A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY INVESTIGATION OF THE UROBILINOIDS

by

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ABSTRACT

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This investigation was concerned with the detection of the urobilinoids (i.e. urobilinogen) by High Performance Liquid Chromatography (HPLC) using a Reversed Phases column. Attempts were unsuccessfully made to crystallize the urobilinoids in order to provide a convenient sample supply for HPLC studies.

Cyclic voltammograms for urobilinogen and the mobile phase (.1 M KCl in 20% MeOH) were taken in order to determine the working potential for the HPLC electrochemical detector. Electrochemical detection was not possible due to the high polarity of the aqueous solvents required by the detector.

Absorption spectra indicated that UV detection was possible. Urobilinogen and bilirubin samples were injected onto the column using various MeOH and MeOH/acetonitrile solvent systems. A separation of bilirubin and urobilinogen was obtained by using a 50% MeOH mobile phase. Bilirubin was not retained on the column for the MeOH/acetonitrile systems, although excellent retention of urobilinogen with the 30:70 MeOH/acetonitrile system was observed. Injection of

samples following an extraction procedure did not yield any peaks, indicating a lack of sensitivity for the UV detection system.

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LIST OF SYMBOLS

SYMBOL	DEFINITION
μA	microamp
μL	microliter
mL	milliliter
g	gram
nm	nanometer
v.	volt
λ	wavelength
HPLC	High Performance Liquid Chromatography
HPLCEC	HPLC With Electrochemical Detection
p.s.i.	Pounds per Square Inch
cm	centimeter
mm	millimeter
m.p.	Melting Point

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CHAPTER I

INTRODUCTION

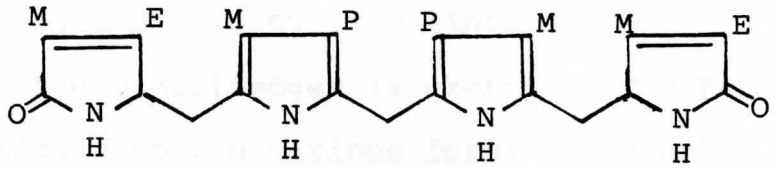
The Nature of the Urobilinoids

The urobilinoids consist of urobilinogen (i-urobilinogen, l-stercobilinogen, and d-urobilinogen) and urobilin (i-urobilin, d-urobilin, and l-stercobilin). The various forms of urobilinogen are colorless and are also referred to as chromagens. The urobilins, which have a yellow color, are sometimes called pigments. All of these substances are found in both urine and feces.

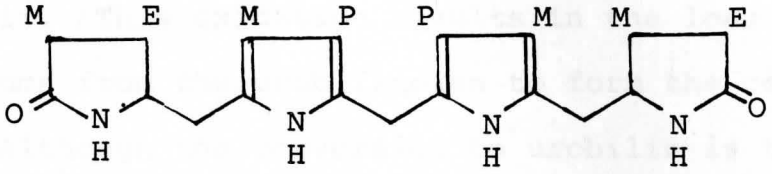
The structures of the urobilinoids are very similar to each other. All of them consist of four five-membered rings which are connected to each other by one carbon atom. The degree of saturation of each substance varies, along with the substituents on the ring. These substituents are made up of methyls, ethyls, vinyls, oxygen, and propanoic acid. The structures are given in Figure 1.[1]

The urobilinogen components are reduction products of bilirubin. The bilirubin itself is formed by a series of reactions of hemoglobin released in red blood cell metabolism.

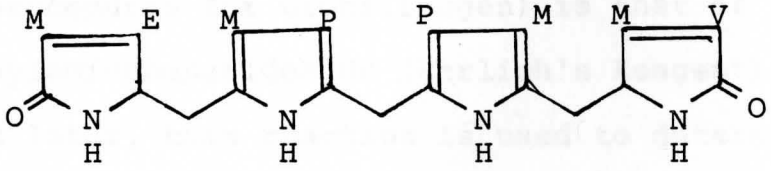
In the intestines, bilirubin is the object of bacterial attack, causing it to be converted to a reduced form. Most of the reduced bilirubin is reabsorbed into the circulatory system while part is removed from the body in the feces. Most



Mesobilirubinogen



l-Stercobilinogen



d-Urobilinogen

P = $-CH_2CH_2COOH$
V = $-CH{=}CH_2$
E = $-CH_2CH_3$
M = $-CH_3$

Figure 1-Structures of Urobilinogen

of the remaining reduction products are kept in the body by the liver. A trace amount (0.5 to 4.0 mg/24 hr.) is excreted in the urine in the form of urobilinogen.

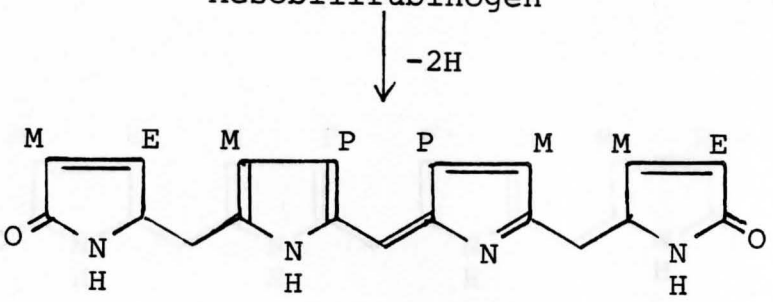
Once the urobilinogen is excreted from the body, it begins to oxidize to the various forms of urobilin. In most cases, l-stercobilinogen is the most common form of urobilinogen.

Reactions of the Urobilinoids

The predominant reaction of the urobilinoids involves the oxidation of urobilinogen to urobilin upon exposure to light and air. This oxidation results in the loss of two hydrogen atoms from the urobilinogen to form the corresponding urobilin. Although the conversion to urobilin is the major reaction sequence for urobilinogen in the urine, it is not the only one. It has been reported that small amounts of urobilinogen in the urine are oxidized to products other than urobilin.[2] The oxidation sequence of urobilinogen is given in Figure 2.[1]

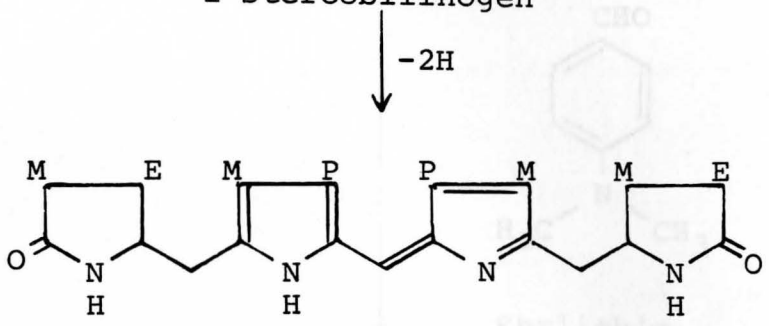
Another reaction (which is crucial in one of the analytical procedures for urobilinogen) is that of urobilinogen and p-dimethylaminobenzaldehyde (Ehrlich's Reagent). As will be discussed later, this reaction is used to determine the amount of urobilinogen present through spectrophotometric methods. Although the exact product of the reaction between Ehrlich's Reagent and urobilinogen is not known for certain, the product gives an intense red color. A possible reaction sequence is given in Figure 3.[3]

Mesobilirubinogen



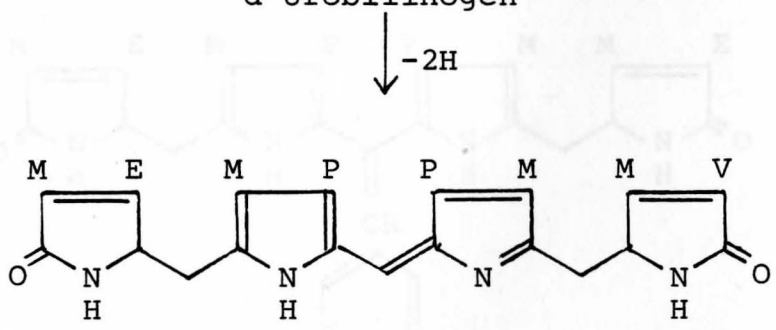
i-Urobilin

l-Stercobilinogen



l-Stercobilin

d-Urobilinogen



d-Urobilin

Figure 2-Oxidation Sequence of Urobilinogen

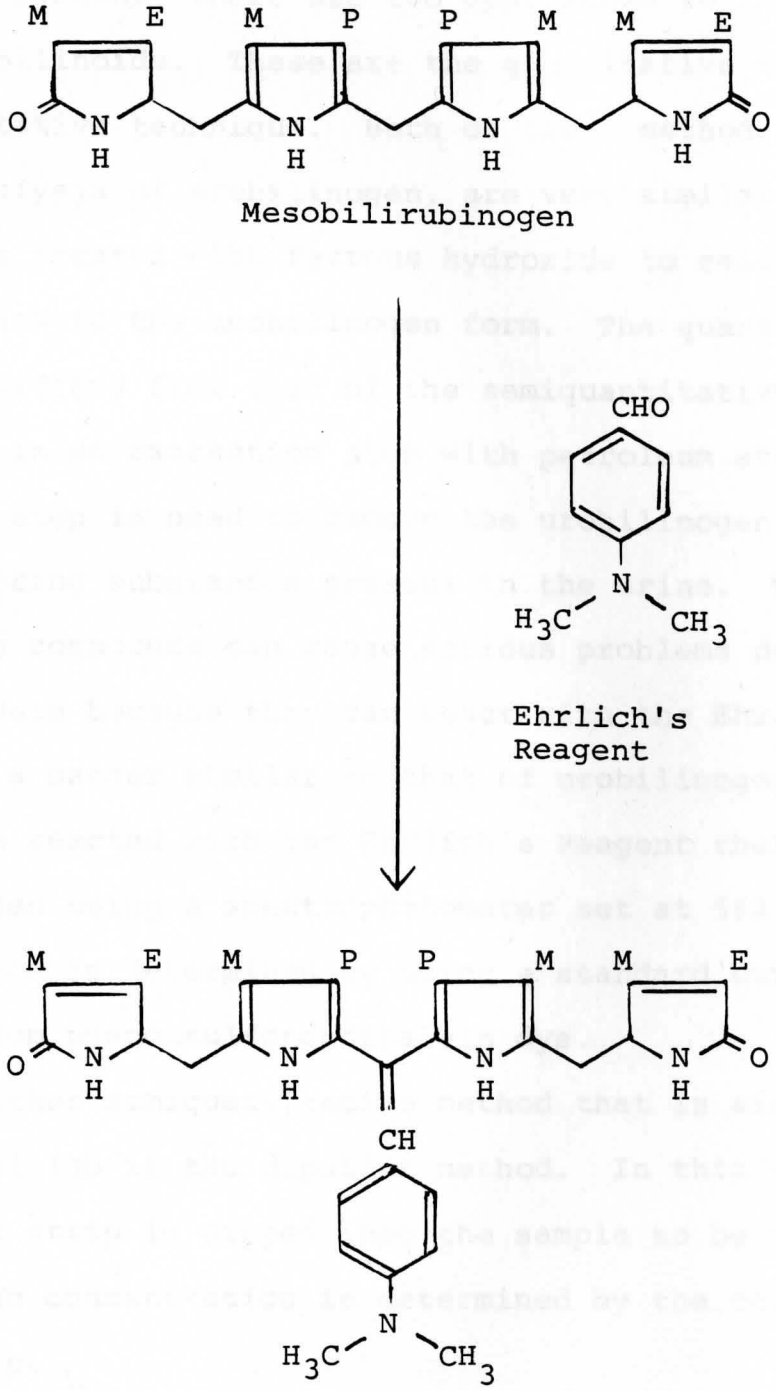


Figure 3-Reaction of Urobilinogen With Ehrlich's Reagent

Present Methods of Analysis

At present, there are two approaches to the analysis of the urobilinoids. These are the quantitative method and the semiquantitative technique. Both of these methods, when used for the analysis of urobilinogen, are very similar. The urine samples are treated with ferrous hydroxide to reduce any urobilin back to the urobilinogen form. The quantitative procedure differs from that of the semiquantitative method in that there is an extraction step with petroleum ether. The extraction step is used to remove the urobilinogen from many of the interfering substances present in the urine. These interfering compounds can cause serious problems during the test procedure because they can react with the Ehrlich's Reagent in a manner similar to that of urobilinogen. Once the samples are reacted with the Ehrlich's Reagent their absorbance is determined using a spectrophotometer set at 562 nm. The concentration is determined by using a standard curve which is prepared from phenolsulfonephthalein dye.

Another semiquantitative method that is widely used in the clinical lab is the dipstick method. In this technique, the reagent strip is dipped into the sample to be tested. The urobilinogen concentration is determined by the color produced on the strip.

In one of the popular reagent strip methods the results can be interpreted by either the lab technician or through the use of an instrument. By using the instrument (Ames "Clini-Tek") the variations among technicians in reading the test strip can be eliminated. Briefly, this instrument operates by

measuring the light that is reflected from the reagent strip. Since an increase in urobilinogen concentration results in a darker color on the strip, the intensity of reflected light is inversely proportional to concentration. In this particular system, there is no bilirubin interference up to 32 mg/L. This method, however, is not suitable for quantitative determination.

Clinical Significance

Testing the concentration of the urobilinoids in urine (usually in the form of urobilinogen) is a very important test for hepatitis. As mentioned earlier, urobilinogen is removed from the portal blood stream by the liver. If a significant number of liver cells are damaged or destroyed, the amount of urobilinogen excreted by the kidneys into the urine increases.

Another disease that can be diagnosed through urobilinogen analysis is obstructive jaundice. Obstructive jaundice involves a decrease or complete stop of bilirubin excretion in the intestines. Without the presence of bilirubin the formation of urobilinogen cannot take place. Thus, there is a decrease in urine urobilinogen concentration.

In general, liver diseases decrease the amount of fecal urobilinogen. In addition, anemias not related to red blood cell destruction cause a decrease in urobilinogen excretion. An exception is hemolytic anemia.

Patients who are undergoing or have just completed antibiotic drug treatment display a decrease in urine urobilinogen. Bilirubin is reduced in the intestinal tract by

bacterial action. Many antibiotics inhibit this reaction, thus the decrease in urobilinogen.

Statement of Problem

As the analysis for urobilinoids is a very important function in the clinical laboratory, any improvement in the analytical procedure for this test would be most desirable. As mentioned earlier, the present methods of analysis are relatively simple but have the problem of poor selectivity. Since concentrations of the urobilinoids are very low in urine, any substance that interferes with the test method can obviously alter the results significantly.

The problems involved in urobilinoid analysis offer an interesting project to the analytical chemist. One method that may possibly be used to overcome the problem of interfering substances in any type of analysis is that of separating the analyte from the materials of no interest. This is accomplished through the use of chromatography.

In this particular case, the use of High Performance Liquid Chromatography (HPLC) for the analysis of the urobilinoids in urine will be investigated. The purpose of this study will be to try to determine the feasibility of using HPLC for a urobilinoid analysis procedure.

Since no reports of work in this area could be found in the literature, the course of investigation of this problem is left to the researcher. In this study, various solvent systems and detection methods are tried in an attempt to

obtain a chromatographic system that allows the analysis of the urobilinoids.

CHAPTER II

HISTORICAL REVIEW

Discovery and Isolation of the Urobilinoids

Maly, working in 1871, first obtained the reduction of bilirubin using sodium amalgam.[4] He was able to isolate a compound which he called hydrobilirubin. This substance resembled a compound found in urine by Jaffe[5] in 1868. He referred to this as urobilin.

In 1911,[6] Fischer was able to isolate crystals using the same sodium amalgam reaction of Maly. In addition to the reduction of the bilirubin, extraction steps including diethyl ether and chloroform were included to purify the product. The end result was the first synthesis of what is now called urobilinogen (or mesobilirubinogen). The product that Maly obtained earlier was probably a mixture of the various reduction products of bilirubin.[7]

This mixture of reduction products was later discovered to contain another distinct component other than urobilinogen. In 1933, Watson[8,9] first obtained urobilin in crystalline form. This was done by allowing the reduced bilirubin to sit in a clear test tube for two weeks, allowing oxidation to take place. The chloroform extract of this yielded the crystals. In 1952, Watson was able to obtain urobilin crystals by oxidizing the urobilinogen with an aqueous iodine solution.[10]

The names used in describing the urobilinoids were very confusing during the early work in the field. For a time, any pigment in the urine with an absorption at 490 nm and an ability to react with Ehrlich's Reagent was referred to as urobilinogen.

Although "urobilinogen" is sometimes used in general terms today to mean all forms of the urobilinoids, it is understood that the three urobilinogens are distinctly different from the urobilins.

Spectrophotometric Methods of Analysis

In 1901, Ehrlich first developed a test for urobilinogen in urine.[11] Working with p-dimethylaminobenzaldehyde (Ehrlich's Reagent) in concentrated HCl, he noticed that when reacted with certain urines, it gave an intense red color. Ehrlich attributed this red color to the reaction between Ehrlich's Reagent and urobilinogen. It was later discovered, however, that indole and skatole derivatives in the urine give the same result.

The basis for today's colorometric methods of analysis was developed by Terwen in 1925.[12] He added several refinements to the work that was done earlier by Ehrlich. He discovered that the Ehrlich's Reagent was more specific if the concentrated HCl were replaced with sodium acetate. This increased the intensity of the urobilinogenaldehyde (the reaction product of Ehrlich's Reagent and urobilinogen) while at the same time decreased the reactions of the indole and skatole

derivatives. Another improvement was the use of ferrous hydroxide as a reducing agent in the urine samples. This converted any urobilin to urobilinogen as well as preventing the oxidation of the urobilinogen.

Up until 1935, diethyl ether was used in an extraction step to remove the urobilinogen from impurities present in the urine. Watson then introduced the use of petroleum ether as a replacement in the extraction.

Henry conducted a study of the quantitative method of analysis and discovered several problems with it.[13] It was found that the possibility exists that the urobilinogen is oxidized to something other than urobilin once it is exposed to air. Thus treatment with a reducing agent such as ferrous hydroxide would not result in the original concentration of urobilinogen. It was also discovered that the extractability of urobilinogen from urine was not constant. This was due to an interfering substance present in the urine which is dependent upon the time of storage before analysis. The reproducibility at low concentrations was found to be erratic. In addition, the quantitative method could not be shown to overcome the problem of interferences with the Ehrlich reaction.

A modification of the quantitative method was developed by Kutter and Humbel[14] in 1973 when they discovered the reaction between urobilinogen and p-methoxybenzene diazoniumfluoborate (p-MDFB).

This procedure is basically the same as the quantitative method developed by Watson and others. The use of the p-MDFB requires that the samples be read at 505 nm. Some substances

that interfere with Ehrlich's Reagent were found not to interfere with the p-MDFB. A high concentration of bilirubin, however, caused an interference. A brief comparison of reported interferences for the spectrophotometric methods is given in Table 1.[15]

TABLE 1

INTERFERENCES FOR SPECTROPHOTOMETRIC METHODS

Substance	Semiquant.	Quant. (Ehrlich's)	Quant. (p-MDFB)
p-Aminosalicylic acid	+	+	-
Antipyrine	+	+	-
Chlorpromazine	+	+	-
Methylglucaminodiatrizoate	+	+	-
Sulfadiazine	+	+	-
Sulfisoxazole	+	+	-
Sulfonamides	+	+	-
Tetracycline	+	+	-
Porphobilinogen	+	+	-
Bilirubin	+	+	+
Nitrites	+	+	+

+ = Interferences

- = No interference

Although the interference problem has been reduced in the p-MDFB method, other complications that occur with the conventional quantitative method are still present. These

problems have led most clinical laboratories to use the less involved semi-quantitative method.

Fluorescent Methods of Analysis

In 1869,[16] Jaffe was the first to observe the fluorescence from the zinc complex formed by mixing alcoholic zinc acetate solution with the urobilin. This green fluorescence was used as a method of identification. The proposed complex is shown in Figure 4.[17]

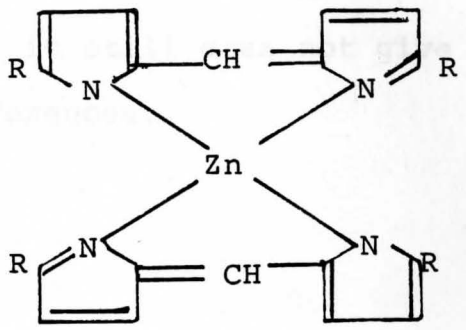


Figure 4-Zinc-Urobilin Complex

Although it has not been used to any great extent, a fluorescent method for the analysis of urobilin has been performed. It involves the oxidation of urobilinogen with iodine or ammonium persulfate to urobilin before complexation with the alcoholic zinc acetate solution. The analysis can be carried out for i-urobilin at 637 nm, 601 nm, 551 nm, and 521 nm. For l-stercobilin, the fluorescent maxima are at 638 nm and 520 nm. This test can be performed on either urine or a chloroform extract.

This test is more sensitive than the Ehrlich's test for urobilinogen and also more selective. Despite these advantages, the zinc fluorescence test procedure has not been developed into a quantitative method.

Dipstick Method of Analysis

In recent years, development of a reagent strip has provided a relatively easy method for screening urines for urobilinogen. Most of these procedures are based on the Ehrlich's reaction. Although refinements of this method are published frequently, it still does not give quantitative results due to interferences.

CHAPTER III

METHODOLOGY

High Performance Liquid Chromatography

High Performance Liquid Chromatography involves the separation of solutes in a mixture by passing them through a chromatographic column. The system is similar to gas chromatography except that the carrier gas is replaced by a liquid mobile phase. HPLC differs from conventional liquid chromatography in that the mobile phase is pumped through the system at very high pressures (usually with an upper limit of 6000 p.s.i.). The packing materials for HPLC columns are small (between 5 and 50 μm) and packed tightly together. Thus, high pressures are required to push the sample through within a reasonable time (usually a maximum of 30 minutes).

A basic HPLC system consists of a pump, injector, column, and detector. The pump must be able to deliver the various solvents at a constant rate. The injection system is used to store the sample before it is injected onto the column, of which there are several types for HPLC. Among the most common types of columns in use today are ion exchange (both anion and cation) and reversed phase. There are several methods of detection for HPLC. These include UV, refractive index, fluorometric, and electrochemical.

In gas chromatography, there are a few carrier gases

and a large number of column packings. In HPLC, the opposite is true. The number of column materials is relatively small compared to the types of mobile phase solvents available.

A particular compound can be identified through HPLC by determining its k' value. The k' value is given in the following expression:

$$k' = \frac{t_r - t_o}{t_o} \qquad (1)$$

where t_r is the retention time(in seconds) of the sample and t_o is the retention time (in seconds) of the solvent peak. Retention times instead of retention volumes may be used with the assumption that the flow rate of the pump system is constant. The k' value will change as the chromatographic conditions change. In order for two or more compounds to separate on the column their k' values must be different.

Reversed Phase HPLC

Reversed phase HPLC involves the use of a non-polar stationary phase with a relatively polar mobile phase. In this manner solutes that are highly polar will tend not to be attracted to the stationary phase as they pass through the column. Thus, they will be eluted first. In other types of column packings, highly polar molecules will be strongly attracted to the column, resulting in long elution times. This problem is overcome by reversed phase HPLC. Non-polar compounds are also separated since they have an affinity for

the column packing.

The most common type of reversed phase column consists of long hydrocarbons bonded to silica. An example of this type of packing is shown in Figure 5.

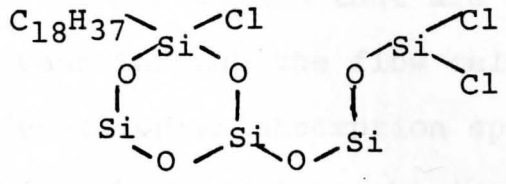


Figure 5-C_{18} Reversed Phase Packing Material

The hydrocarbons vary in size from C_6 to C_{18}. Usually the longer groups show a higher level of selectivity, although separations done on long chain columns can be done on shorter chain packings by increasing the polarity of the mobile phase. The shorter chain materials also seem well suited for separations of highly polar samples.

As mentioned earlier, the composition of the mobile phase (rather then the column) can sometimes be changed to meet experimental needs. This is very important with reversed phase chromatography. The most important solvent variable is polarity. The solvent polarity affects chromatographic performance such that a non-polar sample will be eluted faster by decreasing the polarity of the mobile phase. When using UV detection, the UV cutoff of a solvent can become very important if work is done at the lower end of the UV spectrum.

Methods of Detection for HPLC

In this study the methods of detection used are UV and electrochemical. With UV detection, the absorbance of the materials coming off the column is measured. The detector is set so that the absorbance of the mobile phase is zero. Eluents coming off of the column that are UV active will be detected as they pass through the flow cell of the detector. Before using UV detection an absorption spectrum of the sample in the mobile phase must be obtained in order to determine the optimal wavelength for the sample detection. In the past UV detectors were sometimes limited to two wavelengths--254 nm and 284 nm. Presently, however, variable wavelength detectors are coming into wide use. These instruments offer wavelengths in the UV and visible regions, with UV being preferred because more compounds are UV active and higher sensitivity is obtained.

Another detection method that is gaining popularity is electrochemical detection. This involves an electron transfer reaction at the working electrode involving either oxidation or reduction. The measurement of the current produced by the reaction versus time gives the chromatograph. The concentration of the electroactive species in the detector determines the amount of current produced.

Many of the present day electrochemical detectors use a thin layer flow through cell with a small dead volume. Since the volume of eluents coming off of the column is extremely small, the detector dead volume must also be

negligible in order for separations to be detected.

Depending on the experimental conditions, the type of working electrode in the detector varies. For the thin layer detectors glassy carbon, carbon paste, or mercury are used. The graphite provides an excellent supply of electrons for the oxidation-reduction reactions which take place at the electrode surface.

The working electrode material to be used must be compatible with the mobile phase. Carbon paste, which consists of graphite held together by mineral oil, can be used with aqueous mobile phases containing up to 30% methanol and 10% acetonitrile before the solvents begin to affect the binding material. In addition, the working electrode material can affect the performance of the detector with respect to selectivity. This is illustrated in Figure 6.[19]

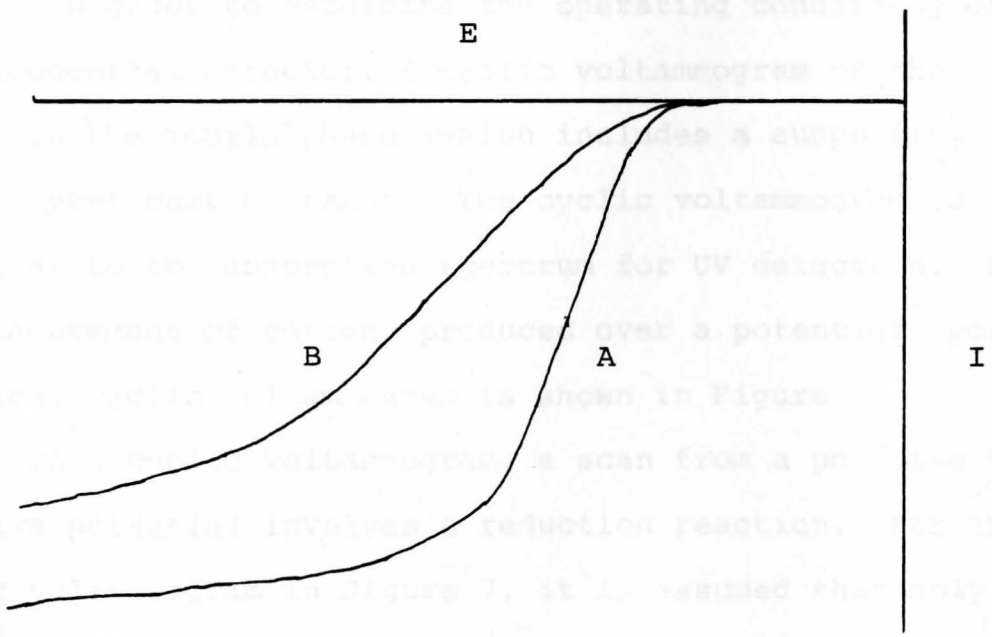


Figure 6-Comparison of Electrode Materials

In the reduction of a sample shown in Figure 6, the voltammogram gives the response of two different electrode materials. Line A does not give a current until immediately before E^0. The sharp rise in current shows that the reaction is very rapid and that no large overpotential is needed. On the other hand, line B indicates a slow electron transfer reaction that does not lend itself to good selectivity.

Electrochemical detectors offer high sensitivity, commonly being able to measure nanogram quantities. Also, since the working electrode is maintained at a controlled potential, only those compounds electroactive at or below that particular potential can be detected. Thus electrochemical detection offers excellent selectivity.

Cyclic Voltammetry

In order to determine the operating conditions of the electrochemical detector, a cyclic voltammogram of the sample in the mobile phase (which includes a supporting electrolyte) must be taken. The cyclic voltammogram is analogous to the absorption spectrum for UV detection. It is the measurement of current produced over a potential cycle. A typical cyclic voltammogram is shown in Figure 7.

In a cyclic voltammogram, a scan from a positive to negative potential involves a reduction reaction. For the cyclic voltammogram in Figure 7, it is assumed that only the reduced form of the sample is initially present. That is why the scan begins in the positive direction. The cyclic voltammogram shown is that of a reversible redox couple.

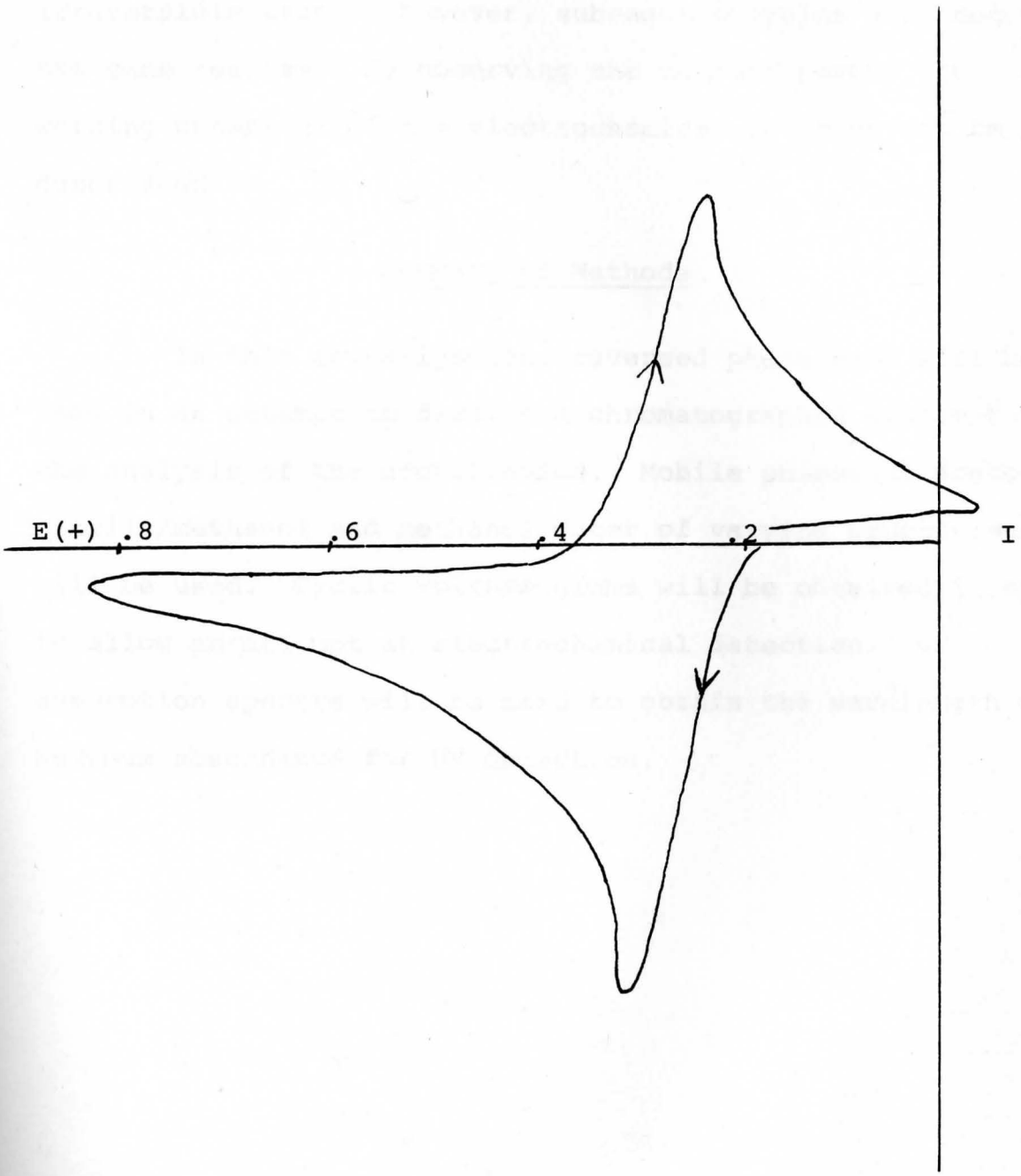


Figure 7-Typical Cyclic Voltammogram

Since the sample is reversible, the voltammogram will be the same no matter how many cycles are run. In the case of an irreversible sample, however, subsequent cycles will not give the same results. By observing the current peaks, the working potential of the electrochemical detector can be determined.

Summary of Methods

In this investigation, reversed phase HPLC will be used in an attempt to develop a chromatographic system for the analysis of the urobilinoids. Mobile phases of acetonitrile/methanol and methanol/water of varying proportions will be used. Cyclic voltammograms will be obtained in order to allow an attempt at electrochemical detection. UV absorption spectra will be used to obtain the wavelength of maximum absorbance for UV detection.

CHAPTER IV

MATERIALS AND APPARATUS

Materials

All materials that were used were taken from the bottle without recrystallization. The grades of the various reagents are given in Table 2.

The pH 5.5 buffer solution was prepared by titrating ammonium hydroxide with glacial acetic acid while observing the pH. The pH meter was standardized using Sargent-Welch standard buffer solutions.

Nitrogen (Airco Co.) was used as an inert atmosphere. For use in the glove box, the nitrogen was dried by passing it through $CaCl_2$ columns.

The bilirubin was of certified grade from Fisher Scientific. It was stored at $0^{o}C$ in a sealed dessicator.

Apparatus

PAR Electrochemistry System

All electrochemical measurements were obtained from a Princeton Applied Research Model 170 Electrochemistry System. This instrument was used in conjunction with the various electrode systems employed during the study. The PAR 170 was equipped with a self-contained x-y recorder.

TABLE 2

LIST OF REAGENTS

Material	Formula	Grade	Manufacturer
Ethyl Acetate	$C_2H_5COOCH_3$	Reagent	Baker & Adamson
Potassium Chloride	KCl	Reagent	Baker & Adamson
Sodium	Na	Reagent	Matheson, Coleman, & Bell
Mercury	Hg	Reagent	Bethlehem
Sodium Carbonate	Na_2CO_3	Reagent	Baker & Adamson
Chloroform	$CHCl_3$	Certified	Fisher
Mineral Oil		Reagent	Eastman
Acetonitrile	CH_3CN	Reagent	Eastman
Methanol	CH_3OH	Certified	Fisher
Methanol	CH_3OH	HPLC Grade	Fisher
Petroleum Ether		Reagent	Matheson. Coleman, & Bell
Ligroine		Practical	Eastman
Sulfuric Acid	H_2SO_4	Analytical Reagent	Mallinckrodt
Ammonium Hydroxide	NH_4OH	Reagent	Matheson, Coleman, & Bell

TABLE 2 (CONTINUED)

Material	Formula	Grade	Manufacturer
Acetic Acid	CH_3COOH	Reagent	Fisher
Graphite	C	Practical	Dixon
Sodium Hydroxide	NaOH	Analytical Reagent	Mallinckrodt

Electrodes and Electrochemical Cells

For electrochemical reduction, a Hanging Drop Electrode (H.M.D.E.) was used (Figure 8). For other studies (such as cyclic voltammograms) a platinum electrode was used. Both of these systems (shown in Figure 9) consisted of S.C.E. reference electrodes and platinum auxillary electrodes. The platinum auxillary electrodes were made up of a Pt wire inserted into a glass tube with a fritted end. This was submerged into the solution. In addition to using these systems for oxidation and reduction reactions, they were also used to obtain cyclic voltammograms. The HPLC electrochemical detector (shown in Figure 10) was also used for the cyclics.

High Performance Liquid Chromatography

A Waters Associates M-6000A pumping system was used for the HPLC studies. The variable wavelength UV detector consisted of a Hitachi Model 100-10 Spectrophotometer connected to an Altex flow cell. Electrochemical detection was provided by a Bioanalytical Systems Inc. TL-3 Plexiglass Thin-layer Detector Cell. A carbon paste working electrode (made from graphite and mineral oil) was used along with a Ag/AgCl reference electrode. A Beckman Ultrasphere C_{18} 15 cm x 4.6 mm I.D. reversed phase column was used.

All injections into the HPLC were made using a Hamilton 10 μL syringe.

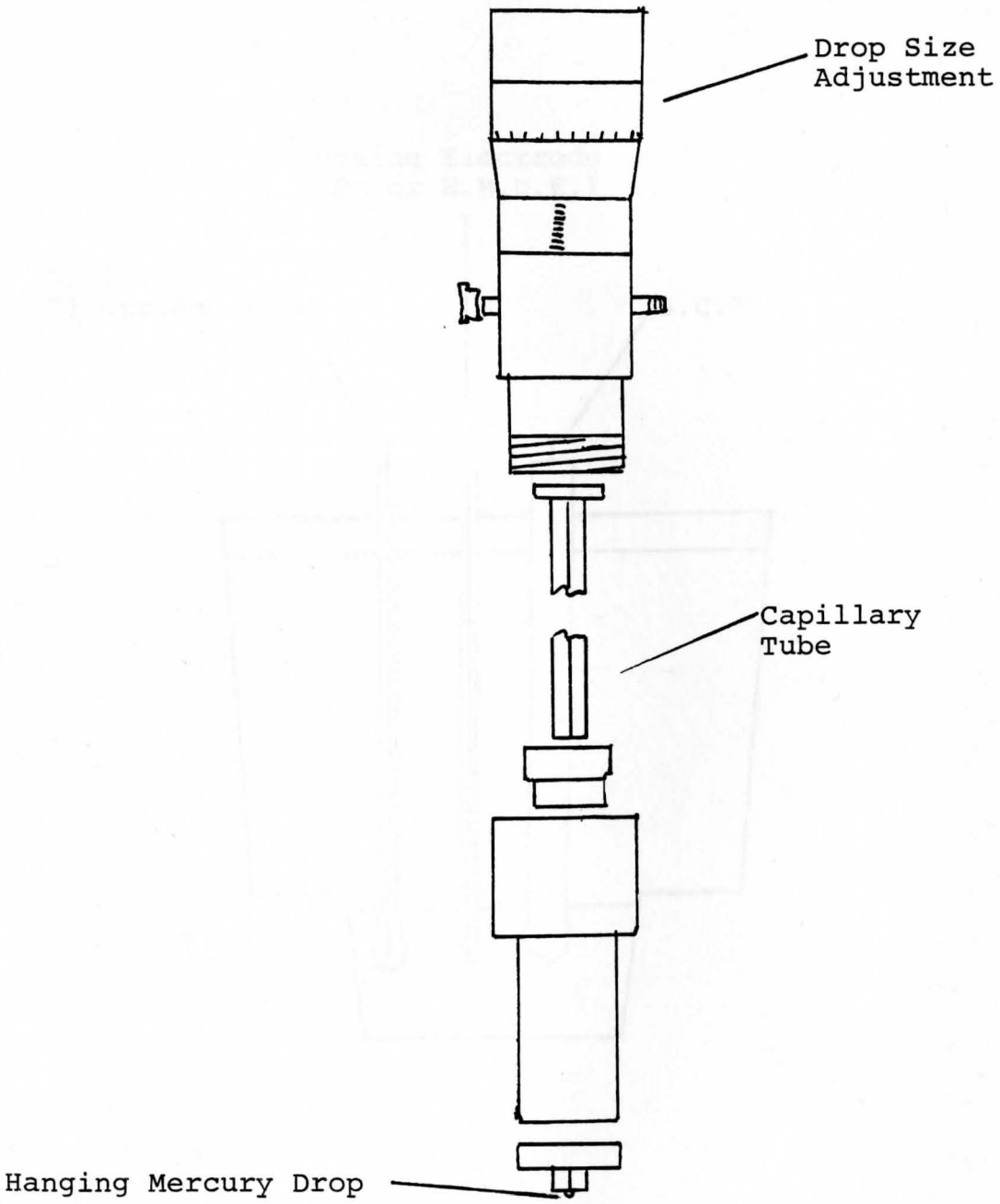


Figure 8-Hanging Mercury Drop Electrode (H.M.D.E.)

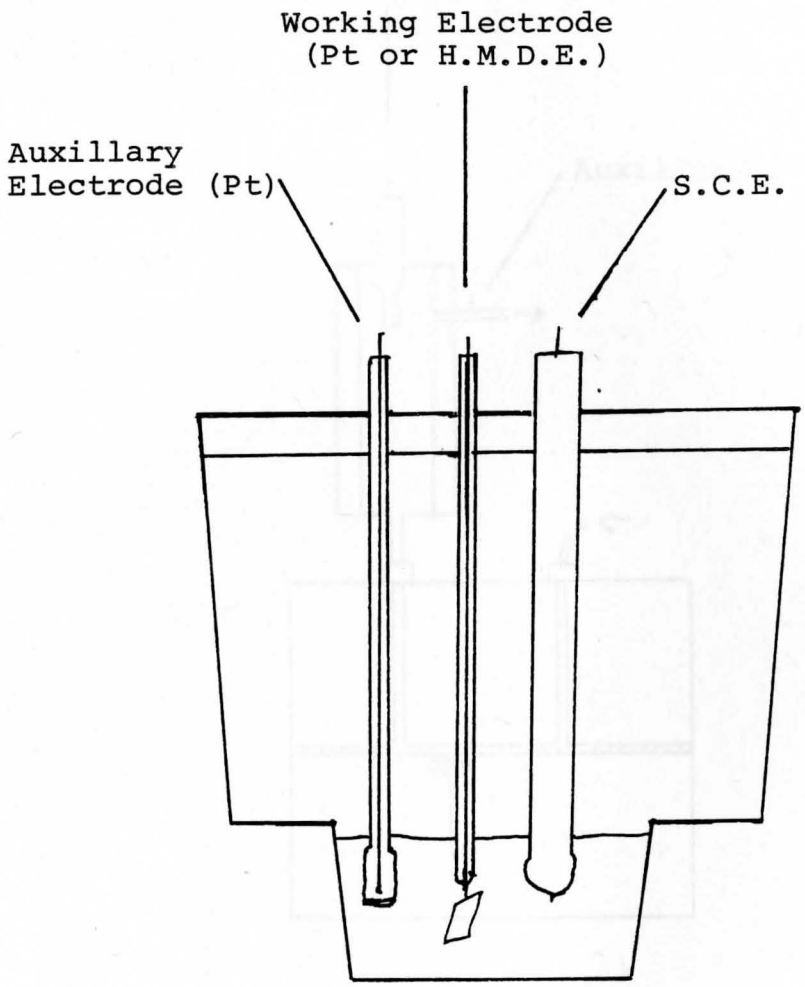


Figure 9-Electrode System

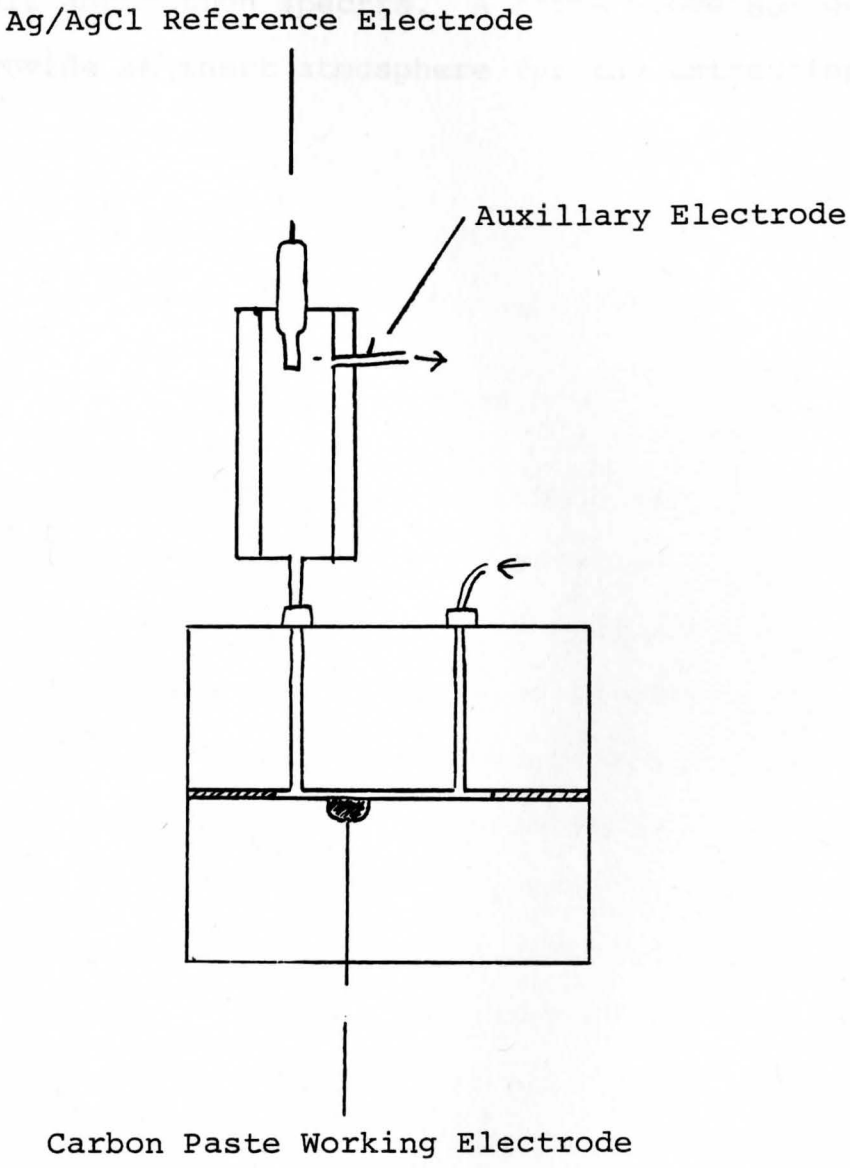


Figure 10-TL-3 Electrochemical Detector

Miscellaneous Apparatus

A Beckman Model 26 Dual Beam Spectrophotometer was used for all absorption spectra. A Forma Glove Box was used to provide an inert atmosphere for the extraction steps.

CHAPTER V

PROCEDURE AND RESULTS

Attempts to Synthesize the Urobilinoids

In this section, attempts at synthesizing urobilinogen and urobilin using various techniques will be described.

Reduction of Bilirubin

The chemical reduction of bilirubin was accomplished by using 3% sodium amalgam. This was obtained by reacting 3 g of pea sized sodium pellets with 75 g of mercury. Stoll and Gray recommended the use of 100 g of mercury but this was found to leave a substantial amount of mercury unreacted. Because of the violent nature of the reaction, it was performed with the protection of a fume hood. In addition, one sodium pellet was added at a time, only after the previous pellet had completely reacted. The reaction mixture was then heated until it was molten, then allowed to cool. After cooling for one hour, the hardened product was broken up and washed with cool water to remove any oxidation products that may have been present. Next, it was washed with small portions of ethanol and ether. Finally, the product was dried under vacuum in a desicator. The final product consisted of silvery particles. The sodium amalgam was stored in a stoppered bottle.

About 50 mg of bilirubin were placed in a container along with .8 mL of water and .2 mL of .1 M sodium hydroxide. The NaOH was necessary to allow the bilirubin to dissolve in the water. To this, 5 g of 3% sodium amalgam were added. The contents were mixed using a magnetic stirrer. The reaction progressed for approximately two hours under nitrogen.

After the reaction was completed the original deep yellow to orange color of the bilirubin was replaced by a colorless solution, indicating the reduction of urobilinogen. Upon exposure to air there was a slight discoloration to a pale yellow.

Attempts to Crystallize Urobilinogen

The first step in the crystallization procedure was to obtain a solution of mesobilirubinogen (urobilinogen) described by Stoll and Gray. The reduced bilirubin was prepared as mentioned above.

The reduced bilirubin was diluted with 20 mL of water and covered with 300 mL of petroleum ether. The aqueous layer was treated with ammonium hydroxide/acetic acid buffer to adjust the pH to 5.5. Both layers were placed in a separatory funnel and shaken vigorously. The petroleum ether was collected and the aqueous layer was washed two more times with 150 mL portions of petroleum ether. The petroleum ether extracts

were collected and washed with two 20 mL portions of water. After filtering, the petroleum ether was washed with 15-20 mL portions of 5% sodium carbonate (which was acidified with 50% H_2SO_4). The Na_2CO_3 solutions were collected and washed several times with 5-10 mL amounts of $CHCl_3$. An absorption spectrum of the $CHCl_3$ solution was taken from 350 nm to 750 nm. In addition, the absorption spectrum for the same region was obtained for bilirubin/$CHCl_3$. The spectra are shown in Figures 11 and 12. The differences in wavelengths of maximun absorbance (shown in Table 3) indicated that the extraction product contained something other than bilirubin.

TABLE 3

WAVELENGTHS OF MAXIMUM ABSORBANCE

Sample	λ_{max}
Bilirubin($CHCl_3$)	450 nm
$CHCl_3$ Extract	495 nm

The $CHCl_3$ extract was then concentrated to 1-2 mL under reduced pressure using the lypholyzer. After concentration, several milliliters of hot acetone were added to induce crystallization. A few crystals of slightly pinkish color were obtained. The melting point was taken but the crystals did not melt within the temperature range of the melting point apparatus (about 280°). The reported m.p. for urobilinogen is 200-202°C.

Another attempt at crystallization was made using a procedure by Fischer. The extraction steps were the same as

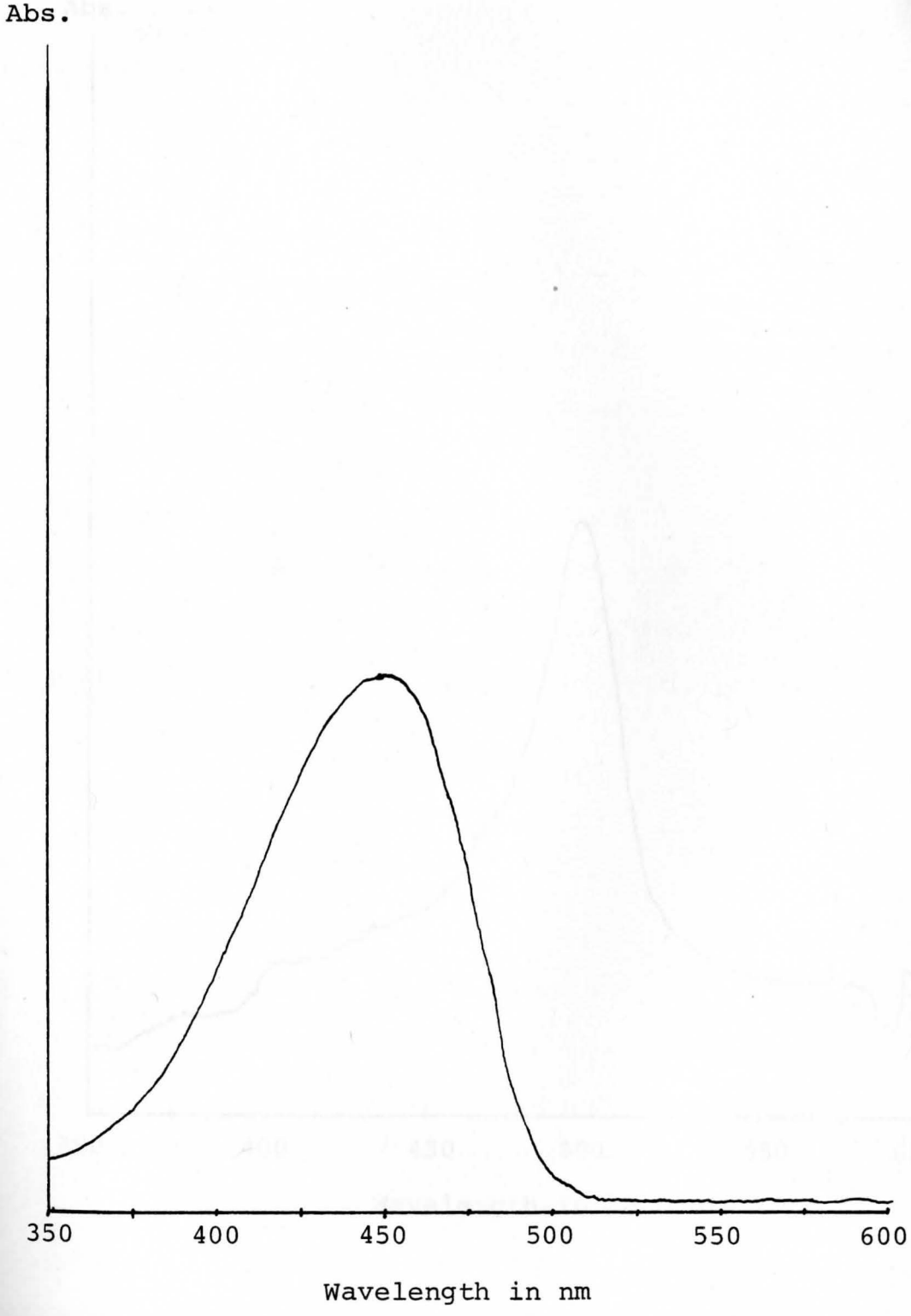


Figure 11-Absorption Spectrum of Bilirubin in $CHCl_3$

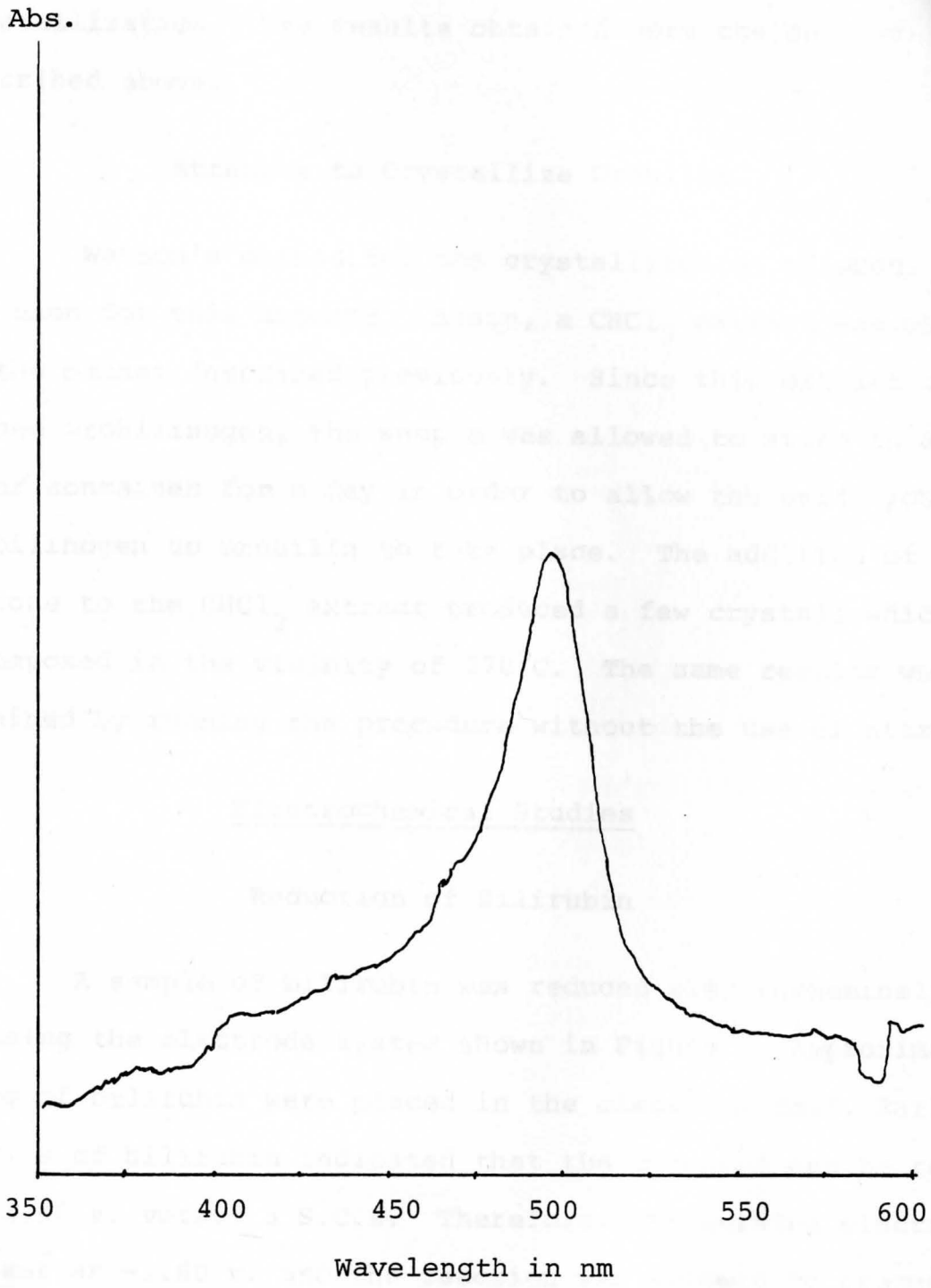


Figure 12-Absorption Spectrum of $CHCl_3$ Extract

those mentioned previously. The concentrated $CHCl_3$ solution was treated with hot ligroine and ethyl acetate to effect crystallization. The results obtained were the same as described above.

Attempts to Crystallize Urobilin

Watson's method for the crystallization of urobilin was used for this attempt. Again, a $CHCl_3$ extract was obtained in the manner described previously. Since this extract contained urobilinogen, the sample was allowed to stand in a clear container for a day in order to allow the oxidation of urobilinogen to urobilin to take place. The addition of hot acetone to the $CHCl_3$ extract produced a few crystals which decomposed in the vicinity of 270°C. The same results were obtained by running the procedure without the use of nitrogen.

Electrochemical Studies

Reduction of Bilirubin

A sample of bilirubin was reduced electrochemically by using the electrode system shown in Figure 9. Approximately 50 mg of bilirubin were placed in the electrode cell. Earlier studies of bilirubin indicated that the compound can be reduced at -1.60 v. versus a S.C.E. Therefore, the working electrode was set at -1.60 v. and the reaction was allowed to proceed for several hours. A color change from orange to yellow was observed. A colorless solution like that obtained with the sodium amalgam reduction was not observed. Thus the reduction product

that was obtained probably consisted of urobilin. Subsequent crystallization attempts of the urobilin solutions did not prove fruitful.

Since the attempts at producing urobilinogen and urobilin crystals failed, it was decided that freshly prepared samples of reduced bilirubin (by the sodium amalgam method) would provide a good supply of urobilinogen for further studies. To increase the volume of sample to work with, the reaction mixture was diluted with 5 mL of water midway through the reaction. All of the reactions proceeded for at least two hours to insure complete reaction of the bilirubin.

Cyclic Voltammograms

As mentioned in Chapter III, HPLC with electrochemical detection (HPLCEC) requires cyclic voltammetry. The PAR-170 was used for the electrochemical measurements. The platinum wire electrode system and the TL-3 detector were used in conjunction with the PAR. The cyclic voltammogram of the mobile phase and of the sample in the mobile phase had to be taken in order to account for the background current. The initial mobile phase was arbitrarily picked to be 20% MeOH. The supporting electrolyte was provided using a .1 M concentration of KCl. The cyclic voltammograms were obtained by impressing a voltage ramp, Figure 13, on the working electrode. The potential range for all of the cyclic voltammograms was -1.0 to +1.0 v. The potential scan rate for all of the cyclic voltammograms was 10 mv/sec. The current range for the TL-3 detector runs was

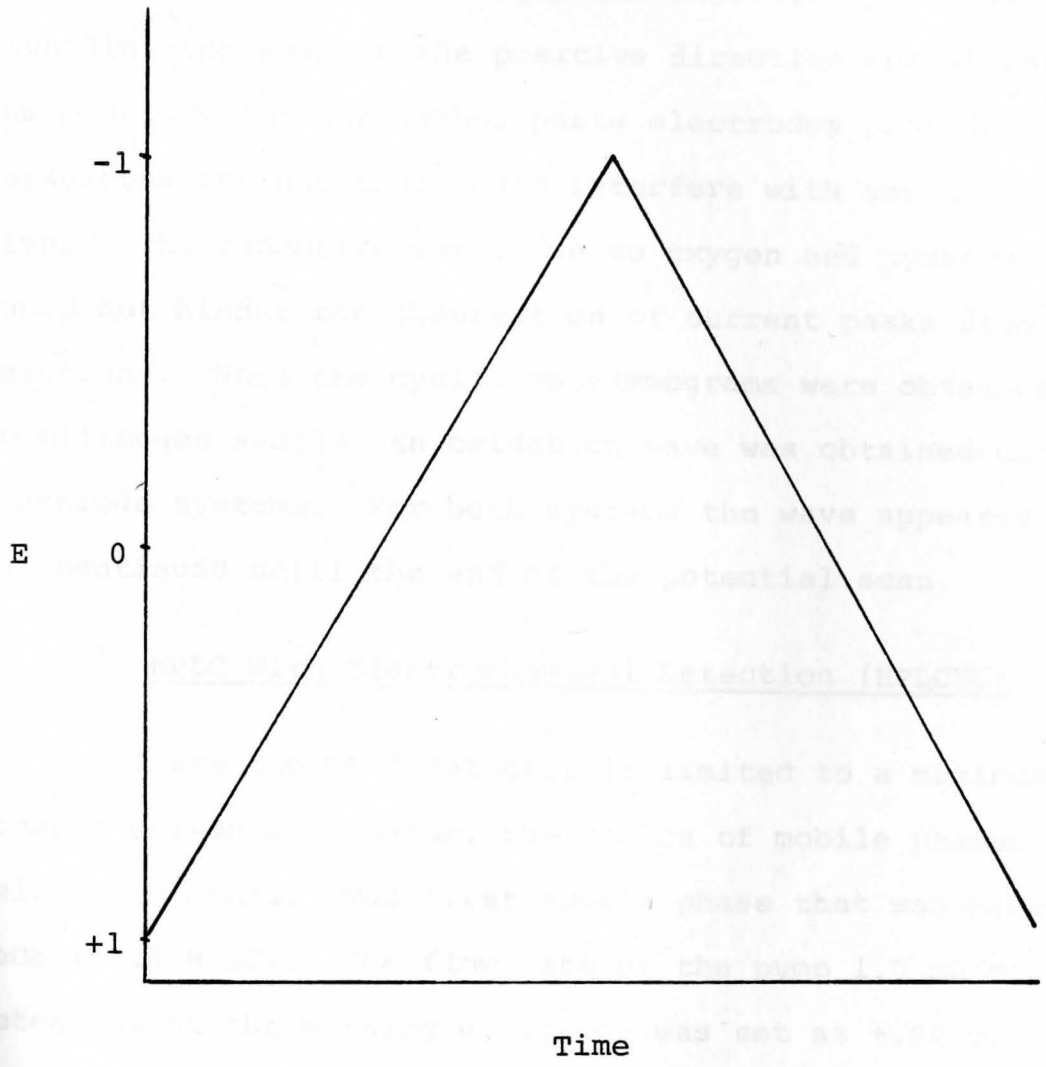


Figure 13-Voltage Ramp For Cyclic Voltammograms

20 μA while for the platinum electrode determination it was 100 μA. The cyclic voltammograms are shown in Figures 14, 15, 16, and 17.

Since the urobilinogen was expected to be oxidized to urobilin, the scan in the positive direction was of interest. The platinum and the carbon paste electrodes provided little background current that would interfere with any oxidation reactions. The reductive waves due to oxygen and hydrogen evolution would not hinder the observation of current peaks from oxidation reactions. When the cyclic voltammograms were obtained for the urobilinogen sample, an oxidation wave was obtained using both electrode systems. For both systems the wave appeared at +0.4 v. and continued until the end of the potential scan.

HPLC With Electrochemical Detection (HPLCEC)

Since the TL-3 detector is limited to a maximum of 30% organic solvents in water, the choice of mobile phases was relatively small. The first mobile phase that was used was 20% MeOH in .1 M KCl. The flow rate of the pump 1.0 mL/min. The potential of the working electrode was set at +.90 v. The current range was 2 μA. Ten uL urobilinogen samples (freshly prepared) were injected onto the column with no discernable peaks observed within 30 minutes.

A less polar mobile phase of 30% MeOH in .1 M KCl was used utilizing the same conditions. Again, no peaks were observed.

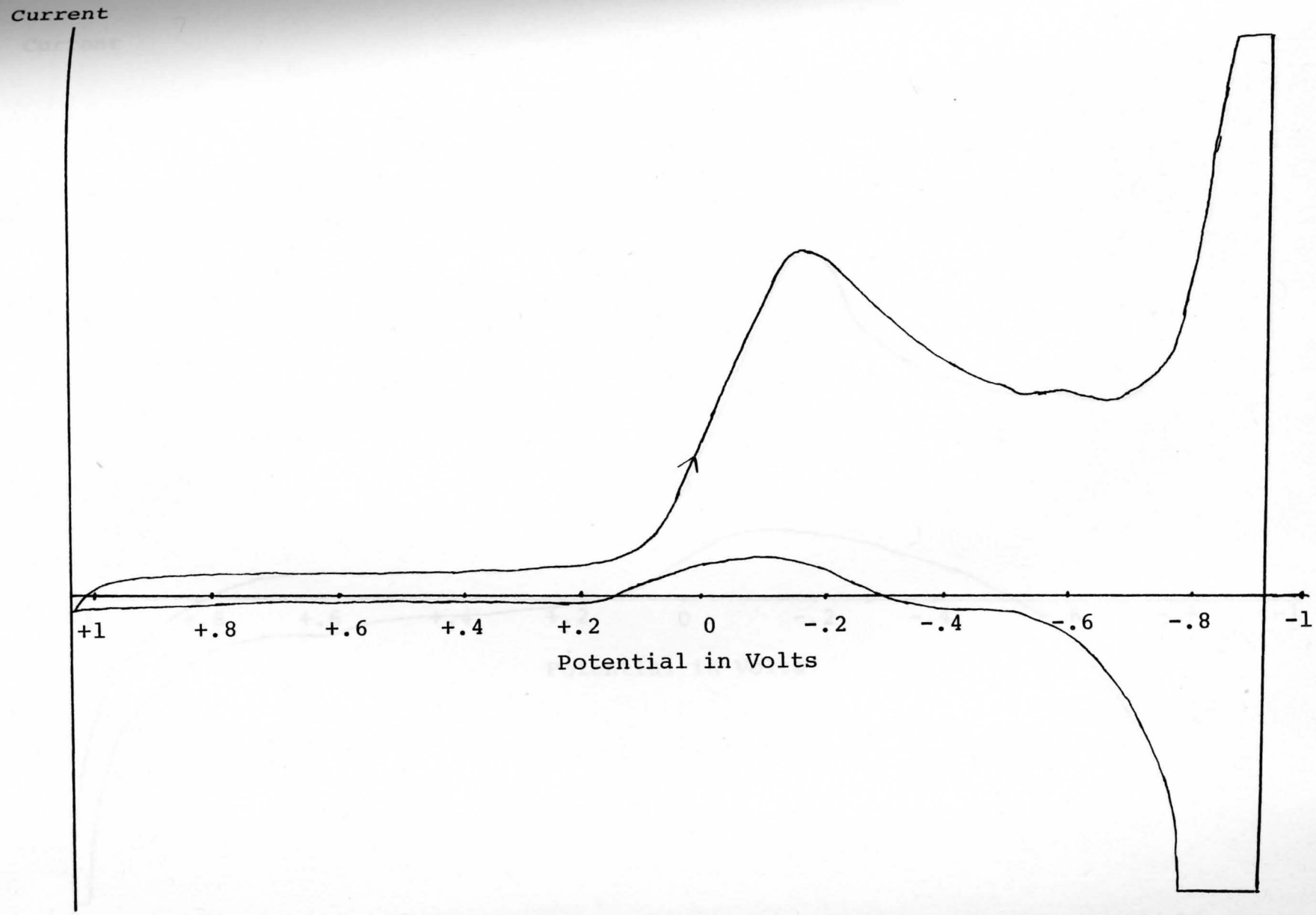


Figure 14-Cyclic Voltammogram of 20% MeOH (.1M KCl) Using Pt Electrode

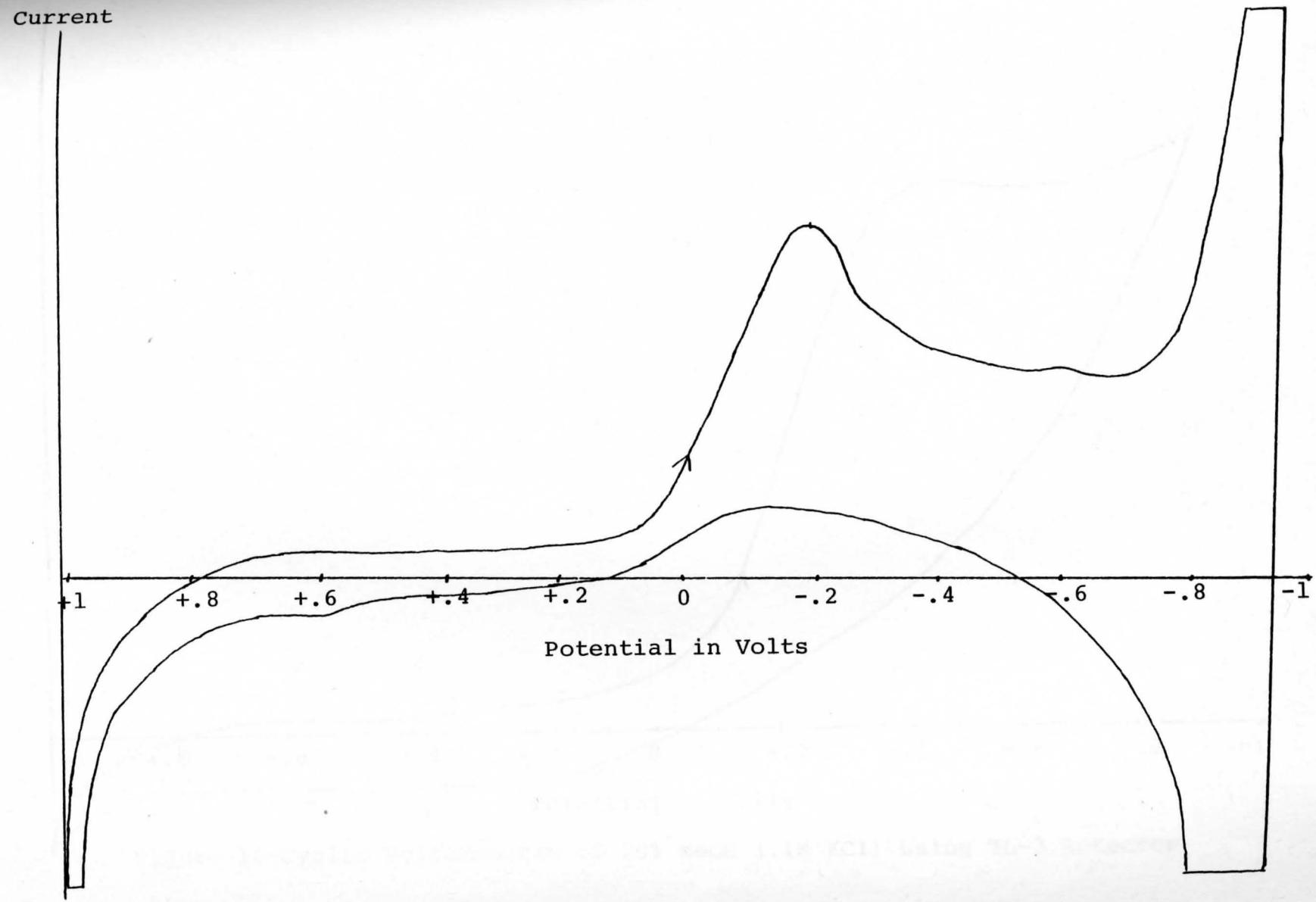


Figure 15-Cyclic Voltammogram of Urobilinogen in 20% MeOH Using Pt Electrode

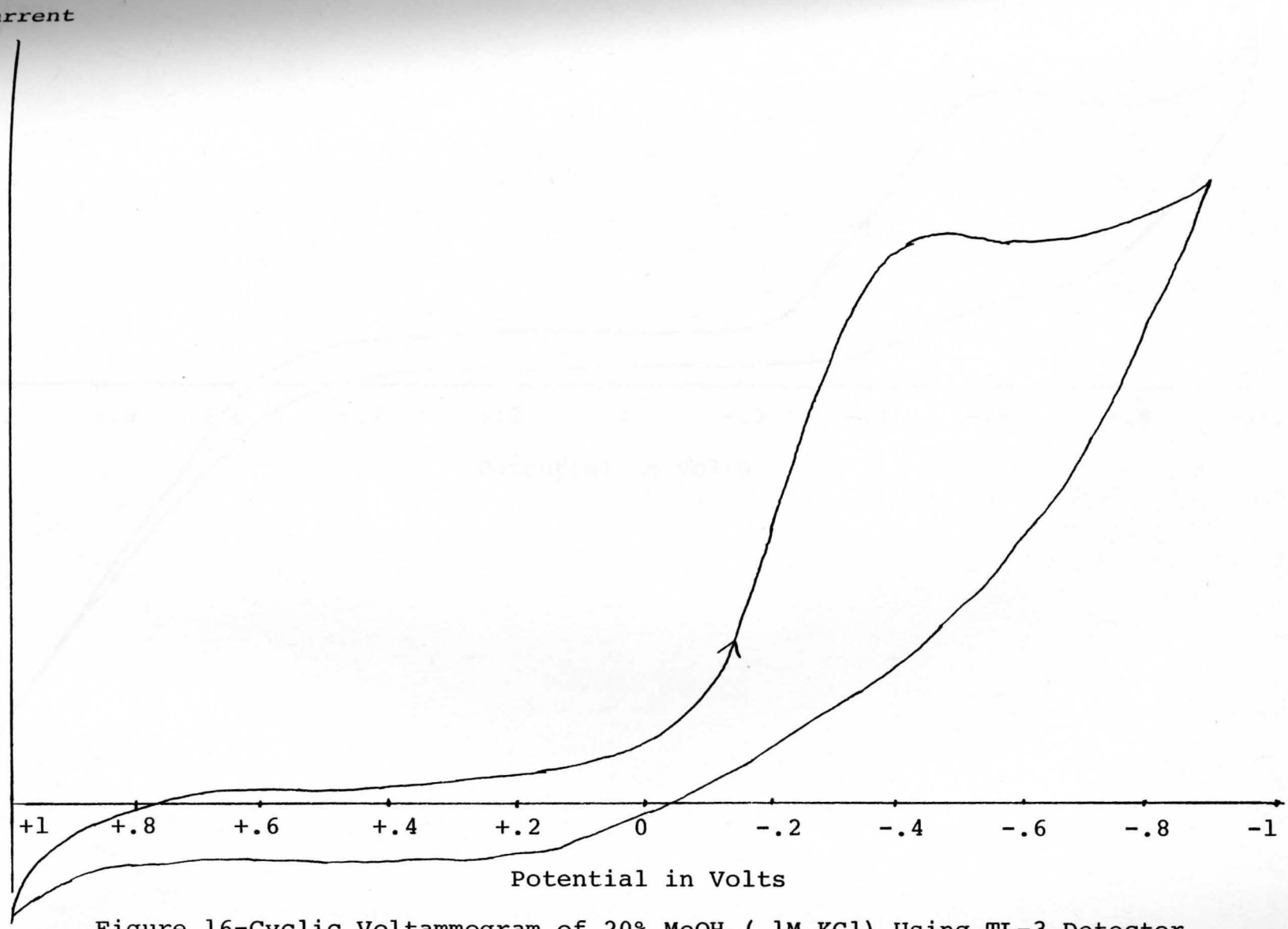


Figure 16-Cyclic Voltammogram of 20% MeOH (.1M KCl) Using TL-3 Detector

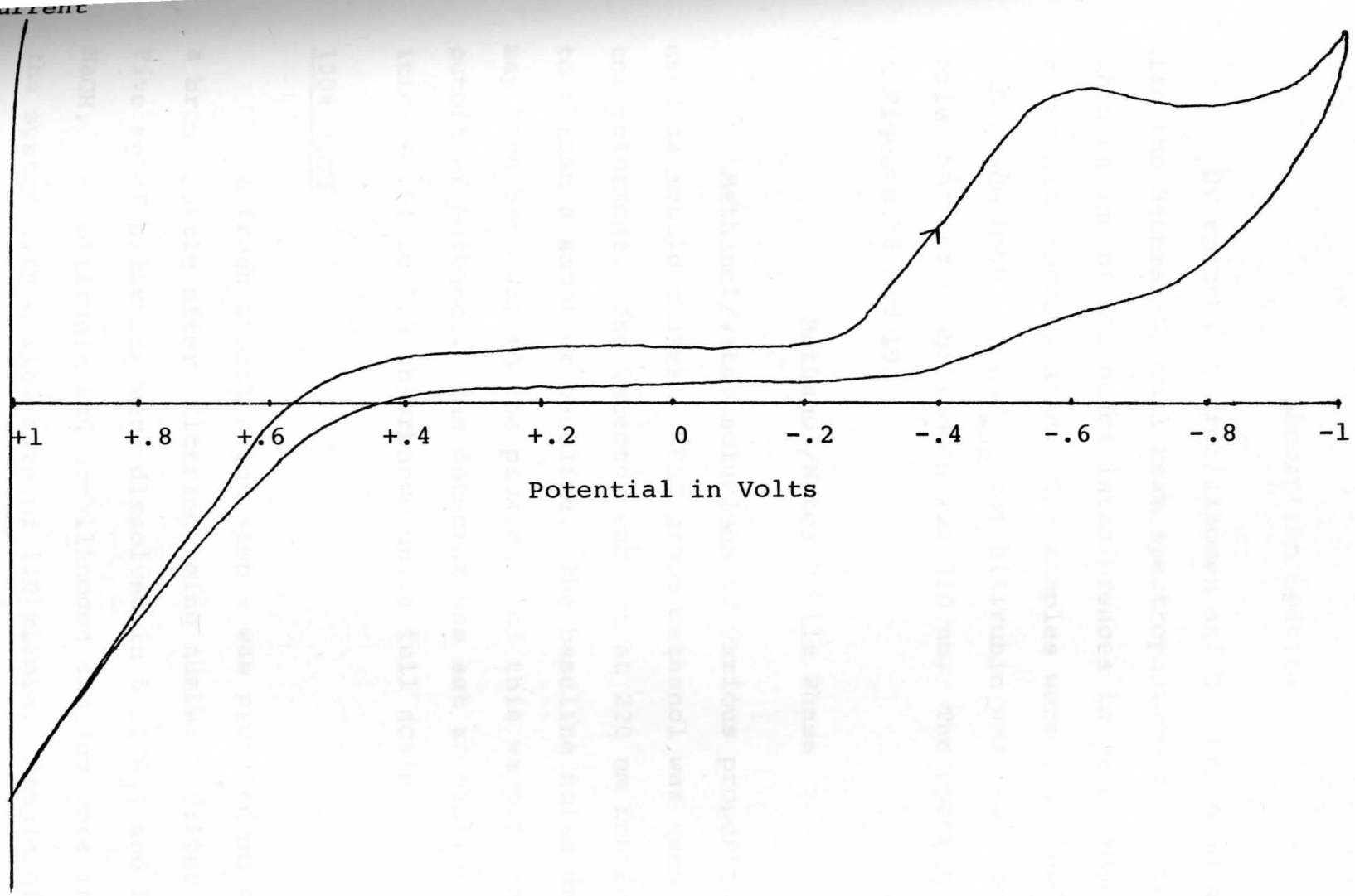


Figure 17-Cyclic Voltammogram of Urobilinogen/20% MeOH Using TL-3 Detector

HPLC With UV Detection

Absorption Spectra

UV spectra of urobilinogen and bilirubin were taken with the Beckman-26 dual beam spectrophotometer. Since bilirubin is one of the major interferences in urobilinogen analysis it was also investigated. The samples were dissolved in 70% HPLC grade MeOH. The λ_{max} for bilirubin was found to be 209 nm while that for urobilinogen was 210 nm. The spectra are shown in Figures 18 and 19.

Methanol/Water Mobile Phase

Methanol/water solutions of various proportions were used as mobile phases. HPLC grade methanol was used for all of the solutions. The detector was set at 220 nm instead of 210 nm to obtain a smoother baseline. The baseline noise at 210 nm may have been due to the proximity of this wavelength to the UV cutoff of methanol. The detector was set at various sensitivities (.01 to 1.0 absorbance units full scale).

100% MeOH

A fresh urobilinogen sample was prepared and stored in a brown bottle after filtering using number 2 filter paper. Five mg of bilirubin were dissolved in 5 mL H_2O and 1 mL of .1 M NaOH. The bilirubin and urobilinogen samples were injected into the system with a flow rate of 1.0 mL/min. Sample sizes ranged from 2 to 5 µL. Both samples were unretained on the column, coming off with the solvent fronts. The solvent fronts were

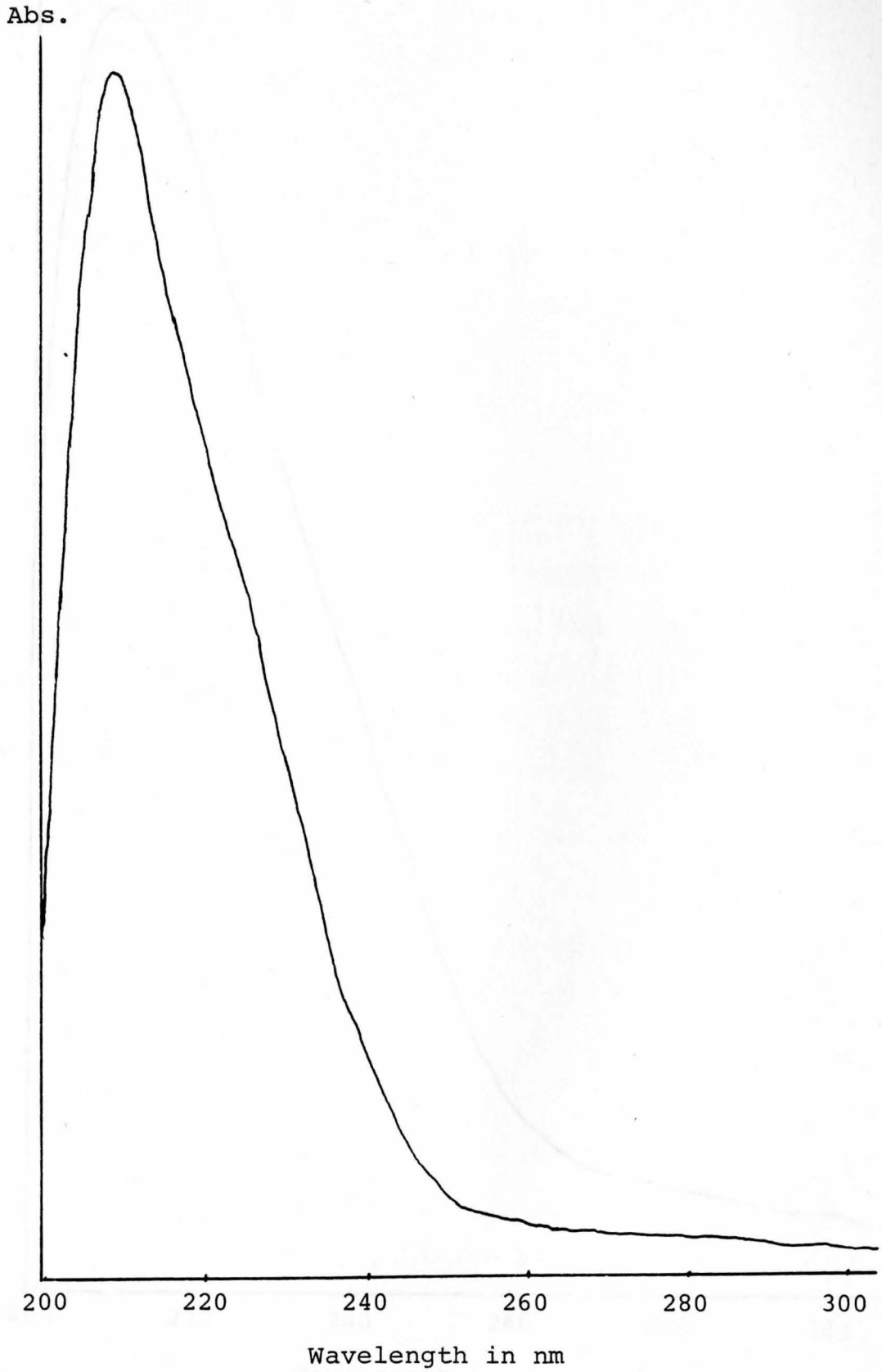


Figure 18-Absorption Spectrum for Bilirubin in 70% MeOH

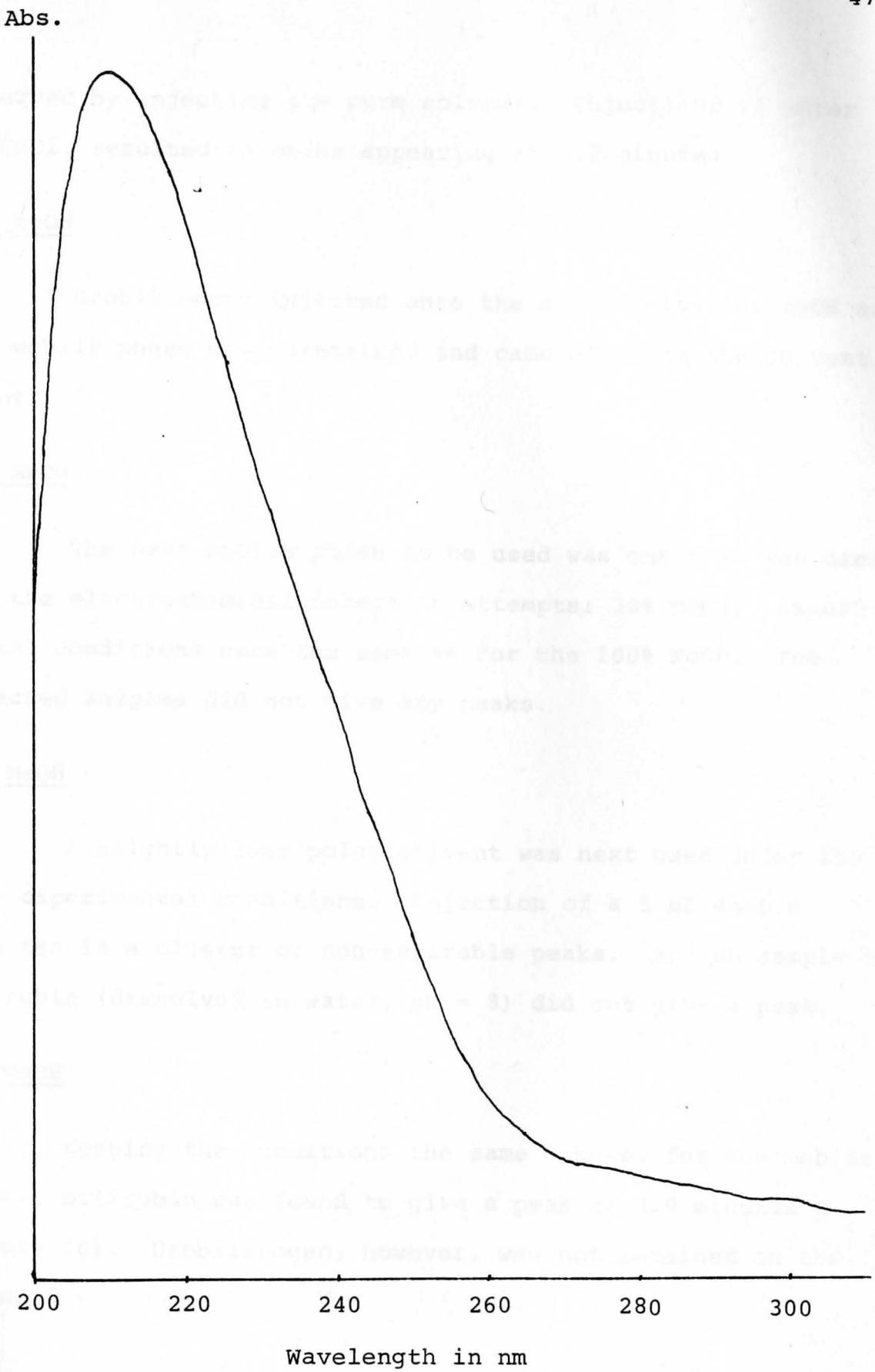


Figure 19-Absorption Spectrum of Urobilinogen in 70% MeOH

observed by injecting the pure solvent. Injections of water or $CHCl_3$ resulted in peaks appearing at 1.2 minutes.

70% MeOH

Urobilinogen injected onto the column with 70% MeOH as the mobile phase was unretained and came off with the solvent front.

30% MeOH

The next mobile phase to be used was one that was used for the electrochemical detection attempts: 30% MeOH. Experimental conditions were the same as for the 100% MeOH. The injected samples did not give any peaks.

40% MeOH

A slightly less polar solvent was next used under the same experimental conditions. Injection of a 5 μL sample resulted in a cluster of non-separable peaks. A 5 μL sample of bilirubin (dissolved in water, pH = 8) did not give a peak.

65% MeOH

Keeping the conditions the same (except for the mobile phase), bilirubin was found to give a peak at 3.9 minutes (Figure 20). Urobilinogen, however, was not retained on the column.

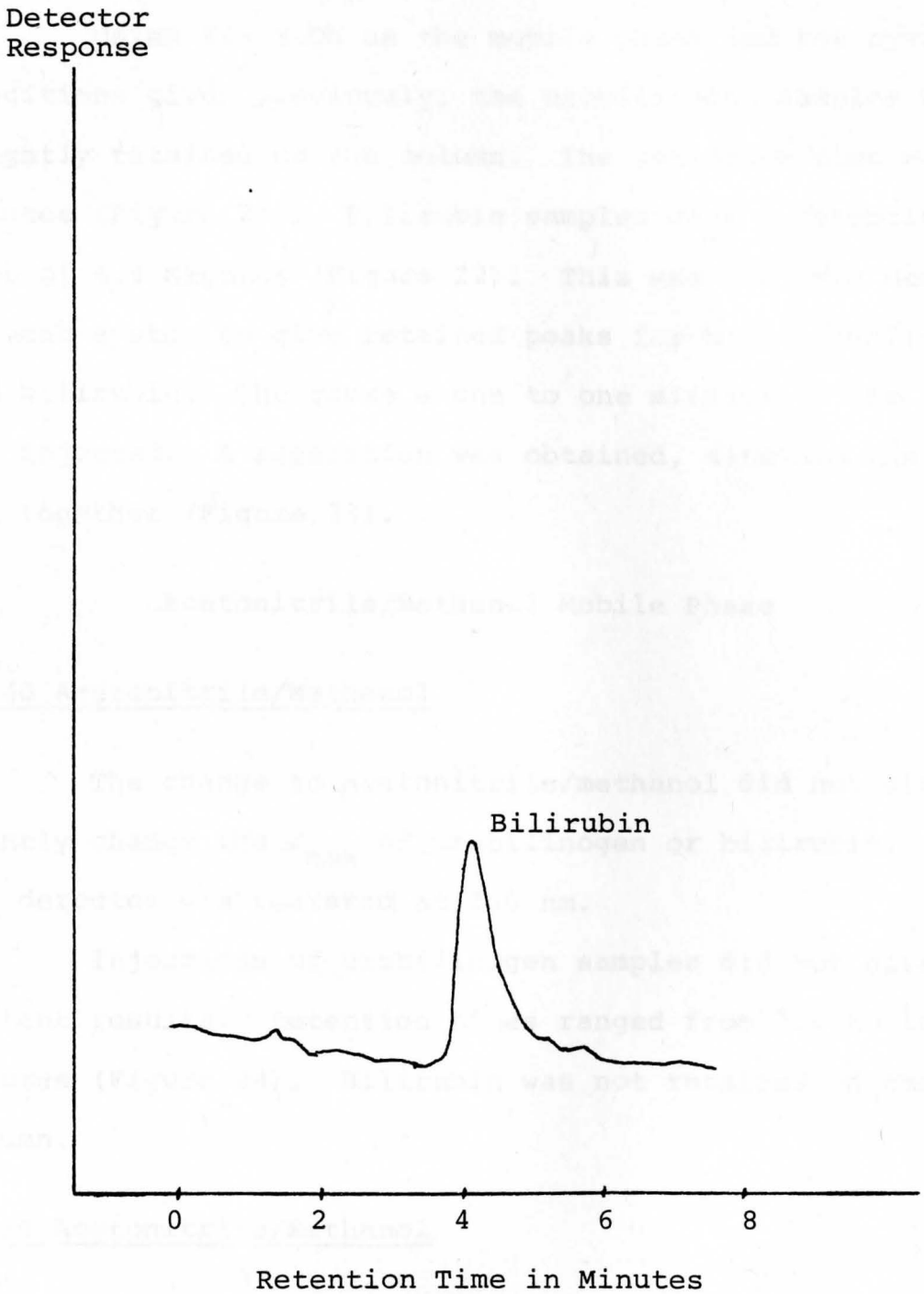


Figure 20-Chromatograph of Bilirubin in 65% MeOH

50% MeOH

Using 50% MeOH as the mobile phase and the system conditions given previously, the urobilinogen samples were slightly retained on the column. The retention time was 2.6 minutes (Figure 21). Bilirubin samples gave a retention time of 4.6 minutes (Figure 22). This was the only MeOH/H_2O solvent system to give retained peaks for both urobilinogen and bilirubin. Therefore a one to one mixture of the two was injected. A separation was obtained, although the peaks ran together (Figure 23).

Acetonitrile/Methanol Mobile Phase

60:40 Acetonitrile/Methanol

The change to acetonitrile/methanol did not significantly change the λ_{max} of urobilinogen or bilirubin. Thus the detector was operated at 220 nm.

Injections of urobilinogen samples did not give consistent results. Retention times ranged from 7.6 to 10.4 minutes (Figure 24). Bilirubin was not retained on the column.

70:30 Acetonitrile/Methanol

Injections of urobilinogen gave a retention time of 10.6 minutes (Figure 25). Bilirubin was not retained on the column.

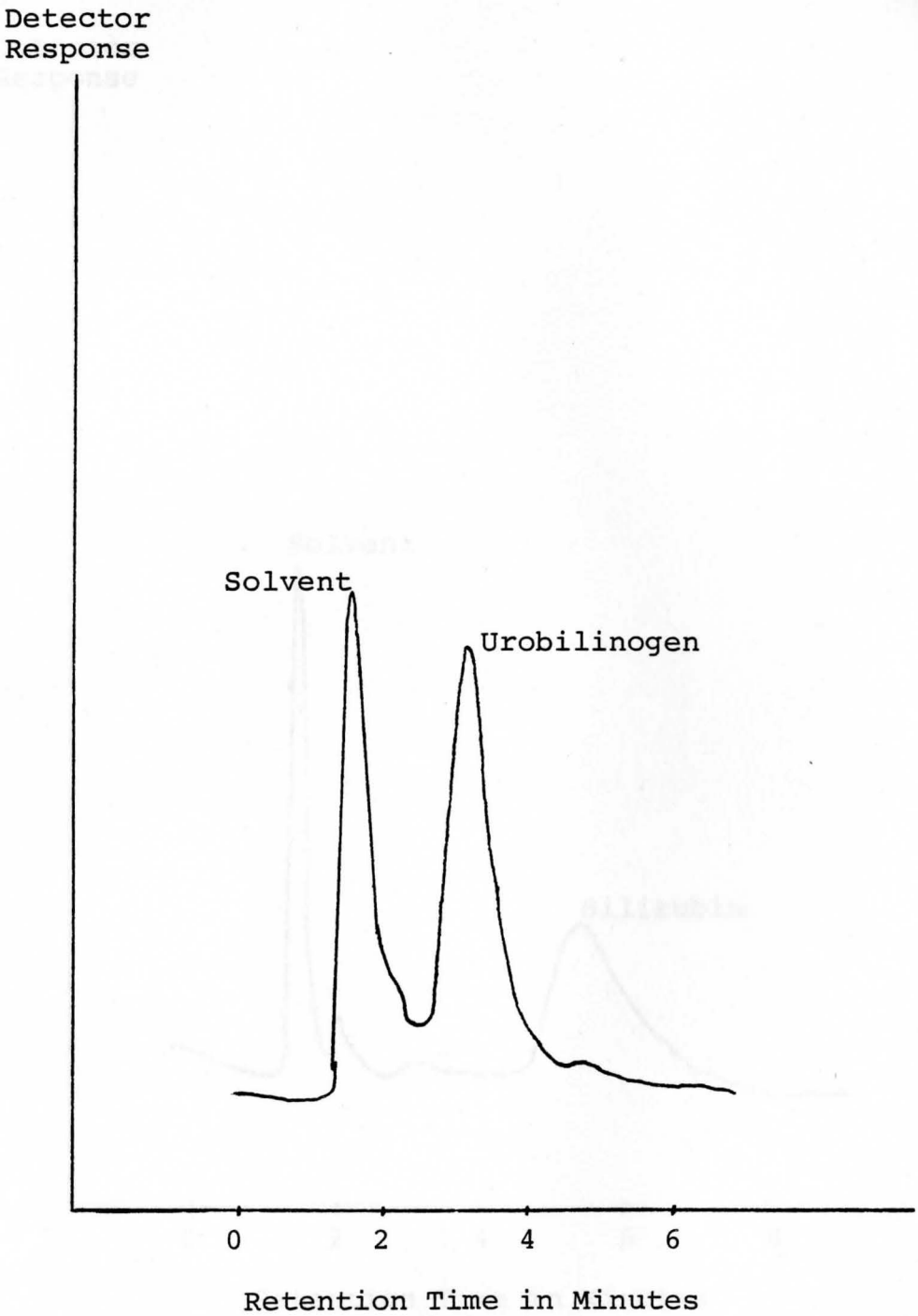


Figure 21-Chromatograph of Urobilinogen in 50% MeOH

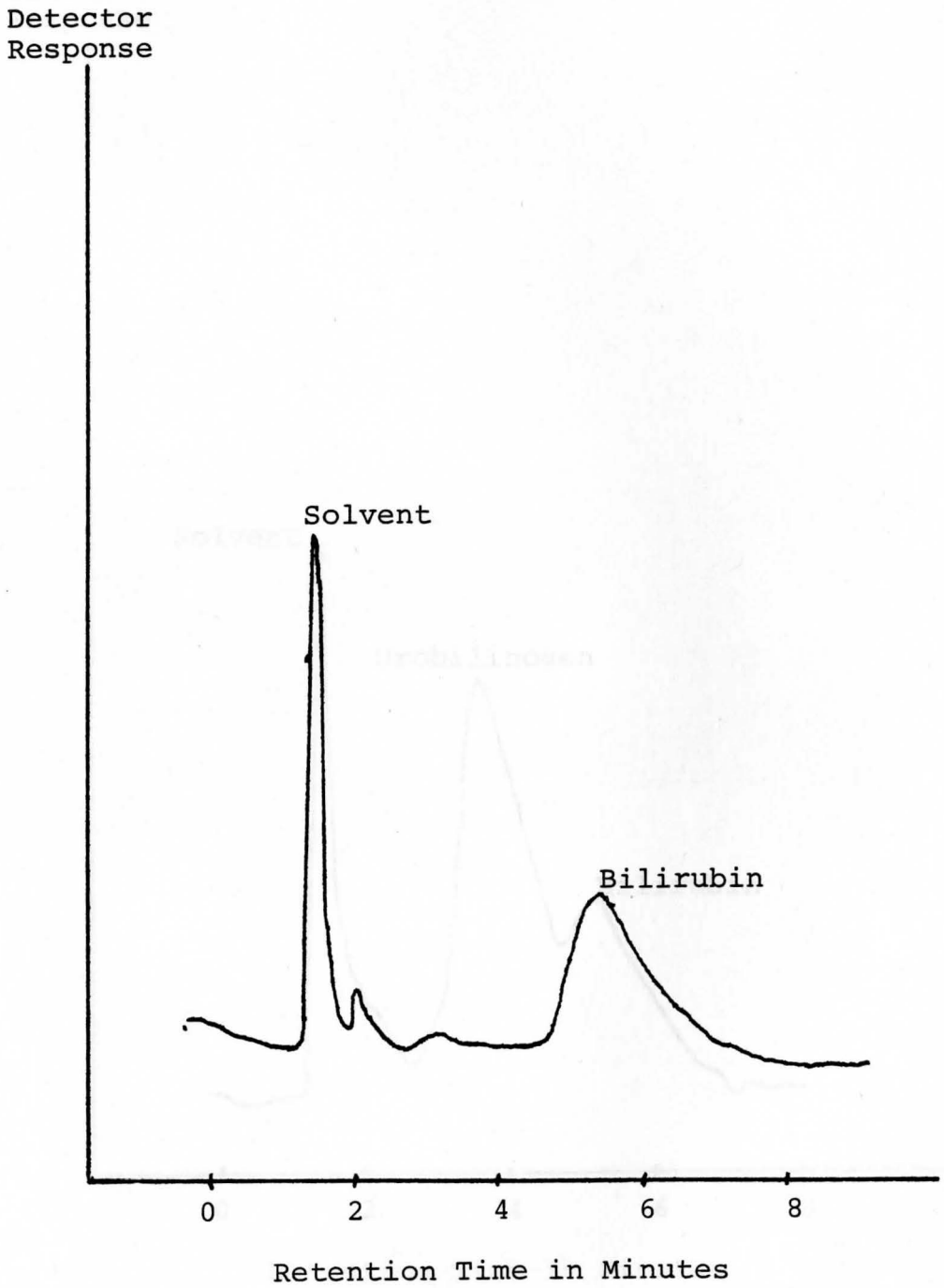


Figure 22-Chromatograph of Bilirubin in 50% MeOH

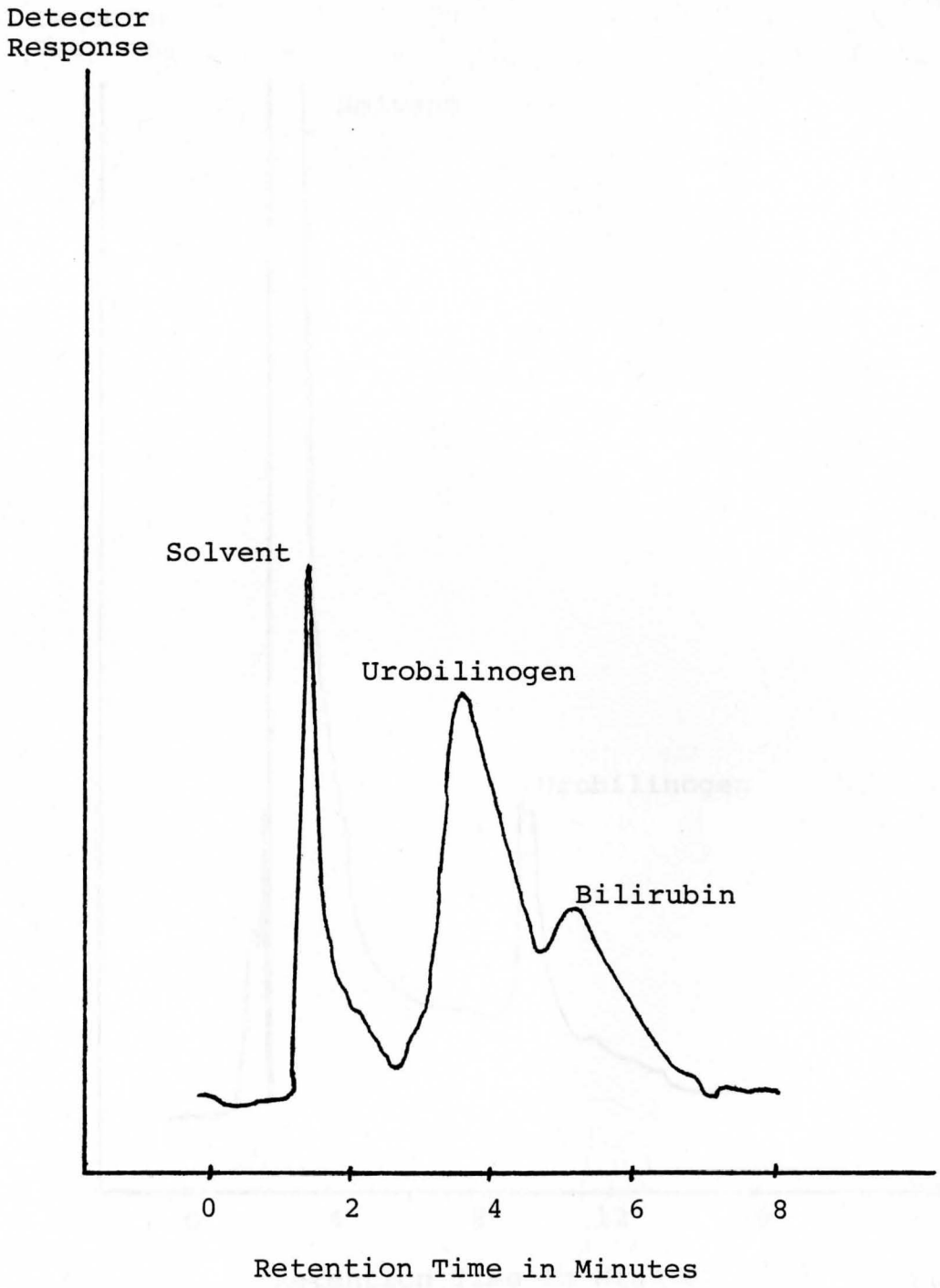


Figure 23-Chromatograph of Mixture in 50% MeOH

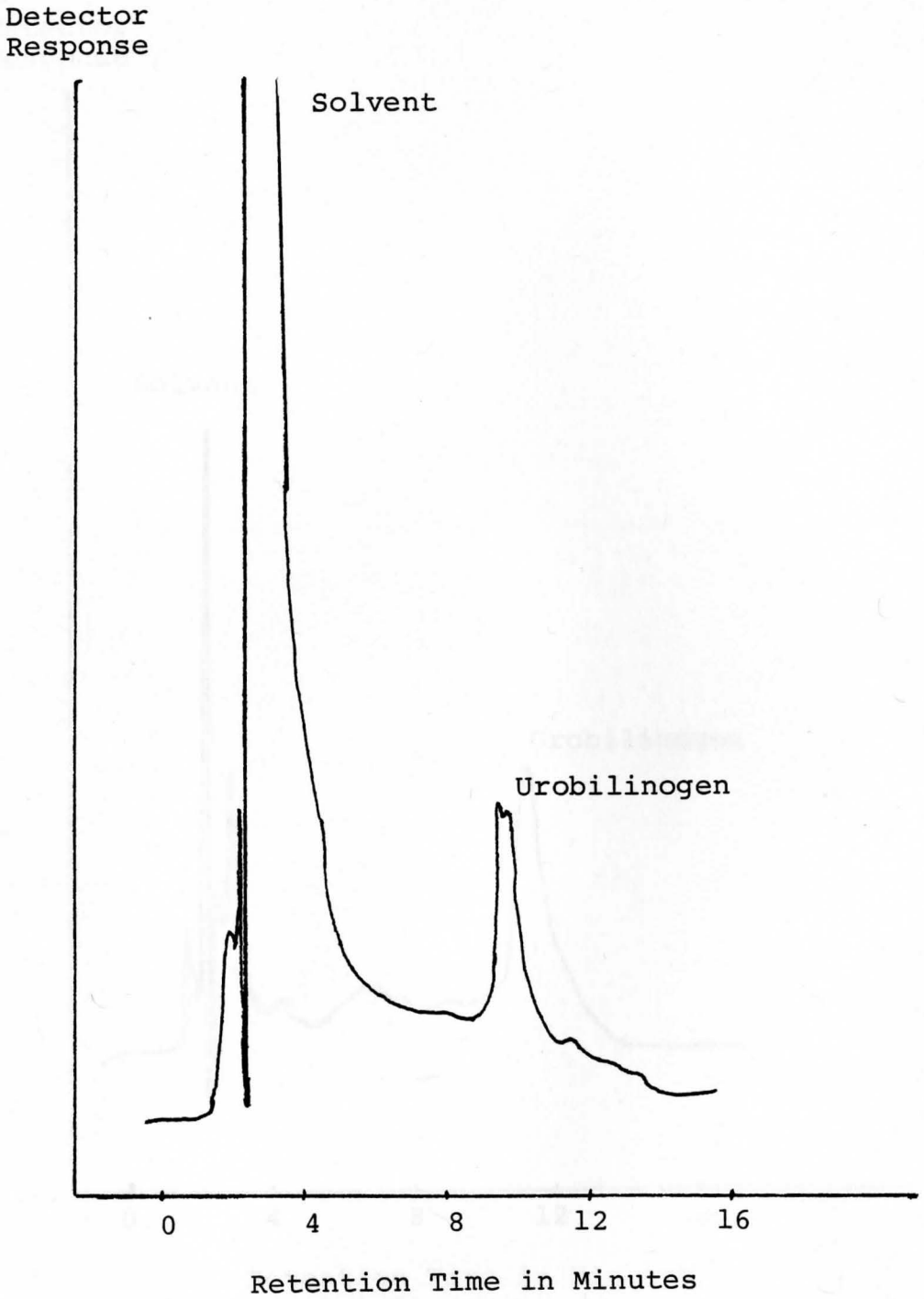


Figure 24-Chromatograph of Urobilinogen
in 60:40 Acetonitrile/MeOH

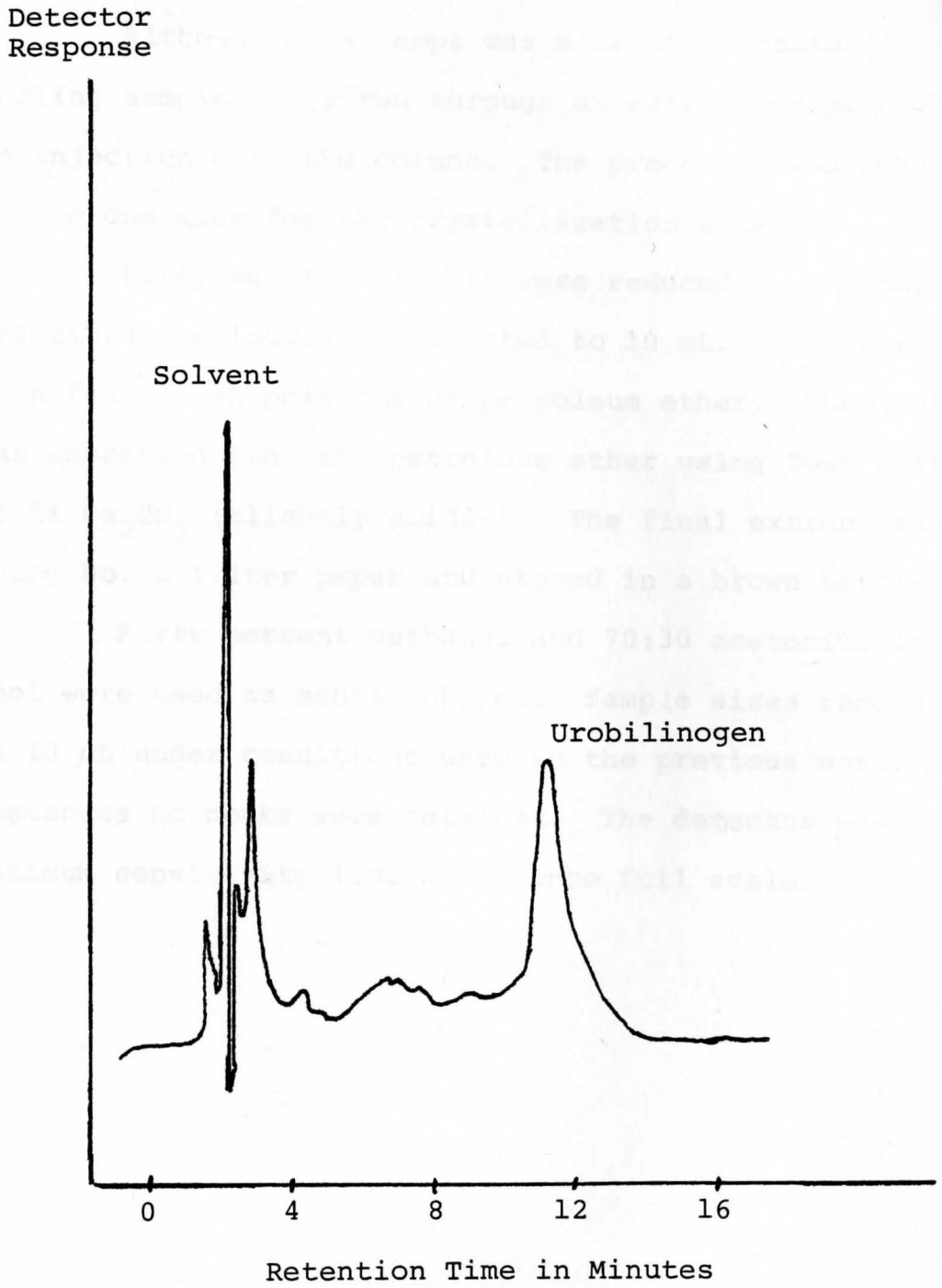


Figure 25-Chromatograph of Urobilinogen in 70:30 Acetonitrile/MeOH

Sample Extraction Procedure

Although no attempt was made at quantitation, the working samples were run through an extraction procedure prior to injection onto the column. The procedure was a modification of the one used for the crystallization attempts.

Fifty mg of bilirubin were reduced in the manner described previously and diluted to 10 mL. This was washed with four 25 mL portions of petroleum ether. The urobilinogen was extracted from the petroleum ether using four 5 mL portions of 5% Na_2CO_3 (slightly acidic). The final extract was filtered using No. 2 filter paper and stored in a brown bottle.

Fifty percent methanol and 70:30 acetonitrile/methanol were used as mobile phases. Sample sizes ranged from 5 to 10 μL under conditions used in the previous work. In all instances no peaks were obtained. The detector was used at maximum sensitivity (.01 absorbance full scale).

CHAPTER VI

DISCUSSION OF RESULTS AND CONCLUSIONS

Attempts to Synthesize the Urobilinoids

Attempts were made to obtain urobilinogen and urobilin crystals. An extraction from a reduced bilirubin solution (using sodium amalgam as the reducing agent) was used to isolate urobilinogen. Subsequent crystallization attempts were unsuccessful. Crystallization of the oxidized urobilinogen extract (urobilin) was also unsuccessful.

Electrochemical Studies

Bilirubin Reduction

Bilirubin was reduced using a H.M.D.E. at -1.60 v. Crystallization attempts of the reduction product were unsuccessful.

Cyclic Voltammograms

Cyclic voltammograms were obtained using a platinum electrode and also the carbon paste electrode of the TL-3 electrochemical detector (Figures 14, 15, 16 and 17). Freshly prepared urobilinogen solutions were used along with 20% MeOH in .1 M KCl. The methanol solution was found to be suitable for a mobile phase since it produced no oxidation wave. Urobilinogen was found to be compatible with electrochemical detection, giving an oxidation peak from .4 to 1.0 v.

for both electrode systems.

High Performance Liquid Chromatography with Electrochemical Detection (HPLCED)

Using a working electrode potential of .90 v., HPLCED was attempted using fresh urobilinogen samples. Mobile phases consisted of 20% and 30% methanol with .1 M KCl as the supporting electrolyte. Injections onto the column did not result in any peaks. Mobile phases of lower polarity using higher proportions of organic solvents were not possible due to detector limitations. The plasticizer contained in the plexiglass components of the detector would be extracted from the plastic by high concentrations (over 30%) of organic solvents. This would result in the destruction of the plastic.

High Performance Liquid Chromatography with UV Detection

Absorption Spectra

Ultraviolet spectra were obtained for bilirubin and urobilinogen in 70% methanol (Figures 18 and 19). The wavelengths of maximum absorbance were 209 nm and 210 nm respectively.

Methanol/Water Mobile Phases

For all of the following systems, the detector was set at 220 nm to give a more stable baseline.

30% Methanol

Injection of urobilinogen samples did not give any discernable peaks.

100% Methanol

To decrease the polarity of the mobile phase, pure methanol was used. Samples were unretained on the column, coming off with the solvent front.

70% Methanol

Samples were not retained on the column.

65% Methanol

Urobilinogen was not retained by the column. Bilirubin, however, gave a peak at 3.9 minutes (Figure 20).

50% Methanol

This was the first system used in which both samples were retained. Urobilinogen had a retention time of 2.6 minutes and bilirubin was detected at 4.6 minutes (Figures 21 and 22). A mixture of the two samples was injected giving a separation (Figure 23). Retention times were not long enough to allow for good separations.

Acetonitrile/Methanol Mobile Phases

60:40 Acetonitrile/Methanol

Bilirubin was not able to be detected with this system. Injection of urobilinogen samples gave inconsistent results, with retention times ranging from 7.6 to 10.4 minutes (Figure 24).

70:30 Acetonitrile/Methanol

This mobile phase also did not allow detection of bilirubin. Urobilinogen, however, gave a peak at 10.4 minutes (Figure 25).

Sample Extraction Attempt

Attempts were made at using an extraction procedure (involving petroleum ether and 5% Na_2CO_3) for the urobilinogen solution. Fifty percent methanol and 70:30 acetonitrile/methanol were both used as mobile phases. Nothing was detected when the samples were injected.

Conclusions

In this study, detection of the urobilinoids by HPLC was investigated. This was the first such study in this area, therefore preliminary work had to be done. This included unsuccessful attempts to crystallize urobilinogen and urobilin to provide a convenient sample supply. The failure to produce crystals may be due in part to the small amounts of material and the low yields (20 to 40% reported by Fisher[6]) involved in the reactions. It was not certain whether the inability to obtain crystals was due to a lack (or small amount) of the desired product in the final extract or inefficiency in the crystallization method.

Electrochemical studies were undertaken in the form of cyclic voltammograms. The data obtained from these indicated that HPLC with electrochemical detection would be

possible with a working electrode potential of +.90 v.

A reversed phase column was used in conjunction with the HPLCED. Due to limitations on the mobile phase able to be used, detection of urobilinogen was not possible with the available detector. Use of a detector with no solvent limitations (such detectors are available) would offer experimental conditions that were not possible in this work (i.e. the polarity range of the solvent would be much greater, although still limited by the solubility of the supporting electrolyte).

Use of a UV variable wavelength detector offered more flexibility in the conditions. Several methanol/water mobile phases were used, with the 50% methanol system giving the best results. Although a mixture of urobilinogen and bilirubin was able to be separated, the short retention time of the urobilinogen (2.6 minutes) would not be conductive to good separation from other interfering substances.

A 70:30 acetonitrile/methanol mobile phase offered a more acceptable retention time for urobilinogen at 10.4 minutes. Although the interference of bilirubin was overcome since it was not detected, a chromatographic separation would have been more desirable. Detection and separation of bilirubin would offer the possibility of its use as an external standard.

The concentrations of the samples used were much higher than would be found in urine. Samples injected into the HPLC had an approximate concentration of 5 to 10 mg/mL

compared to normal urine values of 0.5 to 4 mg per total urine volume for one day. After running the working samples through an extraction procedure, urobilinogen could not be detected on the HPLC. This indicated the possibility that UV detection was not sensitive enough.

Use of other column packings not available during this study would be worthy of future study. More aqueous mobile phases would be permitted, allowing better conditions for electrochemical detection, which in turn offers maximum sensitivity.

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