acid dehydratase activity in erythrocytes. Because this is the enzyme affected by lead intoxication it offers an immediate marker in the diagnosis of acute and chronic lead intoxication. The method involves hemolysis of the blood sample, addition of ALA, and monitoring the PBG formed through the condensation of two molecules of ALA by δ-aminolevulinic acid dehydratase (ALAD) present in the blood. The PBG formed is reacted with Erlich's reagent and the color developed followed spectrophotometrically. Alternatively the PBG formed can be converted to uroporphyrin and the fluorescence emission at 597 nm (excitation at 408 nm) can be followed.

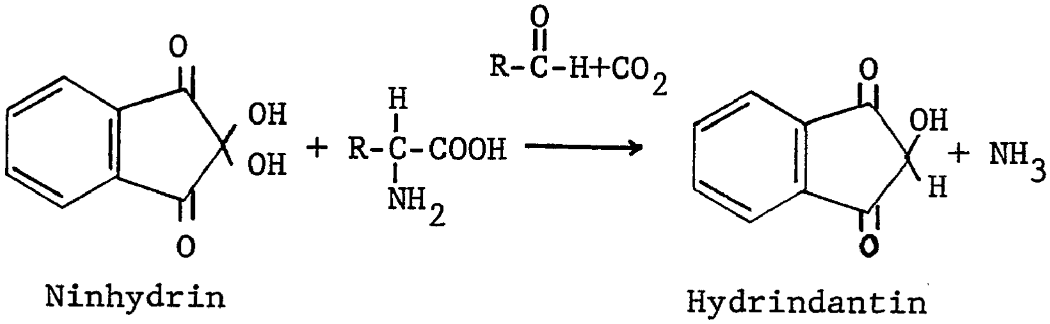
Although the enzymatic method is the most sensitive method in use today it has not enjoyed very wide acceptance because of widely variable inter- and intralaboratory ALAD values and because of the unavailability of the purified enzyme for the determination of quantitative calibration curves (27). These along with the fact that the sample is blood (rather than urine which is easier to collect) add to its unpopularity especially when screening pediatric patients. The ZPP method also suffers from this inconvenience (blood rather than urine).

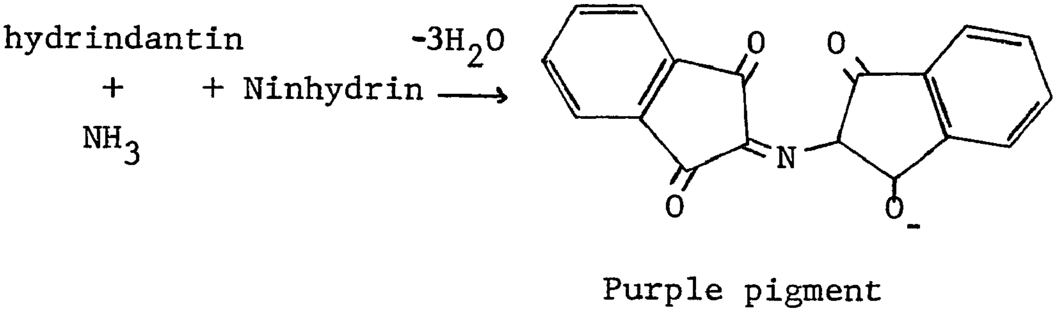
There still appears to be a need for a simple and rapid screening procedure for lead poisoning. More importantly, there is still a need for an improved method for the determination of ALA itself. With this in mind, the following study was undertaken.

Reactions Involving ALA

As indicated earlier, every test in use today involving the measurement of ALA will cause it to react with a molecule of acetylacetone (as in the chemical methods) or another molecule of ALA (as in the enzymatic method). The pyrrole thus formed is then reacted with Erlich's reagent to give a colored product. It is at this point where these methods generally have their weakness, i.e. interference from other Erlich's reactive compounds. It would seem possible to alleviate this problem if one could cause a reaction with ALA and monitor the reaction product without further preparation.

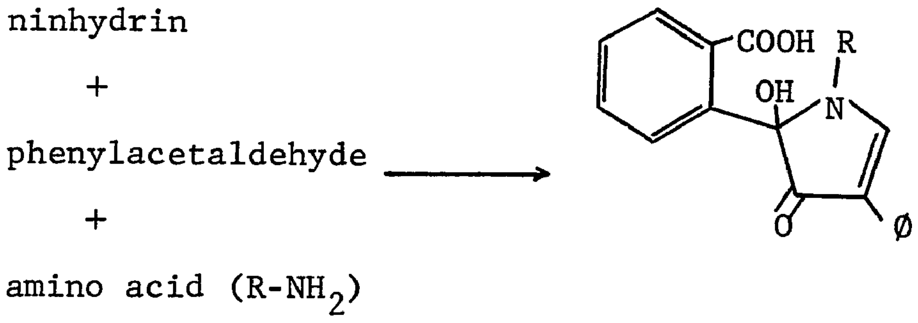
ALA can be correctly described as an amino acid and in particular a δ-amino acid. As such it will undergo the classic ninhydrin reaction.





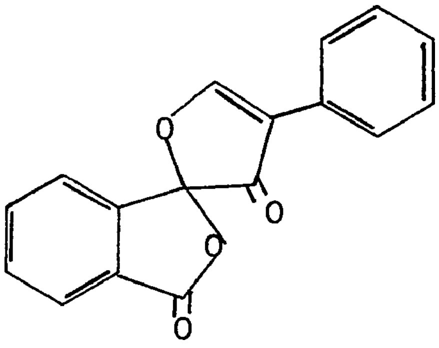
Samuels and Fisher (25) have used the ninhydrin reaction in conjunction with their TLE/TLC procedure and found that ALA reacts with ninhydrin but instead of the characteristic purple color, the reaction product was yellow.

Samejima _et_ _al_. (28) introduced a fluorometric method involving the ninhydrin reaction:



The reaction is limited to non-aqueous systems because the reagent is rapidly destroyed by hydrolysis and the oxidizing properties of ninhydrin often lead to limiting side reactions.

In 1972, Weigele *et al*. developed a reagent, 4-phenylspiro[furan-2(3H),1'-phtalan]-3,3'dione more commonly known as fluorescamine, to replace the fluorometric ninhydrin reaction. Fluorescamine, whose structure is shown below,

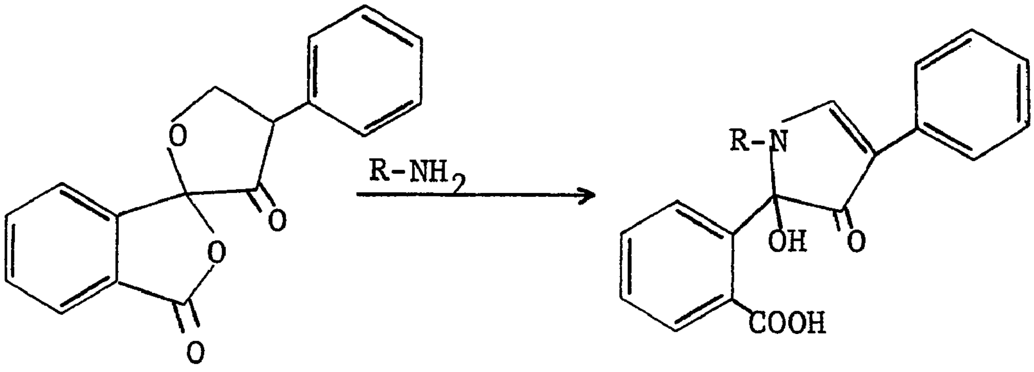


is a nonfluorescent compound which, at alkaline pH, reacts with primary amines almost instantaneously. Excess reagent is hydrolyzed to nonfluorescent water-soluble products while the reaction product fluoresces (390 nm excitation, 490 nm emission). Because fluorescamine is rapidly hydrolyzed the reagent must be added in a nonhydroxylic solvent such as acetone, dioxane, or acetonitrile.

Udenfriend et al. (30) in studying the kinetics of the fluorescamine-amine reaction found that at pH 9 the reaction proceeded at room temperature with a half-time of between 100 and 500 msec. They also found that the excess fluorescamine was hydrolyzed to its nonfluroescent products with a half-time of between 5 and 10 sec. Further studies indicated that the reaction between fluorescamine and primary amines was first order with respect to the amine and the hydrolysis of excess fluorescamine was first order with respect to the reagent (31).

Peptides in general yield greater fluorescence intensity than their component amino acids when reacted with fluorescamine. Peptides also yield their maximum fluorescence at pH 7 whereas amino acids yield their maximum fluorescence at pH 8-9. The relative fluorescence intensity of various fluorescamine-amine derivatives is constant between pH 4 to 10 but the quantum yield of the different fluorophors is dependent on the reactivity of each individual amine at specified pH's.

The reaction of fluorescamine with primary amines



yields the same fluorophors as are generated in the ninhydrin-phenylacetaldehyde reaction. Derivatives studied by previous workers all show the same emission maximum at 490 nm when excited by 390 nm radiation.

ALA was reacted with fluorescamine and found to yield a fluorescent product with emission at 490 nm (390 nm excitation):

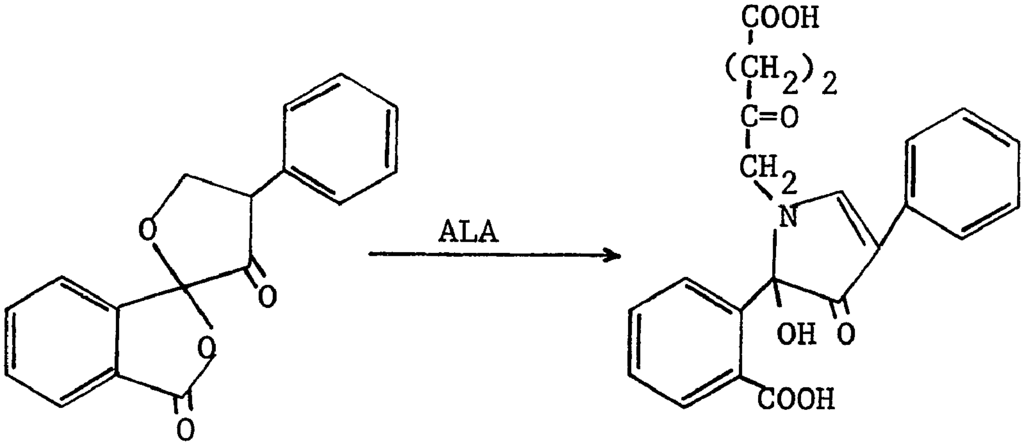


Figure 16 shows a typical emission spectrum of the fluorophor generated from fluorescamine and ALA. The standard reaction mixture consists of: 1 ml fluorescamine reagent (in acetone), 1 ml amine, and 2 ml buffer (or 1 ml buffer and 1 ml water). The amine and buffer are added to the reaction vessel and continuously mixed as the fluorescamine reagent is added. The mixture is allowed to stand at room temperature for one minute prior to the luminescence measurement. The above reaction was run at different buffers to determine the optimum reaction pH. The results of those experiments are shown in Figure 17. For comparison two other amines were also tested and the results are also shown in Figure 17. It is apparent from the data presented thus far that ALA shows the same characteristics as other amino acids when reacted with fluorescamine, i.e. 390 excitation with 490 nm emission and an optimum reaction pH of 8.5. This does differ from peptides which have an optimum reaction pH of 7.

As mentioned previously, there is still a need for a fast and accurate procedure to quantitate ALA. Fluorescamine may offer an alternative. In the procedures mentioned earlier one must first convert ALA to a pyrrole followed by conversion of the pyrrole to a measurable product. With fluorescamine one can measure ALA directly. The reaction is fast, requires

Figure 16. Typical fluorescence emission spectrum of fluorescamine-ALA reaction.

0.95 μmol fluorescamine, 1.0 μmol ALA, pH = 8.6, 390 nm excitation, sensitivity X10.

Figure 16

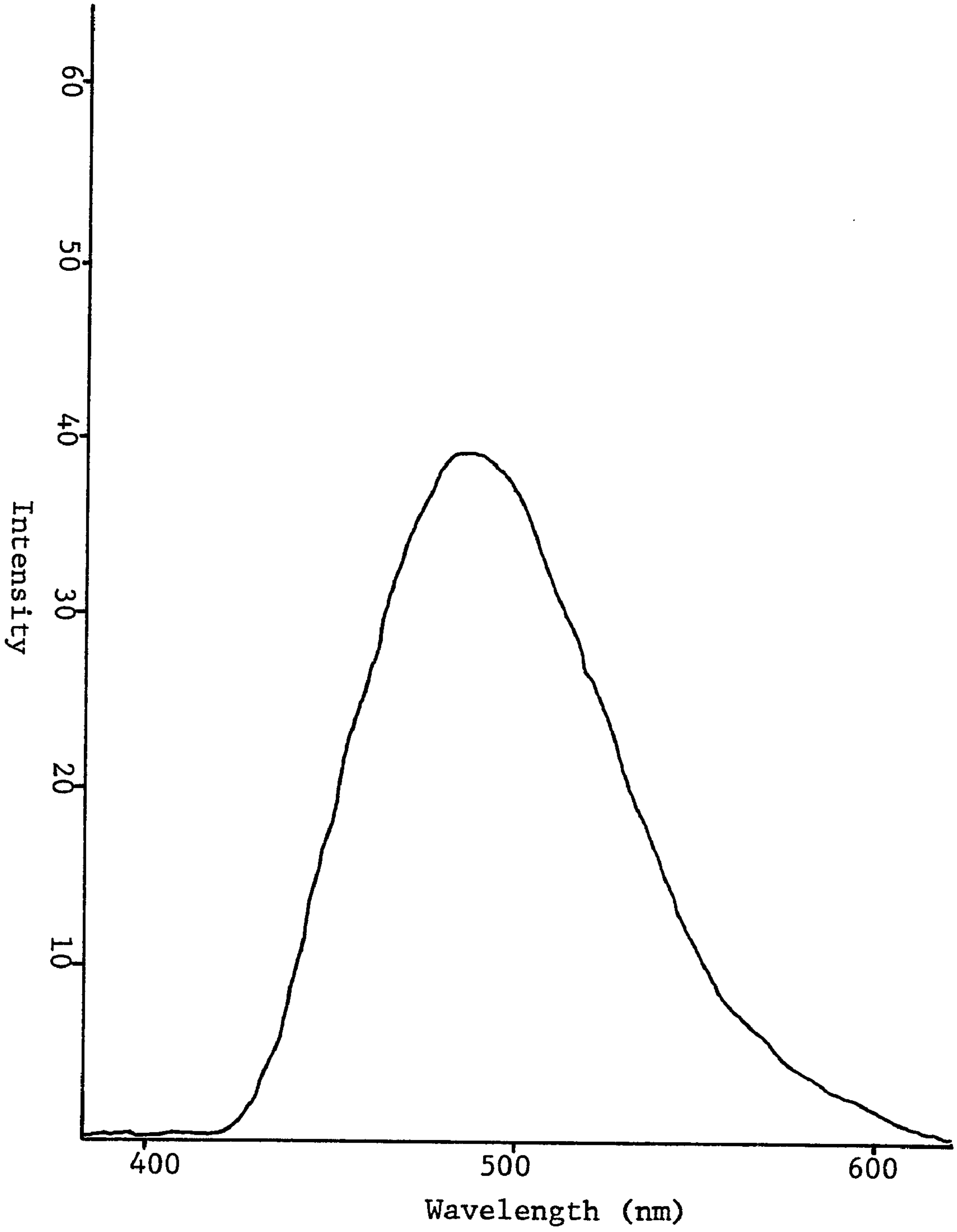
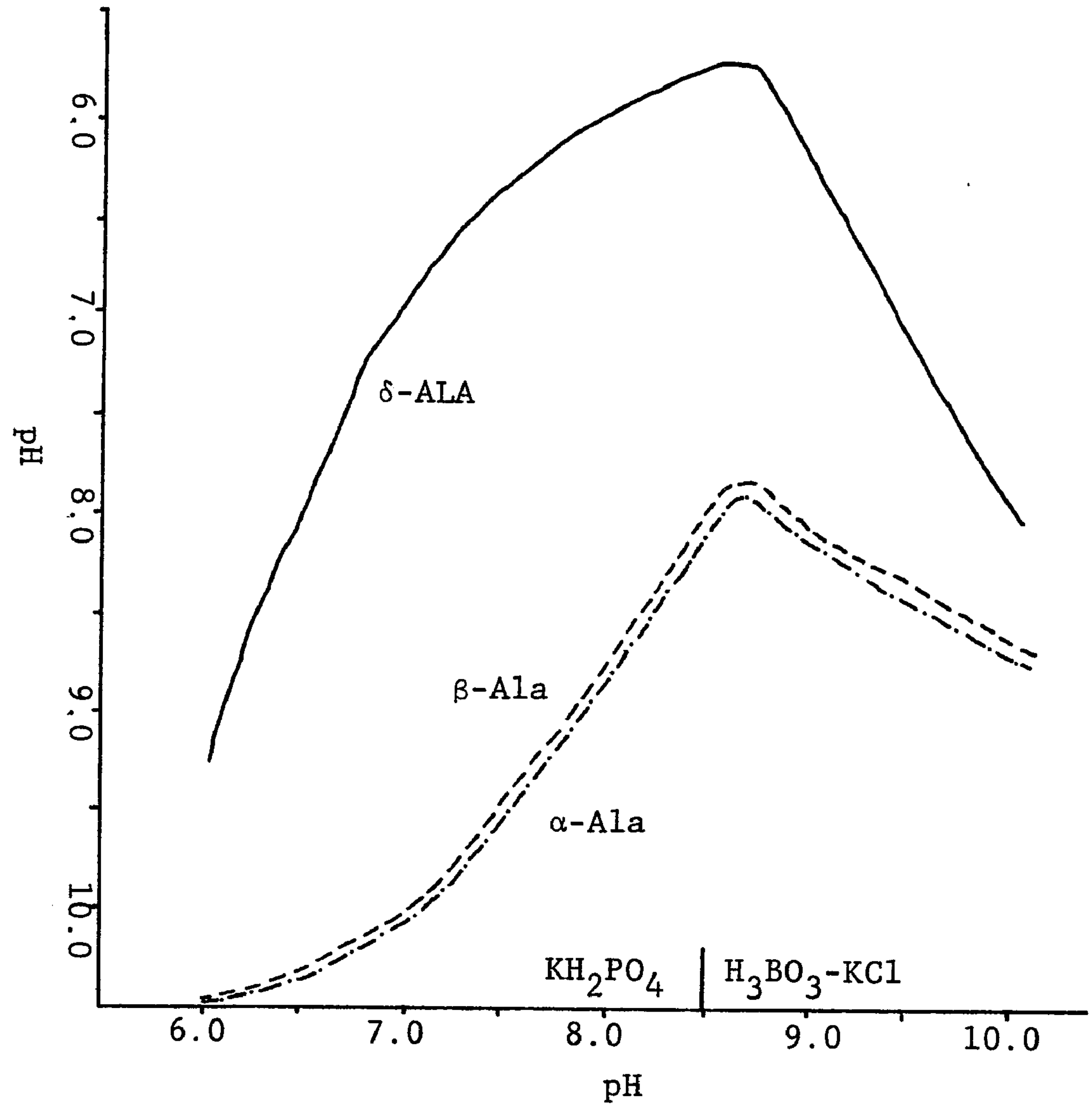


Figure 17. Effect of pH on fluorophor formation in the reaction of fluorescamine with primary amines.

0.9 μmol fluorescamine, 1.0 μmol amine, pH buffer 390 nm excitation, sensitivity X10.

Figure 17


δ-ALA
β-Ala
α-Ala
KH_2PO_4
H_3BO_3-KCl
6.0
7.0
8.0
9.0
10.0
pH
6.0
7.0
8.0
9.0
10.0
pH

no unusual reaction conditions, and is more sensitive than existing colorimetric methods (luminescence measurements are generally more sensitive than absorption methods).

Figure 18 shows a representative calibration curve obtained with the standard fluorescamine-ALA reaction. ALA ranged between 6.6 nmols and 160 nmols per ml with a 5.7% relative standard deviation. Greater sensitivity can be achieved by excitation of the fluorophor by 365 nm radiation which yielded about a four fold increase in the emission intensity.

It is quite evident that the fluorescamine-ALA method presented is capable of measuring low levels of ALA in aqueous solutions. The sensitivity is at least equal to that claimed by Mazerall and Granick (24) and the reproducibility is good. Twelve week reagent stability has been reported (33) while in the present study a fluorescamine solution was used for one month with no detectable alterations.

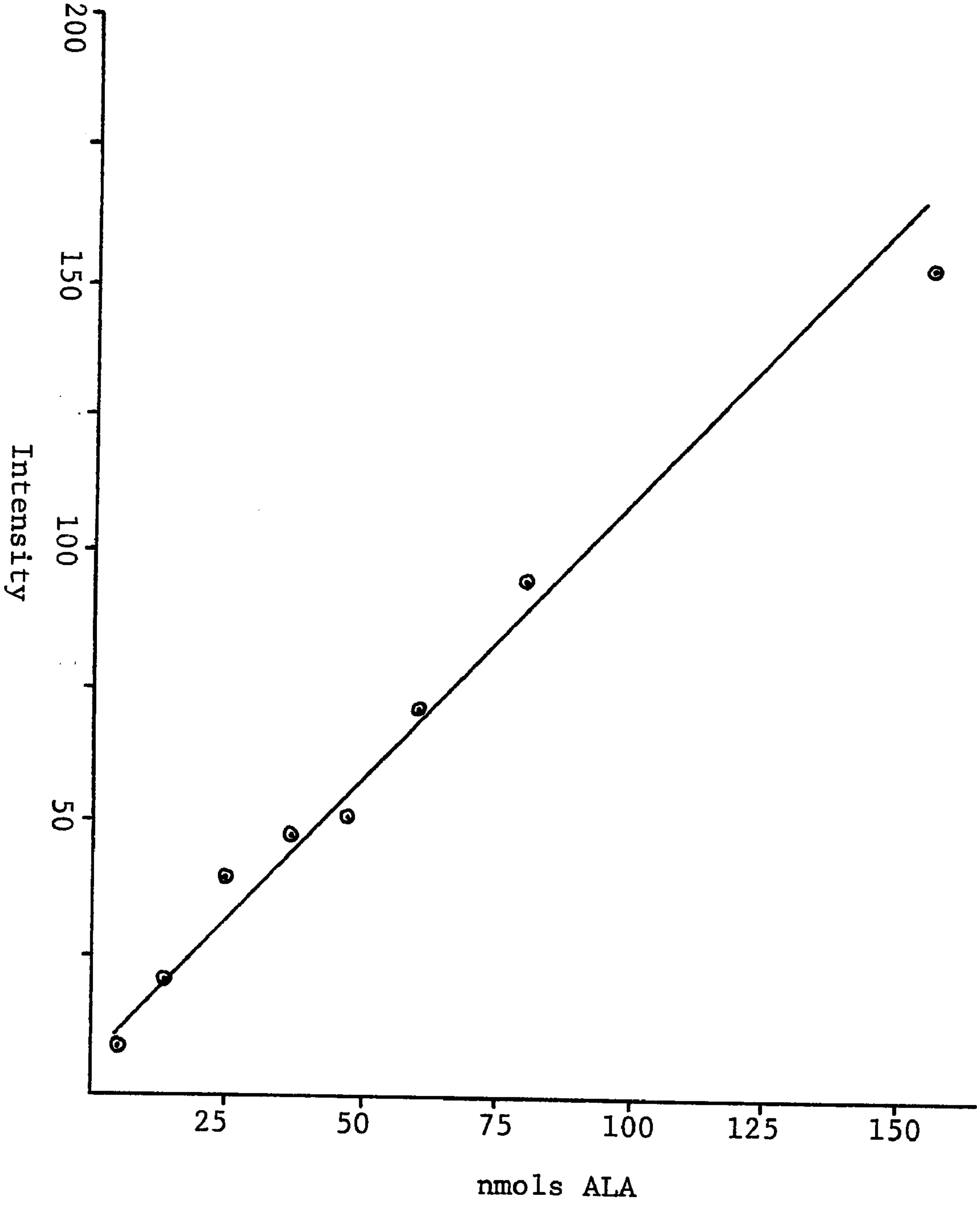
Detection of ALA in Urine

For the method presented above to be of any practical value it must be shown that it is capable of measuring ALA in real samples. Using fluorescamine to measure urinary ALA presents some rather large problems. As previously state, fluorescamine was developed as an

Figure 18. Representative calibration curve for ALA analysis using fluorescamine.

3.59 μmol fluorescamine, pH 8.6 buffer, 390 nm excitation, sensitivity X1.

Figure 18

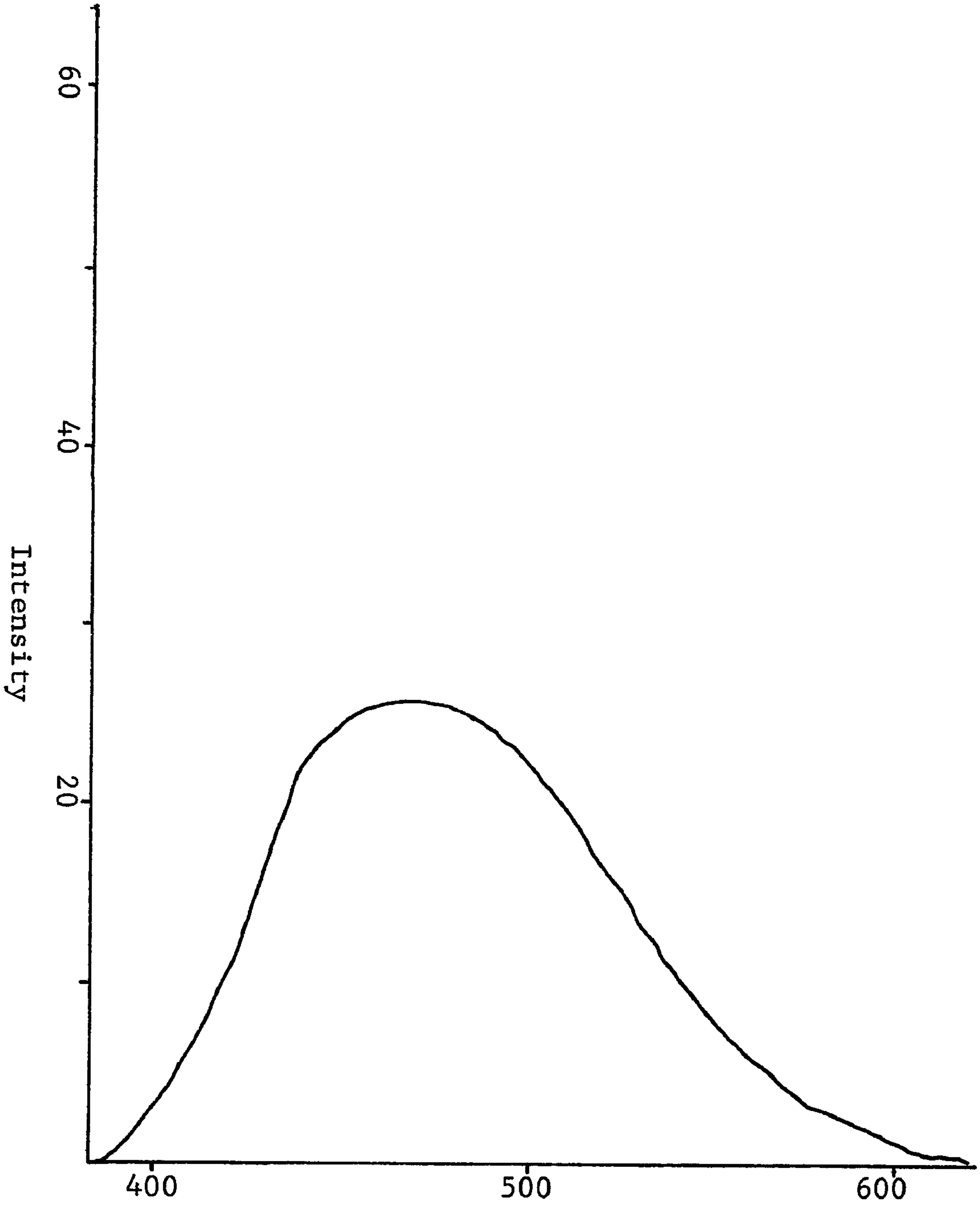


alternative to the ninhydrin-phenylacetaldehyde reaction for amino acids and is therefore specific for primary amines.

Urine shows a native luminescence extending from 400 nm to 550 nm (excitation at 390 nm). This accounts for the scarcity of acceptable luminescence assays in this spectral ragion. Figure 19 shows the background luminescence of urine. In addition to this, urine contains large numbers of amines. The most immediate source of interference in the use of fluorescamine for urinary ALA comes from amino acids. Measured by nonspecific techniques, values for urinary amino acids of 200 to 700 mg per 24 hours are obtained. Roughly one-third are present in the free form with the rest occurring as peptides or in some other conjugated form (32). Further sources of interference can come from γ-aminobutyric acid, β-alanine, histamine, catecholamines, amino sugars, spermine, and spermidine. These compounds are usually present in negligible quantities, but will become significantly elevated in various disease states. Fortunately urea, creatinine, urate, and ammonium ion which are major constituents of urine do not react with fluorescamine. It therefore becomes imperative that in some way ALA must be separated or distinguished from other urinary primary amines. The following study was undertaken in an attempt to differentiate the fluoresca-

Figure 19. Luminescence spectrum of undiluted urine. 390 nm excitation, sensitivity X10.

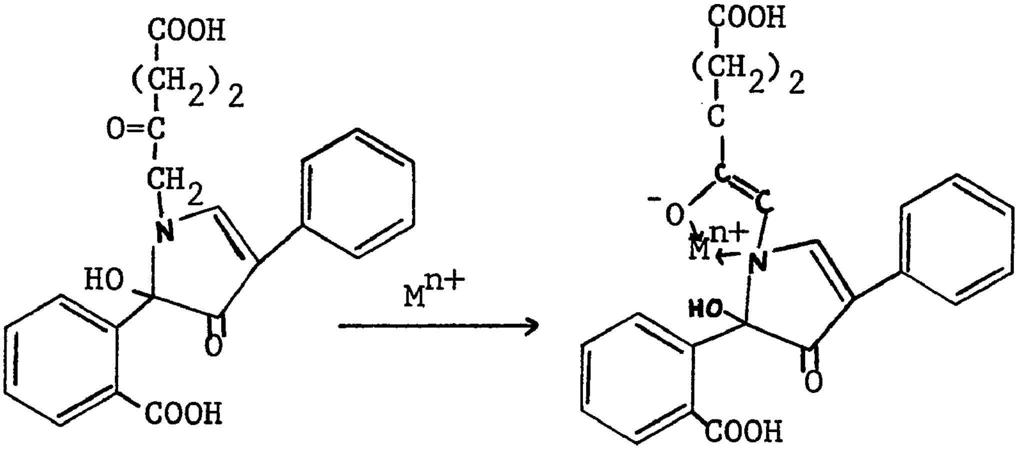
Figure 19



mine-amine products which might be produced in a urine assay.

It has been shown that paramagnetic metal ions will often quench fluorescence of organic molecules. High molecular weight diamagnetic metal ions will also cause fluorescence quenching but there are numerous cases where low atomic weight diamagnetic metals will enhance fluorescence of organic molecules and even cause non-fluorescent organic molecules to fluoresce through metal-ligand complex formation. An example is 8-hydroxyquinoline which by itself is non-fluorescent. Coordination of a low atomic weight diamagnetic metal, such as Al^{3+}, with 8-hydroxyquinoline will cause the formation of a highly fluorescent metal-ligand complex.

Considering the proposed structure of the fluouscamine-ALA product the possibility exists that it could act as a ligand.



If this metal-ligand complex formation is possible there would be no need for time consuming separations in a procedure for measuring urinary ALA levels. Table 9 shows the results of a series of experiments where different metal salts were added to a solution containing the fluorophor generated by the reaction of fluouscamine with ALA. Figure 20 represents the fluorescence spectrum of an ALA-fluorescamine reaction mixture before and after the addition of zinc.

It appears from the data presented that the fluorescamine-ALA product generated in the above reaction is capable of complexing a diamagnetic metal and thereby increasing the fluorescence of the fluorophor. It was found in experiments using various amino acids in place of ALA that the fluorescence enhancement did not occur. It should be noted that the reaction described in Table 9 took place in a pH 6.0 phosphate buffer. This serves two purposes. First, the intensity of the ALA fluorophor is much greater than the intensity of the amino acid fluorophor a pH 6.0. In fact, the fluorophor generation (fluorescamine-amino acid reaction) seems to be minimal. This in itself aids in the differentiation of the fluorophor generated by the reaction of fluorescamine with ALA and those generated by the reaction of fluorescamine with amino acids (see Figure 17). The use of a metal to form the metal-ligand complex and

TABLE 9

Effects of Metals on the Fluorescence Intensity of the Fluorescamine-ALA Fluorophor*

Metal Salt	Intensity (pH 6.0)	% Increase
1. None	252	
2. $ZnSO_4$	304	21
3. $ZnCl_2$	311	23
4. $CdCl_2$	310	23
5. NiSO	268	6
6. $NiCl_2$	256	2

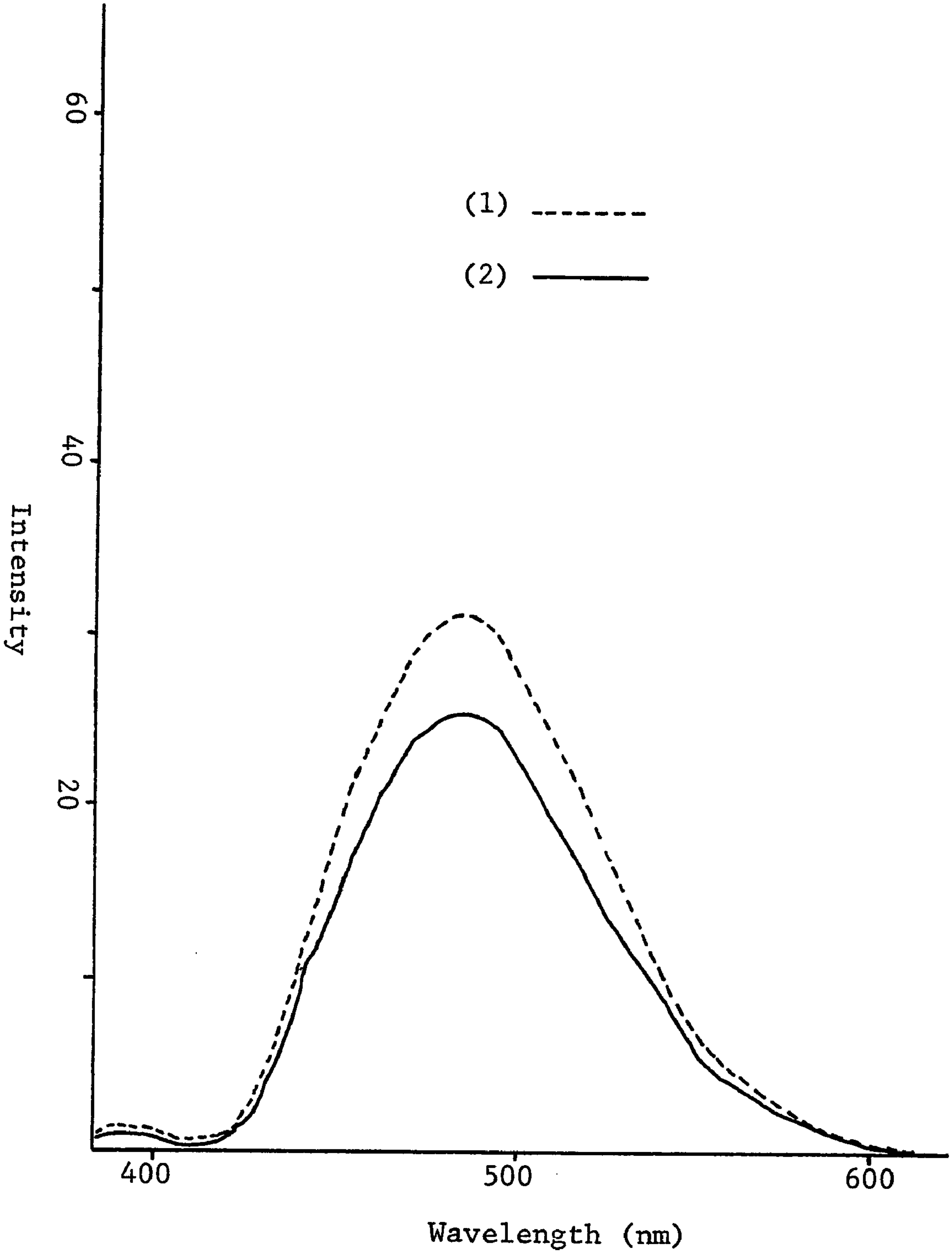
* Each solution contains 1 ml (0.95 *mM*) fluorescamine, 1 ml (0.68 *mM*) ALA, 1 ml buffer, 1 ml (1 *mM*) metal; reaction run at room temperature; excitation at 390 nm with emission at 490 nm. The blank contains 1 part water instead of metal.

Figure 20. Effect of Zn^{2+} on the fluorescence emission of the fluorescamine-ALA fluorophor.

(1) pH 6.0, 1 μmol Zn^{2+} present

(2) pH 6.0, no Zn^{2+} present

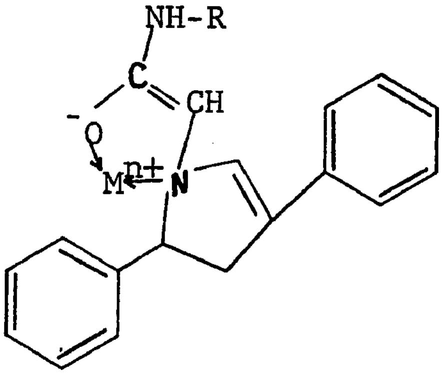
Figure 20

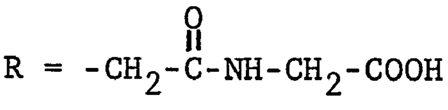


enhance the fluorescence is an added advantage. Secondly, the k_{sp} of $Zn(OH)_2$ is 1.2×10^{-17}. This means that at the concentration of zinc used in the experiments described in Table 9 one must be careful of $Zn(OH)_2$ precipitation and thus light scattering. Calculations show that at pH 6.0 the k_{sp} for $Zn(OH)_2$ has not been exceeded and one should not expect to see a light scattering effect. At pH 8.6 the k_{sp} is exceeded and the precipitation of $Zn(OH)_2$ is expected. Experiments show that when the reaction described in Table 9 is run in a pH 8.6 buffer you do get an increase in the fluorescence intensity of the fluorophor, but only half as much (11.6% as compared to 23% in pH 6.0 buffer). Further, the emission spectrum of the pH 8.6 reaction shows the appearance of a band at 390 nm which could indicate precipitate formation and a light scattering of λ_{exc} effect. Since the extent of reaction between fluorescamine and primary amines is a function of pH and you would expect to have at pH 8.6 the greatest extent of fluorophor formation it would be reasonable to assume that the enhancement of fluorescence should be greatest at pH 8.6. Because this is not the case, $Zn(OH)_2$ formation is all the more reasonable.

Adjustment of the pH in addition to the formation of metal-ligand complex appears to offer a method by which one can differentiate between the fluorescamine-ALA fluorophor and the fluorescamine-amino acid fluorophors. Complications arise because only about one-third of the average daily output of urinary amino acids are in the free form. Experiments were conducted to study the effect of zinc on the fluorescence intensity of the fluorophor generated in the reaction between fluorescamine and triglycine. Triglycine is a peptide consisting of three glycine moieties and was chosen as a representative of the small peptides normally excreted in urine.

It has been found by previous workers that the optimum reaction pH for the fluorescamine-peptide reaction is 7.0 (30). It was also found that the fluorescence intensity of the fluorophor generated in the flourescamine-peptide reaction was much greater than the intensities of the amino acid fluorophors (33). This study showed consistent results. When zinc was added to a solution containing the fluorescamine-triglycine fluorophor (same reaction conditions and concentrations as previously described) there was also an enhancement of the fluorescence intensity at 490 nm. This indicates that the fluorescamine-triglycine fluorophor can act as a ligand in a similar manner to that of the ALA fluorophor. The structure of the triglycine fluorophor shows similarities to the ALA fluorophor.





$R = -CH_2-\overset{\overset{O}{\|}}{C}-NH-CH_2-COOH$

Both structures have a carbonyl beta to the reactive (terminal) nitrogen. The addition of zinc to the triglycine fluorophor solution gave an average enhancement of the fluorescence intensity at 490 nm of 19%. In addition, the triglycine fluorophor yielded a greater fluorescence intensity at pH 6.0 than did the ALA fluorophor (∿18% greater).

It appears that the formation of metal-ligand complex in the ALA-fluorescamine reaction would not help differentiate the ALA fluorophor from that generated from fluorescamine and peptides. Therefore, in order to use fluorescamine successfully as a urinary ALA reagent, ALA must be physically separated from the interfering amines.

D. Separation and Quantification of ALA from Urine

There have been two methods presented for the separation of ALA from urine. The first is that proposed by Mauzerall and Granick (24) and the second is that proposed by Samuels and Fisher (25). The method of Samuels and Fisher involves the separation of ALA by high voltage thin-layer electrophoresis followed by thin-layer chromatography. As previously discussed, this is a fast and efficient separation. The problem of sensitivity could easily be overcome by using fluorescamine as the detecting reagent. Budgetary considerations did not allow further study with this technique.

The method of Mauzerall and Granick accomplishes the isolation of ALA by use of a Dowex 50 cation exchange column. The following procedure was used to determine its applicability to the fluorescamine-ALA reaction.

1. A 1 cm diameter chromatographic column was packed to 5 cm depth with Dowel 50 W-X8 resin in the hydrogen form. The column was then washed with 30 ml of water to remove any unbound hydrogen ions.
2. 1 ml of (1 m<u>M</u>) ALA standard was added to the top of the column and allowed to drain at a rate of 6-8 drops per minute.
3. The column was then rinsed with 50 ml of water followed by 3 ml of 0.5 <u>M</u> sodium acetate which

was allowed to drain to the top of the column.

4. 7 ml of 0.5 M sodium acetate was added and the eluate was collected.
5. The eluate was tested for the presence of ALA by the use of fluorescamine.

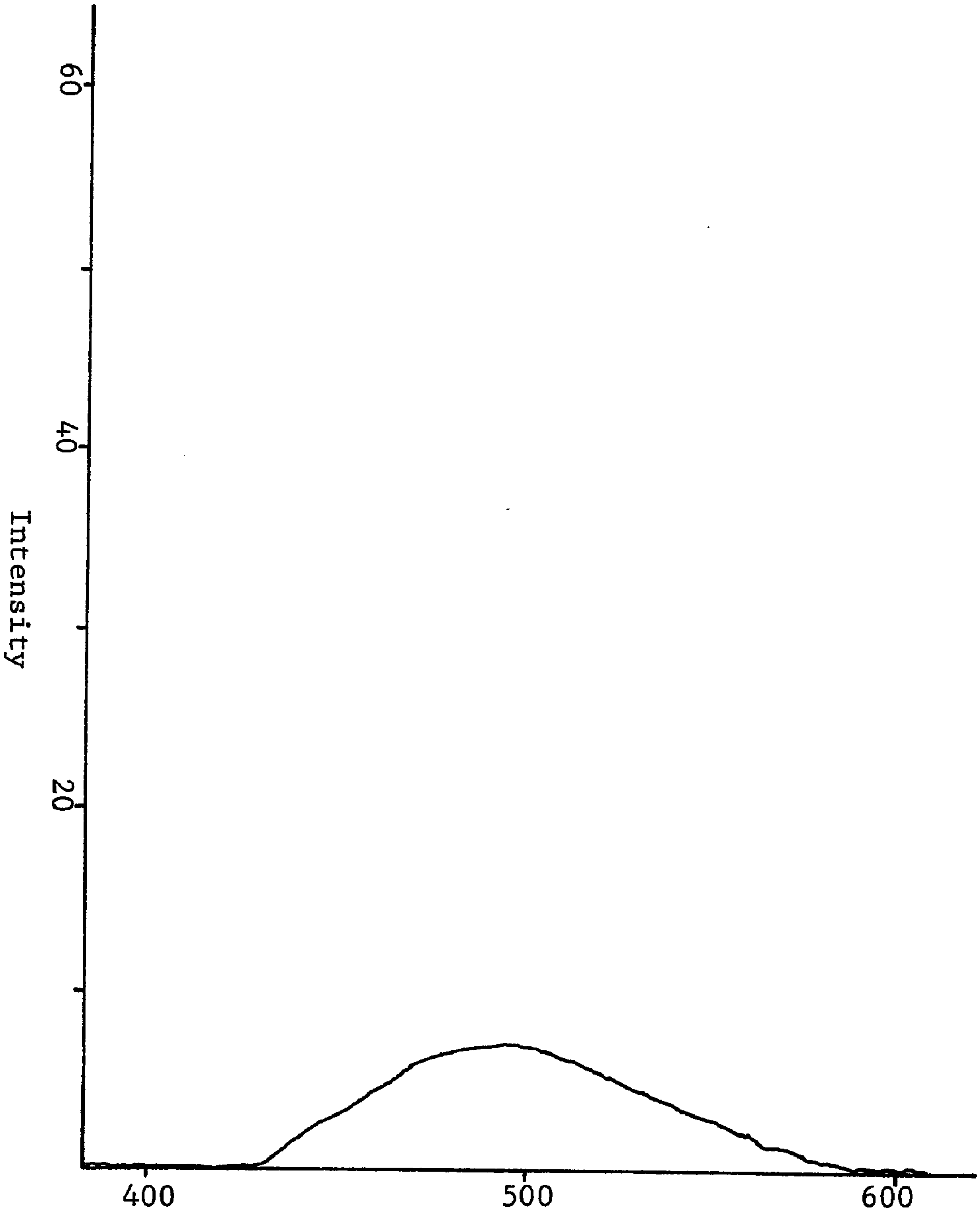
It was hoped that this procedure would accomplish two things: first that the background luminescence of urine could be removed and secondly that there would be separation of ALA from the amino acids and peptides present in urine. Dowex 50 W-X8 resin has been used previously in the separation of amino acids (34). The procedure takes 72 hours for completion, but excellent separation was reported. The method was applied to urine samples, but no supporting data were given.

Figure 21 represents the fluorescence spectrum obtained from the reaction of fluorescamine with the eluate collected from the Dowex 50 W-X8 column after passage of 1 μmol of ALA through it. Seven ml of the eluate were collected and diluted to 10 ml with pH 8.6 buffer. Assuming 100% recovery of the added ALA one would expect a fluorescence intensity corresponding to ∿100 nmoles of ALA after reaction of the diluted eluate with fluorescamine. The fluorescence intensity obtained in no way approaches that value (actual intensity is 7 units). If the reaction was carried out in pH 6.6 buffer

Figure 21. Fluorescence emission spectrum of fluorescamine-eluate reaction:

1 μmol ALA added to Dowex 50 column, 7 ml eluate collected and duluted to 10 ml. 1 ml diluted eluate reacted with 3.59 μmol fluorescamine at pH 8.6. 390 nm excitation.

Figure 21



an identical spectrum is obtained. The blank value corresponded to an intensity of 6 units at 490 nm. This indicates that the ALA added is not being eluted from the column. Similar findings were reported by Flood (35).

A random urine was collected and a 1 ml aliquot was reacted with fluorescamine at pH 8.60. The resulting solution showed a very pronounced inner filter effect. It was necessary to make a 1:25 dilution on the urine before an acceptable fluorescence spectrum could be obtained. Another 1 ml aliquot of the urine was treated with 0.8 μmols of ALA and diluted to 25 ml. One ml portions of the final 1:25 dilutions were then reacted with fluorescamine at pH 8.60. A 1:2 dilution of the resulting mixture was used for the luminescence measurements shown in Figure 22. The difference in intensities at 490 nm was 34 units (taking into account the 1:2 dilution). The expected difference, when refereing to Figure 18, is 37 units. The observed difference was within one standard deviation of the expected value. The quantity of ALA that was added to the urine corresponds to an ALA output of about 13.4 mg per 100 ml of urine or about 3.5 g per 24 hours. When dealing with tenths of a milligram output per 100 ml of urine (normal ALA levels) the above experiments indicate that the method of standard additions would be useless since you must add excessively large quantities of ALA to achieve a

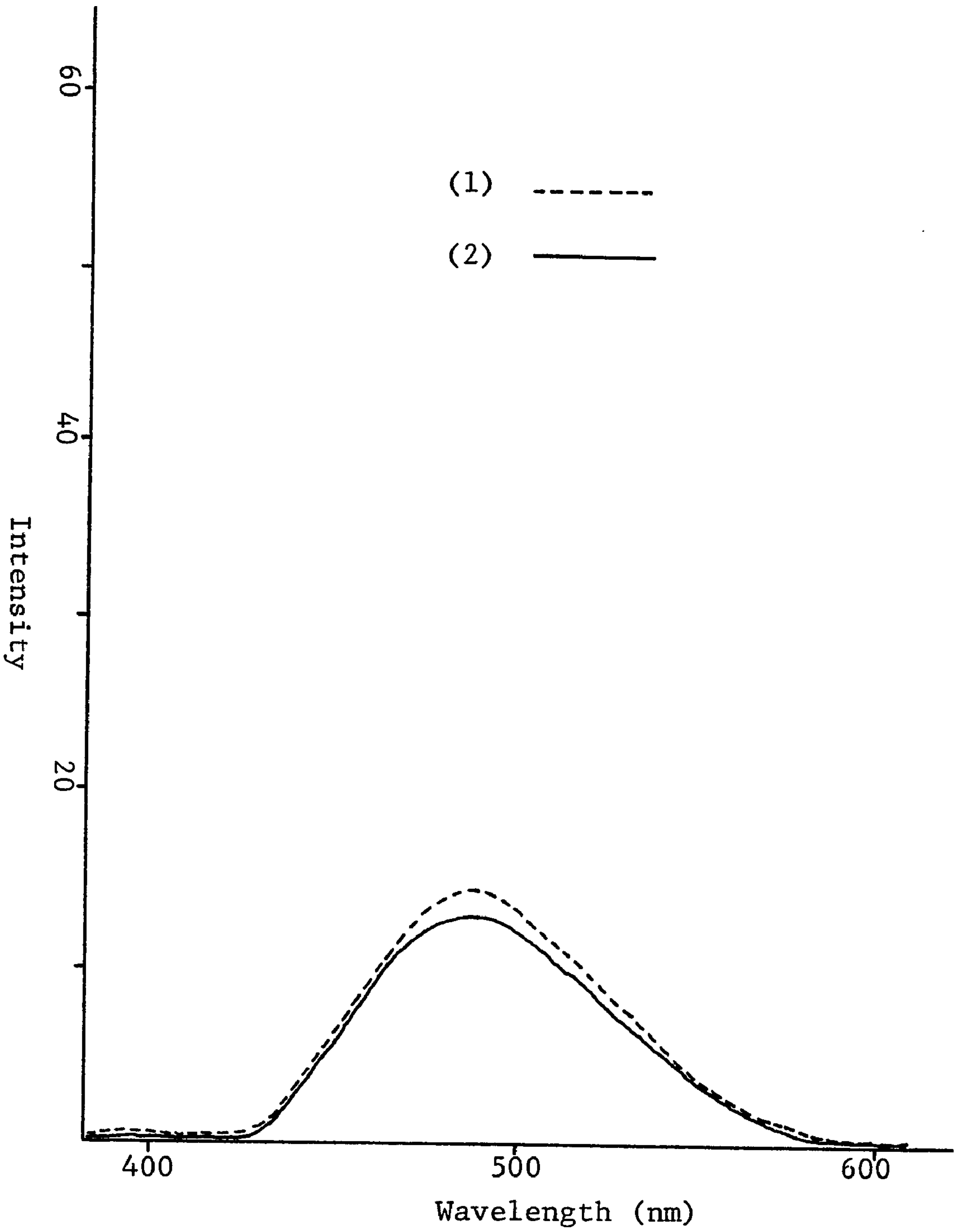
Figure 22. Fluorescence emission spectrum of fluorescamine-urine reaction.

(1) urine diluted 1:25

(2) urine + 0.8 μmol ALA diluted 1:25

1 ml of diluted sample reacted with 3.7 μmol ALA at pH 8.6, 390 nm excitation. Reaction solutions diluted 1:2 prior to spectral recording.

Figure 22



reasonable response. Further experiments showed no difference in intensities under the conditions previously described when normal quantities of ALA were added (normal with respect to the average output of urinary ALA).

E. Summary

Fluorescamine has proven to be a very sensitive reagent in the detection of primary amines. Its usefulness is totally dependent upon the degree with which one can separate the analate from potential interferences. Fluorescamine has been shown capable of quantitating extremely low amounts of ALA so long as the analate system is "clean." Its use in the measurement of urinary ALA levels has not been successful because of the lack of a good ALA separation procedure.

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VITA

Dennis Michael Todd was born on October 8, 1948, in Allentown, Pennsylvania, the son of Charles and Dolores Todd.

He attended the public schools of Linden, New Jersey, and was graduated from Linden High School in 1966. In 1970, he received a B.A. in Secondary Science Education from Newark State College.

1970 through 1973 he served on active duty in the United States Army.

He entered Lehigh University in 1973 as a graduate teaching assistant. He received a M.S. in Chemistry in 1977.

He was married to the former Linda Pieretti of West Orange, New Jersey, in July of 1971. Their first son, Damian Joseph Todd, arrived in December of 1975.

Mr. Todd is a member of the American Chemical Society and the American Association for Clinical Chemistry.

His publications are:

1. "Correlation of Plasma Luminescence Intensity with the Clotting Process," with James G. Flood and William E. Ohnesorge, 9th International Congress on Clinical Chemistry, Toronto, Canada, July 1975.
2. "Simplex Optimization in Drug Design," with George E. Barringer and Robert S. Rodgers, 9th Central Regional Meeting, American Chemical Society, Charleston, West Virginia, October, 1977.

Appendix A

Trigylceride analysis

The procedure involved for the triglyceride analysis is as follows:

1. To 1.0 ml of 4 x $10^{-2}\underline{M}$ H_2SO_4 add 0.2 ml of sample.
2. Add 5.0ml of extraction reagent to each tube and mix for 15 sec.
3. After separation of the two phases, pipet 0.5 ml of the upper phase into a new test tube.
4. Add 0.5 ml of the hydrolizing reagent to each of the new tubes containing the upper phase and allow to stand for 5 minutes after mixing.
5. Add 0.5 ml of periodate reagent to each tube and allow to stand for 5 minutes after mixing.
6. To each tube add the appropriate quantity of tris-(2,2'-bipyridine) - iron (II) reagent (quantity depends on the concentration of the reagent).
7. Transfer mixture to a 50 ml volumetric flask and add phosphoric acid to give a final acid concentration of between $1\text{x}10^{-3}$ $\underline{M}$ and $2\text{x}10^{-3}$ $\underline{M}$; dilute with water to 50 ml.

8. Transfer flasks to 100°C water bath and heat reaction solutions for 5 minutes.

9. After 5 minute heating cool solutions to room temperature and read absorbance at 522 nm.

ALA ANALYSIS

The following procedure applies to the ALA analysis involving fluorescamine:

1. To a clean acid washed test tube add 1.0 ml of the sample.
2. Add 2.0 ml of phosphate or borate buffer depending on the pH desired.*
3. Add 1.0 ml of fluorescamine reagent and mix for 10 seconds.
4. Allow solution to stand 1 minute prior to fluorescence measurement.

*When using Zn^{2+} to differentiate ALA from amino acids, add only 1 ml of buffer. After addition of fluorescamine (step 3) add 1.0 ml of Zn^{2+} reagent to form the metal-ligand complex. Continue with Step 4.