ELECTROCHEMICAL PRECOLUMN CONDITIONING OF BILIRUBIN FOR THE INVESTIGATION OF UROBILINOGEN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

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ABSTRACT

ELECTROCHEMICAL PRECOLUMN CONDITIONING
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The utilization of precolumn electrochemical cells in High Performance Liquid Chromatography (HPLC) can be used to generate compounds that are difficult to obtain by more conventional means. The application of this principle during a recent study led to the design of an electrosynthesis cell capable of reducing bilirubin to urobilinogen.

Urobilinogen is a highly labile organic compound with clinical significance. Because of its instability no workable standard exists. This study demonstrated the ability of a flow-through electrosynthesis cell to produce quantities of urobilinogen sufficient for analysis.

As the electrosynthesis cell did not provide 100 % conversion of bilirubin to urobilinogen in a flowing system, it was necessary to develop a method of separating and detecting both species.

Experimental results revealed three possible methods of detection (electrochemical detection, fluorometry, and UV spectrometry) for urobilinogen, but only one for bilirubin (UV spectrometry). Therefore, UV spectrometry was employed as the detector system throughout the remainder of the project.

Reverse phase HPLC was used with several different mobile phases in an attempt to separate the two species. Systems explored

included various ratios of methanol/water and acetonitrile/DMSO/ water. A separation of bilirubin and urobilinogen was obtained with 50 % methanol and water at a flow rate of 1.0 mL/minute. However, this separation was not sufficient for a quantitative measurement. No separation was obtained with the acetonitrile/DMSO/water mobile phases.

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Finally, I wish to thank my parents for their understanding and constant support during this time and always.

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LIST OF SYMBOLS

SYMBOL	DEFINITION
ma	milliamp
µl	microliter
mL	milliliter
g	gram
mg	milligram
nm	nanometer
v	volt
HPLC	High Performance Liquid Chromatography
cm	centimeter
M	molar
µA	microamp
d	dextrorotatory
l	levarorotatory

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CHAPTER 1

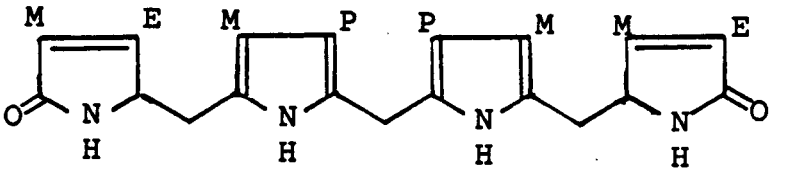
Introduction

The Nature of Urobilinogen

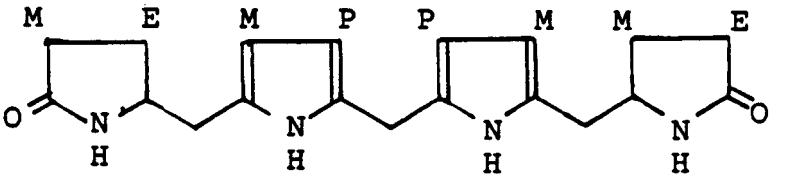
Urobilinogen is a term given to three different chemical species. While these species are not isomers, they are grouped together because of their common origin, similar structure, and almost identical physical properties. The three forms are Mesobilirubinogen, l-Stercobilinogen, and d-Urobilinogen.[1]

The structures of the urobilinogen components are given in Figure 1.[2] From this it can be seen that the structures differ only in their degree of saturation and the positioning of substituent groups on the rings. Each consists of 4 pyrrole rings joined by methene bridges. The various substituent groups are comprised of vinyls, methyls, ethyls, oxygen, and propanoic acid.

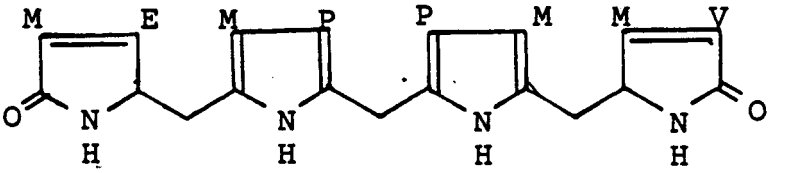
The urobilinogen components are reduction products of bilirubin. The bilirubin itself is produced by the normal metabolism of red blood cells, and like urobilinogen has three different forms. These are bilirubin, dihydrobilirubin and mesobilirubin. In the body, bilirubin is reduced in the intestines by bacteria to d-urobilinogen, which is further reduced to mesobilirubinogen. This can, in turn, be reduced to l-stercobilinogen. Most of this urobilinogen is reabsorbed by the circulatory system or removed from the body in feces. A trace amount (0.5 mg to 4.0 mg/24 hr) is excreted in urine. The relationship between the three forms of bilirubin and the three forms of urobilinogen are shown in Figure 2.



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

The three forms of urobilinogen are generally referred to collectively and shall be for the remainder of this work.

Reactions of Urobil

Urobilinogen is an extremely labile compound that undergoes spontaneous oxidation to urobilin when exposed to light and air. The oxidation reaction involves the loss of two hydrogen atoms from urobilinogen. This conversion is easily detected by the change from colorless urobilinogen to the intensely yellow urobilin. The oxidation reaction is given in Figure 2.[3]

The other reaction of major importance is that of urobilinogen with Ehrlich's reagent (p-dimethylaminobenzaldhyde). This reaction is of great importance in several analytical procedures that will be discussed later. Although it is used in both qualitative and semi-qualitative methods, the exact reaction product is as yet unknown. Ehrlich's reagent reacts readily with urobilinogen to give an intense red color. A probable reaction sequence is shown in Figure 3.[4]

Present Methods of Analysis

At the present time no reliable quantitative method of urobilinogen analysis exists. There are, however, qualitative and semi-qualitative procedures involving the extraction of urobilinogen from urine with petroleum ether. The ether extracts are then reacted with Ehrlich's reagent and the absorbance determined at 562 nm. The concentration is then determined by using a standard curve which is prepared from phenolsulfonephthalein dye. There are three basic reasons why it has been difficult to obtain quantitative results from this procedure, the primary reason being incomplete removal of

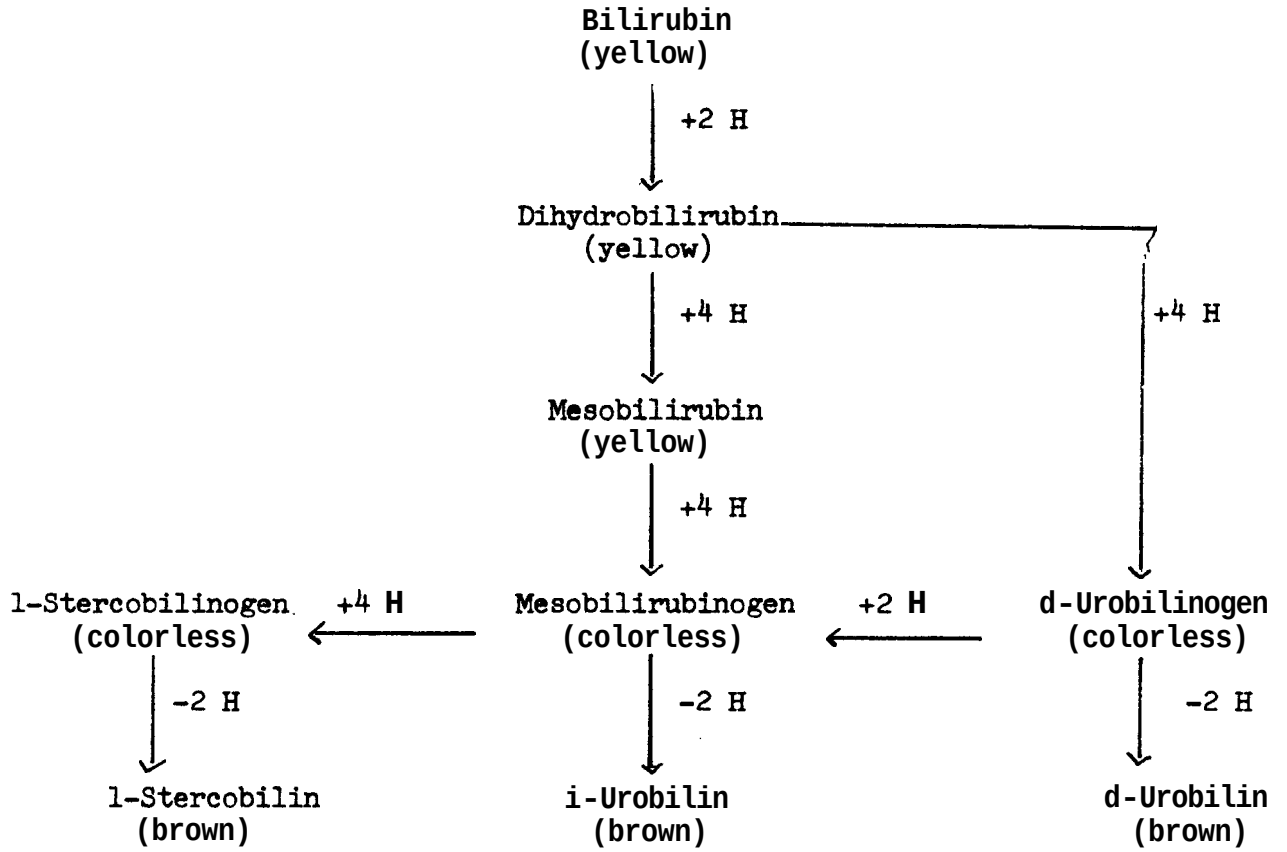


Figure 2-Bilirubin Metabolism

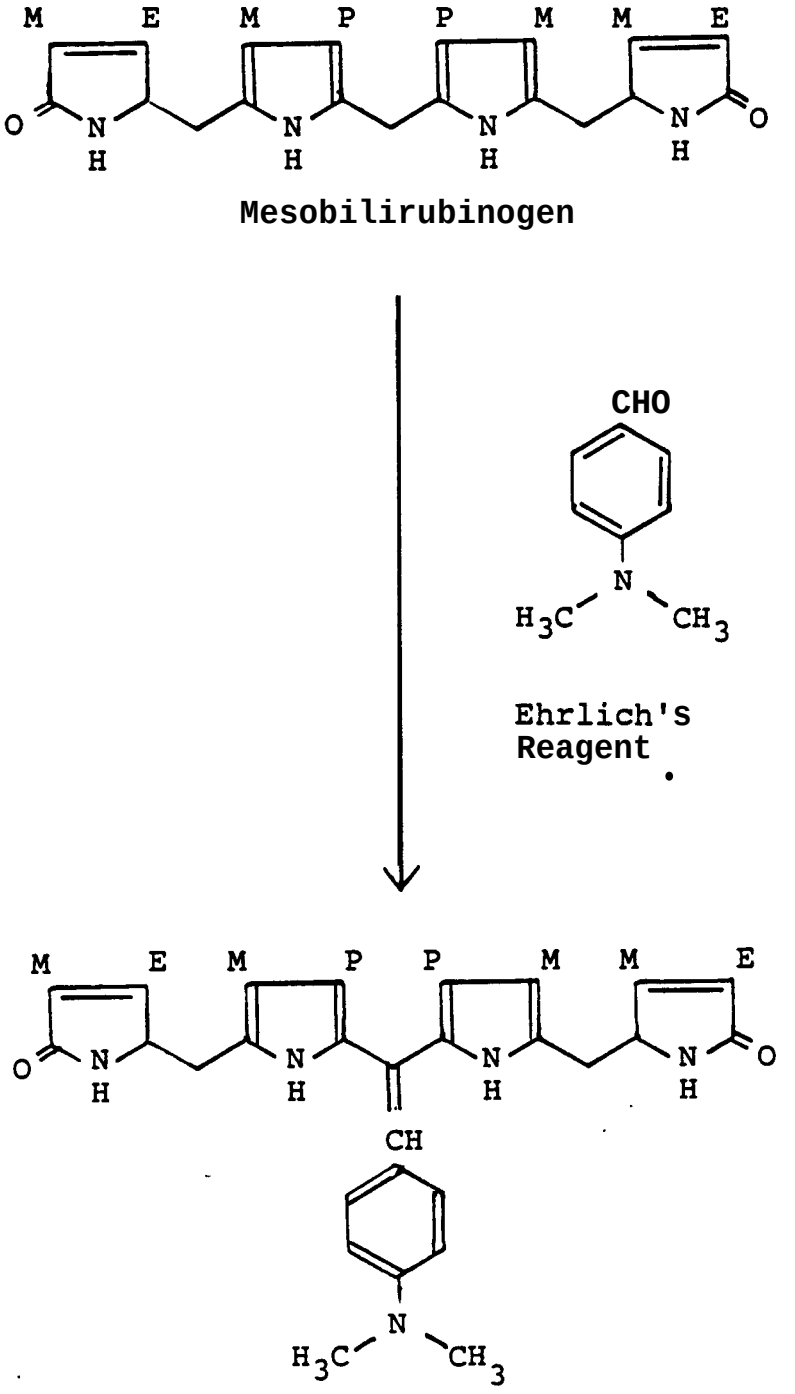


Figure 3-Reaction of Urobilinogen With Ehrlich's Reagent

interfering substances during the extraction. Secondly, there is an appreciable loss of urobilinogen due to incomplete removal by the organic phase. Third, the standard curve used to determine concentration is not prepared from a true urobilinogen standard, but is estimated using the phenolsulfonephthalein dye.

Another method that is employed primarily as a qualitative test, but can also be used for semi-quantitative measurement, is the reagent strip (dipstick). In this technique the commercially available reagent strip is "dipped" into the sample to be tested. The presence of urobilinogen is then determined by the appearance of the appropriate color.

A recently-introduced reagent strip allows the semi-quantitative measurement of urobilinogen. This procedure makes use of an instrument (Ames "Cliniter") to read the reagent strips, thereby eliminating variations among technicians in interpreting results. The instrument measures the light that is reflected from the reagent strip. Since the color of the strip becomes darker as the concentration of urobilinogen increases, the amount of light reflected is inversely proportional to the concentration. Although this method is an improvement over previous methods, it has not, to this date, become a quantitative measurement due to the presence of interfering substances.

Clinical Significance

An increase in the concentration of urobilinogen in urine is often indicative of a liver disorder such as hepatitis. In a non-diseased state the liver serves to remove urobilinogen from the bloodstream. However, if a large number of liver cells are damaged, the urobilinogen will pass to the kidneys to be subsequently removed and excreted in

the urine. The urobilinogen level in urine can then be monitored to evaluate the effectiveness of a particular drug in the treatment of a liver disorder.

While an increase of urobilinogen in urine often points to liver or hemolytic diseases, a decrease in urobilinogen can also be significant. Anemias not caused by red blood cell destruction, as well as obstructive jaundice and antibiotic drug treatment can cause a decrease in urine urobilinogen.

It is obvious that urobilinogen testing is of great importance, both in the initial detection and confirmation of a specific disease state and in the monitoring of a patient's response to treatment.

Statement of the Problem

The analysis of urobilinogen in the clinical laboratory suffers from two basic problems, the first being the presence of interfering substances. Since the reagents used in detection (primarily Ehrlich's reagent) have poor selectivity, a great number of competing reactions can take place. This, coupled with the fact that urobilinogen concentrations are low, allows the presence of interfering substances to significantly alter results. The second main problem is the lack of a urobilinogen standard. Without such a standard it is virtually impossible to quantify chemical or spectrometric methods of analysis.

This project addresses these two main obstacles separately. First, the presence of interfering substances was dealt with by the use of High Performance Liquid Chromatography (HPLC). This allows the separation of the analyte from materials of no interest. Second, since no commercially produced standard is available and no dependable

method of synthesis exists, an attempt was made to produce urobilinogen electrochemically. This process involves the reduction of bilirubin, a stable precursor of urobilinogen. Thus, an electrochemical cell capable of reducing bilirubin to urobilinogen in a flowing system was designed. Since urobilinogen readily oxidizes to urobilin, the cell was designed to function at the precolumn side of the HPLC, thereby ensuring no delay between synthesis and analysis of the urobilinogen.

CHAPTER II

Historical Review

Discovery and Isolation of Urobilinogen

In 1871 Maly accomplished the reduction of bilirubin using sodium amalgam.[5] The reduction product was referred to as hydrobilirubin. As this reaction took place without the benefit of a dry nitrogen atmosphere, the product was probably a mixture of various reduction products of bilirubin including urobilinogen.

Urobilinogen was first isolated in 1911 by Fischer.[6] In his study the sodium amalgam reduction of bilirubin was coupled with several extraction steps, which were carried out primarly with diethyl ether and chloroform. The product of this first synthesis was what is now called mesobilirubinogen.

The literature citations on the early work involving urobilinogen suffer from contradictory nomenclature. Originally, the term urobilinogen referred to any pigment in urine that reacted with Ehrlich's reagent to give an absorbance at 490 nm. In other cases, urobilinogen was used to refer to all of the urobilinoids (urobilinogen and urobilin). Much of the experimental work conducted on "urobilinogen" was actually work conducted on mixtures of urobilinogen and other urobilinoids or on different species altogether. For this reason, extreme caution must be exercised when examining these studies.

t l of Anal

The first test for urobilinogen was developed in 1901 by Ehrlich.[7] While working with p-dimethylaminobenzaldhyde (Ehrlich's reagent)

in concentrated HCl, he noticed that an intense red color developed with certain urines. Ehrlich correctly attributed this color to the reaction of Ehrlich's reagent with urobilinogen. It was later discovered that other substances in urine (indole and skatole derivates) gave identical results.

Several improvements were added to the procedure by Terwen in 1925.[8] By replacing concentrated HCl with sodium acetate, the reaction with Ehrlich's reagent was found to be more specific. Sodium acetate intensified the reaction product of Ehrlich's reagent and urobilinogen (urobilinogenaldhyde) while decreasing the reactions between Ehrlich's reagent and the derivatives of skatole and indole. Another improvement was the use of ferrous hydroxide as a reducing agent in the urine samples. This prevented the oxidation of urobilinogen during the analysis procedure.

In 1935 another improvement came when Watson replaced diethyl ether with petroleum ether in the extraction step.[9] This allows for a more complete removal of urobilinogen with a lesser extraction of interfering substances.

Although there have been several improvements, the analysis of urobilinogen with Ehrlich's reagent still has many problems.[10] The major problem stems from the reaction of interfering substances with Ehrlich's reagent. Other problems include lack of reproducibility at low urobilinogen concentrations and incomplete extration of urobilinogen from urine.

To overcome the problem of interfering substances, p-methoxybenzene was substituted for Ehrlich's reagent,[11] and the complex which forms absorbs at 505 nm. Several substances that interfere with Ehrlich's

reagent were found not to interfere with p-methoxybenzene.

Although the interference problem is reduced with p-methoxybenzene, other complications that occur with the conventional method still exist. The complexity and unpredicatabilty of both of these methods have prevented their widespread use in the clinical laboratory.[12]

Dipstick Method of Analysis

The dipstick method makes use of the reaction of Ehrlich's reagent with urobilinogen. The dipstick is impregnated with p-dimethylaminobenzaldhyde and an acid buffer. Ascorbic acid is added to the sample as a reducing agent to stabilize the urobilinogen-aldhyde complex. A sodium acetate solution is also added to increase the pH and minimize the production of a colored product with indole and skatole. The color of the strip is then measured spectrometrically or visually by comparing it with a standard phenolsulfonephthalein (PSP) solution. The method is semi-quantitative, since pure urobilinogen cannot be obtained as a standard.

CHAPTER III

Methodology

Procedure

Cyclic voltammetry, electrochemical synthesis, electrochemical detection, fluorometry, UV spectrometry, and High Performance Liquid Chromatography (HPLC) were employed during the course of this work. Although all methods were not employed in the final phase of the project, they did serve to characterize and identify the chemical species under study. Therefore, these various procedures will be discussed.

Cyclic Voltammetry

Cyclic voltammetry is a versatile electroanalytical technique that is used as a means of studying redox states. This technique allows a wide potential range to be scanned for reducible or oxidizable species. The electrode potential at which a species undergoes reduction or oxidation can be rapidly and precisely located using cyclic voltammetry.[13] This is often the first method used in any project involving electrochemical detection or synthesis.

Cyclic voltammetry consists of cycling the potential of an electrode, suspended in an unstirred solution, and measuring the resulting current. The potential at this electrode (working) is controlled relative to a reference electrode (such as a silver-silver chloride electrode), and can be considered the potential excitation signal.[14] The excitation signal first scans the potential in one

direction (negative or positive) until it reaches a preselected value. At this point, the scan direction is reversed and the scan then returns to the original potential. This is a complete cycle, but single or multiple cycles can be used. The potential range that is scanned, as well as the scan rate itself can easily be varied to accommodate different chemical systems.

The information gathered by cyclic voltammetry is represented in the form of a cyclic voltammogram. The resulting voltammogram is analogous to the absorption spectrum for UV spectrometry in that it conveys information as a function of an energy scan. A cyclic voltammogram is obtained by measuring the flow of current between the working and auxiliary electrodes as a function of the applied voltage. When a chemical species present in the solution undergoes oxidation or reduction, there is an increase in the flow of current. Thus, the potential (excitation signal) at which a reduction or oxidation occurs can be identified by an increase in current (response signal).

Electrochemical Synthesis

Electrochemical synthesis is the latest application of controlled potential electrolysis. Although electrolysis has been used extensively for more than a century in the synthesis of metals from their salts, application to other areas of chemical synthesis have been extremely limited. It should be noted that until recently the fine control of the potential needed to obtain the desired product was not possible. In classical electrolysis the only control available was provided by appropriate selection of electrode material, solution acidity, and supporting electrolyte.[15] Today, the use of modern potentiostats and related instruments allow the effective control of the potential at the

working electrode. This new technology makes possible the use of a wide range of solvent systems and supporting electrolytes. By optimizing the selection of supporting electrolyte, pH and electrode material, electrochemical synthesis (or electrosynthesis) can provide a relatively pure product.

A more recent development in the field of electrosynthesis is the advent of the flow-through electrochemical cell. The flow electrolysis cell is used to apply a controlled potential to a continuously flowing solution, resulting in high efficiencies and rapid electrolysis.[16] The benefits of such a cell are maximized when it is coupled with other instrumentation, such as HPLC.

The design of the cell must take into account several factors. Since the working electrode is the basic variable that controls the degree of completion of a electrolytic process, selection of a working electrode material is critical. The electrode selected must provide a uniform charge density over a large surface area and the desired potential must be within its potential window. Other requirements include a stable reference electrode, carefully placed to minimize uncompensated resistance effects. The auxiliary electrode must also be carefully situated to provide uniform current distribution across the working electrode. This is usually accomplished by placing the auxiliary electrode in a compartment isolated from the working electrode by an ion-porous membrane.

The adaptation of a flow-through electrochemical cell to the pre-column of HPLC provides the ability to study compounds difficult to obtain by more conventional means. In many cases commercially prepared samples of unstable species are not available. Therefore,

all samples must be prepared, when needed, by the reduction or oxidation of a stable precursor. The electrosynthesis of these species by a flow-through cell provides a superior pathway by offering samples which are relatively pure and easily synthesized.

High Performance Liquid Chromatography

High Performance Liquid Chromatography accomplishes the separation of solutes in a sample by passing it through a chromatographic column. HPLC was developed from classical liquid chromatography as the available technology grew in sophistication. In the original liquid chromatography experiment, the mobile phase was percolated through the packing by gravity alone. Flow rates were, at best, a few tenths of a milliliter per minute; this made separations extremely time consuming.[17] As technology progressed pumps were added and particle size decreased, giving better and faster separations. Needless to say, the instrumentation involved is much more complex that the simple devices of original liquid chromatography.

A basic HPLC system consists of a pump, injector, column and detector. The pump supplies a constant flow of solvent (mobile phase) at pressures up to 6000 psi. In order to obtain reproducible results the pump must maintain the flow within $\pm$ 2 %. The injector is designed to introduce a sample of constant volume into the flowing solvent system without interrupting the flow rate. The column is by far the most important part of any HPLC system. By choosing the correct type of column packing material (ion exchange, reverse phase, etc.) separations can be made on the basis of different charges, shapes, or molecular weights depending on the constituents to be separated. The detector

can also be tailor-made for the species under study. Some common detectors include UV/Vis spectrophotometers, fluorometers, mass spectrometers, and electrochemical cells.[18]

Another main feature of HPLC is the mobile phase. The composition of the mobile phase, rather than that of the column, can be varied to meet specific experimental situations. Depending on the type of column employed, changing the polarity, ionic strength, or viscosity of the mobile phase can have a dramatic effect on the retention time and the separation of solutes.

Reverse Phase *HPLC*

Reverse phase *HPLC* is most often employed in situations involving organic molecules. A reverse phase *HPLC* techniques involves the use of a non-polar stationary phase with a polar mobile phase. This has the effect of allowing polar molecules to pass through the column quickly since they are not attracted to the stationary phase. The less polar organic species are retained on the column longer since they are attracted to the stationary phase.

The most common type of reverse phase column is composed of long chain hydrocarbons bonded to silica. An example is shown in Figure 4.[19]

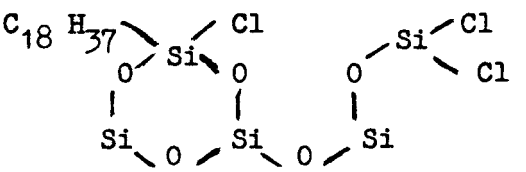


Figure 4-Reverse Octadecylsilica Phase Packing Material

The hydrocarbons vary in length from C_4 to C_{18}, though longer chain molecules tend to give better separation with longer retention times. The C_{18} column is used extensively in the separation of closely related biological species.[20]

Methods of Detection

In this study three methods of detection for HPLC were examined; UV spectrophotometry, fluorometry, and electrochemistry. A great deal of work involving the use of spectrophotometry and HPLC in the analysis of bilirubin and related species has been conducted.[21-23] Before using UV spectrophotometry in the detection mode, an absorption spectrum of the sample in the mobile phase must be obtained to determine the optimal wavelength for measurement. The detector must be equipped with a flow-through cell so that the eluent coming off the column can be measured. This type of detection offers several advantages, including low detection limits and simple operation. The main disadvantage of a UV detector is poor selectivity, since many compounds have UV absorbances at the selected wavelength.

Another method that was examined was fluoroscence. The basic differences between fluorometric and spectrometric detectors are selectivity and sensitivity. Fluorometric methods may be effectively applied to samples of much lover concentrations.[24] In fluorometry, it is possible to increase the concentration related parameter independent of the power source. In spectrometry, this is not possible since the concentration related parameter is the ratio of the measured power to the power of the source. Usually, this gives fluorometry a sensitivity of two to four orders of magnitude greated than spectrophotometry.

Although it is known that an impressive number of biochemical substances fluoresce[a5], no work involving bilirubin or urobilinogen could be located. To determine if bilirubin and urobilinogen fluorescence spectrum must be run for each species to determine the optimal excitation and detection wavelengths. As in spectrophotometry, a fluorometer with a flow-through cell is needed to measure the fluorescence of the eluting molecules.

The final method of detection to be examined is electrochemical. An extensive treatment of electrochemical detection coupled with HPLC is available in the literature.[26-28] Briefly, electrochemical detection involves an oxidation or reduction of the species under study at the working electrode. The measurement of the redox current versus time gives the chromatogram. The concentration of the electroactive species is related to the amount of current produced.

The cell requirements are very similar to those of an electrosynthesis cell. The working electrode material must be carefully selected to function with the mobile phase in use, the supporting electrolyte used in the electrosynthesis and to provide the needed potential window. The cell must have a stable reference electrode, a suitable auxiliary electrode, and also contain a low dead volume.

Electrochemical detectors are often able to measure nanogram quantities, rendering this method extremely sensitive. Since the working electrode is maintained at a controlled potential, only compounds electroactive at or below that potential can be detected. This, then, is what gives electrochemical detection the capability of excellent selectivity.

Summary of Methods

Cyclic Voltammetry was used to develope the electrochemical parameters needed for electrosynthesis and electrochemical detection. A pre-column electrochemical cell was constructed in an attempt to synthesize urobilinogen from the reduction of bilirubin. Reverse-phase HPLC with various mobile phases was used in an attempt to separate urobilinogen from bilirubin and other species present in urine. UV spectrometry, fluorometry, and electrochemical detection were explored as methods of detection.

CHAPTER IV

Material and Apparatus

Materials

All chemicals were A.C.S. reagent grade or better and were used without further purification. The mercury used in the hanging mercury drop electrode (HMDE) and the electrosynthesis cell was Bethlehem Triply Distilled Mercury (Bethlehem Apparatus Co., Hellertown, Pa.)

Nitrogen (Airco, Co.) which was used in the chemical reduction of bilirubin was dried by passing it through $CaCl_2$ columns.

The bilirubin was a certified grade obtained from Fischer Scientific Co. (Fischer Scientific Co., Fair Lawn, N.J. 07410). It was stored at 0°C in a dessicator.

Apparatus

All cyclic voltammograms were generated using a BAS CV-1B cyclic voltammogram (Bioanalytical Systems Inc., West Lafayette, Ind. 47906) connected to an x-y recorder. The electrode system employed in the reduction studies consisted of a HMDE working electrode (Figure 5), a platinum auxiliary electrode, and a silver/silver chloride (Ag/AgCl) reference electrode. For the observation of oxidation reactions, the working electrode was changed to a platinum electrode while the auxiliary and reference electrodes remained the same.

High Performance Liquid Chromatography

A Beckman Model 110A HPLC pump coupled with a Beckman Model 210 sample injection valve (Beckman Scientific Co., Fullerton, Ca. 92634)

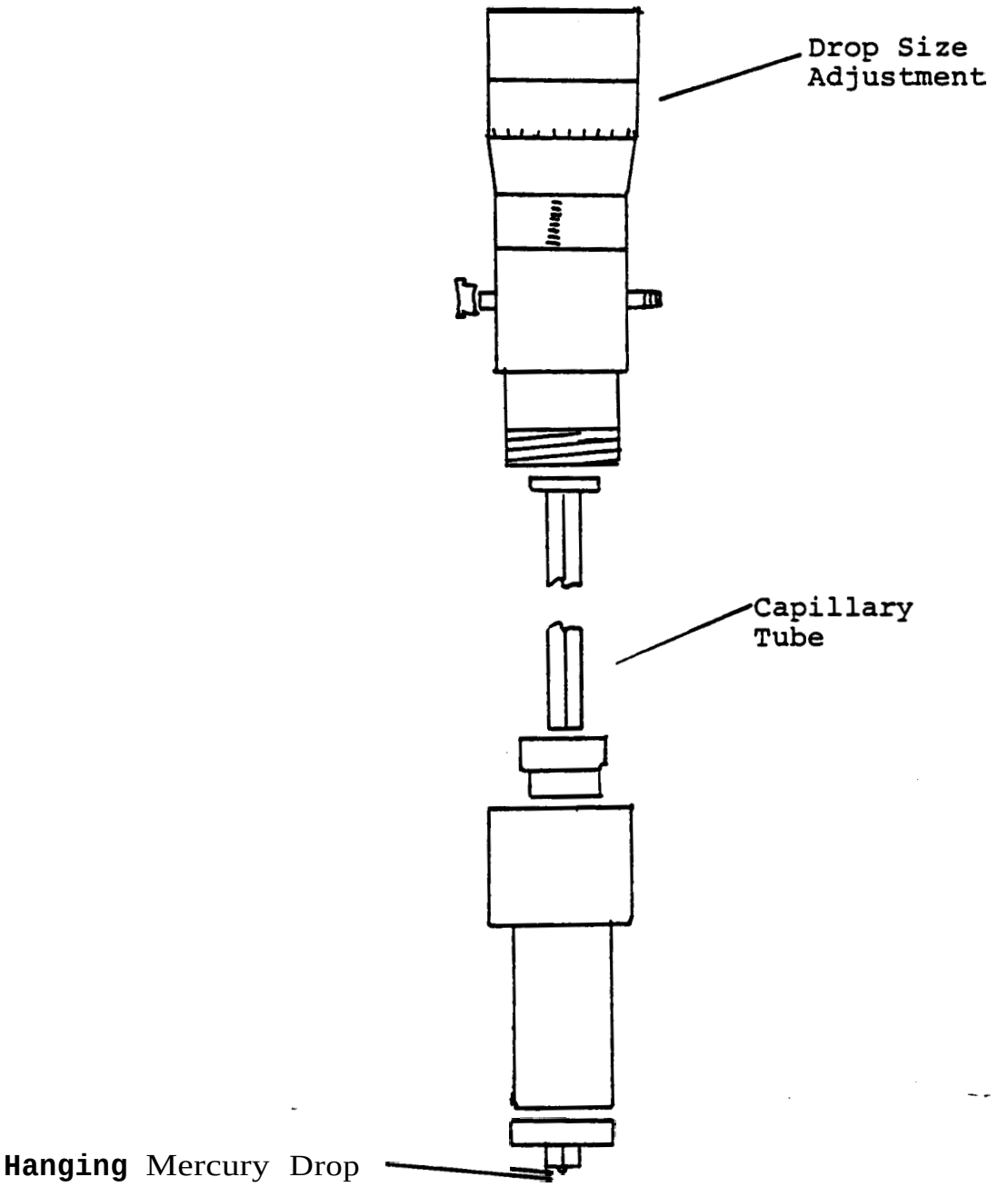
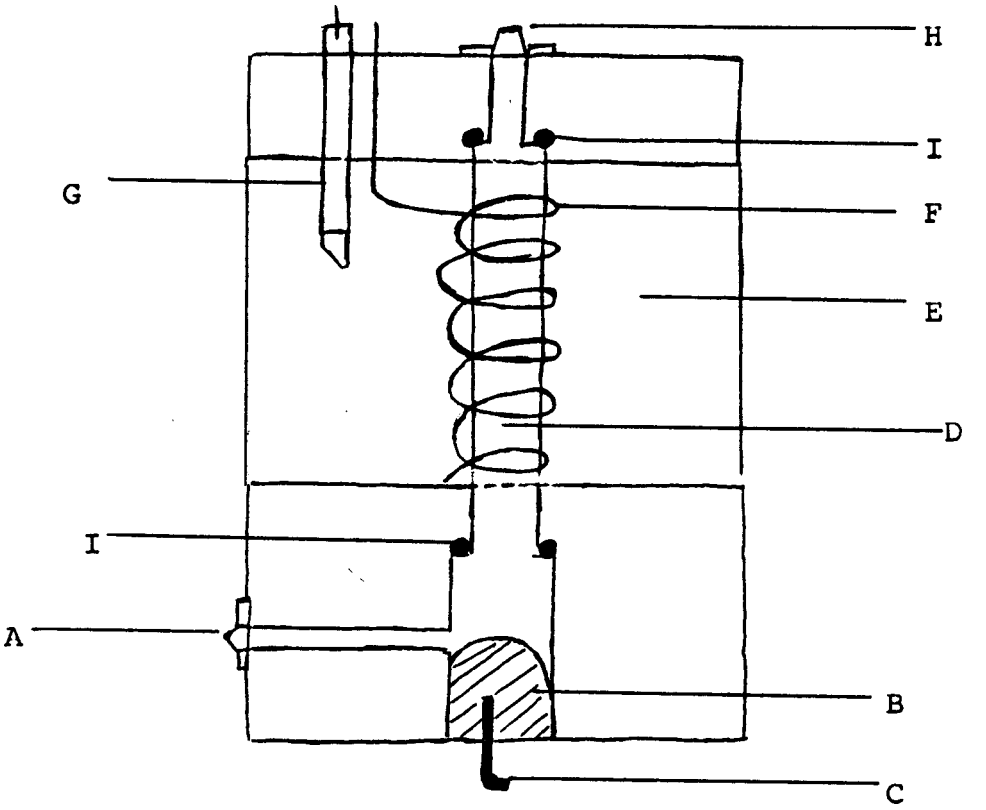


Figure 5–Hanging Mercury Drop Electrode (H.M.D.E.)

were used for all HPLC studies. A prepacked C_{18} reverse-phase column and a MPLC guard column from Brownlee Labs (Brownlee Labs, Inc., Santa Clara Ca. 95050) were employed. Detection was provided by a Hitachi Model 100-10 W-Vis spectrophotometer(Hitachi LTD, Tokyo, Japan). The mobile phases were filtered through a 0.5 μm Millipore filter prior to use.

Electrosynthesis Cell

The electrochemical cell shown in Figure 6 was constructed of two 2-inch (5.1 cm) diameter plexiglass cylinders and a plexiglass tude (5.1 cm outer diameter and 4.4 cm inner diameter with a length of 4.6 cm). The top plexiglass cylinder was cut to a length of 3.2 cm and the bottom cylinder to 5.1 cm. Approximately 1.6 cm of each cylinder was machined to a diameter slightly less than 4.4 cm, allowing them to fit into the plexiglass tube. A 1/5 inch (0.51 cm) diameter channel was drilled through the center of each cylinder. 1.6 cm of this vertical channel was then enlarged to a diameter of $\frac{1}{4}$ inch (0.64 cm) on the machined end of each cylinder. A 1/5 inch (0.51 cm) diameter horizontal channel was made 3.0 cm from the base of the bottom plexiglass cylinder. This horizontal channel progresses through the cylinder until it intersects the vertical channel. A $\frac{1}{4}$ inch (0.64 cm) vertical channel is made through the top plexiglass cylinder to accommodate the Ag/AgCl reference electrode. Next a 4.6 cm length of vycor tubing (0.6 cm outer diameter, 0.4 cm inner diameter) sealed with two o-rings is inserted into the vertical channels on the machined end of each plexiglass cylinder. The vycor tubing is then wrapped with 25-gauge silver wire and the entire assembly is held together with three stainless steel bolts (not shown). The joints formed between the



A. HPLC Fitting: sample solution inlet

B. Mercury Pool: working electrode

C. Platinum Wire: lead to working electrode

D. Vycor Tubing

E. 1.0 M KC1 solution

F. Silver Wire: auxiliary electrode

G. Silver-Silver Chloride Electrode: reference electrode

H. *HPLC* Fitting: solution outlet

I. O-rings

Figure 6-Electrosynthesis cell for *HPLC*
(cell shown without bolts used for compression)

plexiglass cylinders and the plexiglass tube are sealed with parafin wax. The inlet and outlet ports of the cell were made with two stainless steel HPLC fittings (Upchurch Scientific, Oak Harbor, Wa. 98277). The horizontal channel is capped by a small cork with a platinum wire through the center of it.

Once the cell is assembled the horizontal channel is filled with mercury until the level reaches the inlet port. Next the chamber created by the plexiglass tube is filled (through the reference electrode channel) with 1.0 M KCl. The reference electrode is then inserted, making sure its tip is submerged in the KC1 solution. Connection with the inlet and outlet ports is made with Autoanalyzer pump tubing (Technicon Corp., Tarrytown, N.Y. 10591). The flow through the cell was generated by an Autoanalyzer Proportioning Pump (Technicon Instruments, Co., Tarrytown, N.Y. 10591). All potentials were applied with a BAS SP-Z Potentiostat (Bioanalytical Systems, Inc., West Lafayette, Ind. 47906).

Miscellaneous Apparatus

A Beckman Model 26 Dual Beam W-Visible Spectrophotometer (Beckman Scientific, Co., Fullerton, Ca. 92634) was used for all absorption spectra.

A Turner Spectrofluorometer, Model 430, (G.K. Turner Associates, Inc., Palo Alto, Ca,) was used to determine the absorption and fluorescence spectra of urobilinogen and bilirubin.

CHAPTER V

Experimental

Cyclic Voltammetry

The first experiment conducted during the development of an electrosynthesis procedure was cyclic voltaxnetry. It was used to determine the most suitable supporting electrolyte, the electrode system needed for synthesis and the potential at which synthesis occurs.

To determine the best possible combination of working electrode material and supporting electrolyte, cyclic voltammograms were performed on several different combinations. Cyclic voltammograms were first obtained using each supporting electrolyte-working electrode combination studied to account for any background current. Bilirubin was then added to each system and reduction attempted.

Bilirubin solutions used in the cyclic voltammetry studies were prepared by dissolving 10 mg of bilirubin in 100 mL of supporting electrolyte. The pH of the supporting electrolyte was raised to 8.0 with NaOH to increase the solubility of bilirubin.[29,30] All solutions were deoxygenated by bubbling dry nitrogen through them for approximately thirty minutes.

The auxiliary and reference electrodes, platinum and Ag/AgCl respectively, were used for all systems. The potential scan rate used for all cyclic voltammograms was 0.1 V/minute.

The three working electrode materials used were platinum (Pt), Glassy Carbon, and Mercury. The supporting electrolytes employed were

1.0 M KCl, 1.0 M KNO_3, and 0.5 M Na_2SO_4. The results are listed in Table 1.

These results served to establish the parameters needed to synthesize urobilinogen by reducing bilirubin. It was determined that 0.5 M Na_2SO_4 should be the supporting electrolyte and that the working electrode should be constructed of mercury. The cyclic voltammograms of 0.5 M Na_2SO_4 with and without bilirubin are shown in Figures 7 and 8.

Electrosynthesis Cell

The electrosynthesis cell was constructed as described in Chapter III and shown in Figure 8. Once the cell was completed, a series of tests were conducted to determine if the cell was functioning and if urobilinogen was indeed being produced.

A cyclic voltammogram utilizing the electrosynthesis cell as the electrode system was performed. A solution of 10 mg/100 mL of bilirubin was prepared using 0.5 M Na_2SO_4 (pH=8.0). The inlet port of the cell was sealed and filled with the bilirubin/Na_2SO_4 solution which had been previously deoxygenated. The potential scan rate was 0.1 V/minute. The resulting cyclic voltammogram is shown in Figure 9. It was learned while attempting this procedure that the cell takes approximately 15 minutes to equilibrate after the solutions and reference electrode are in place.

To confirm that urobilinogen was actually being produced a series of absorption spectra were performed. A solution of 1 mg/100mL bilirubin was prepared in 0.5 M Na_2SO_4 (pH=8.0) and a spectrum scanned from 600 nm to 210 nm was obtained. The same solution was then placed in the electrosynthesis cell(with the inlet port still sealed) and

TABLE 1

WORKING ELECTRODE - SUPPORTING ELECTROLYTE COMBINATIONS

Working Electrode Material	Supporting Electrolyte	Cathodic Limit	Reduction of Bilirubin Observed	Potential at Reduction
Platinum	1.0 M KCl	-0.8 V	No	------
Platinum	1.0 M KNO_3	-0.8 V	No	------
Platinum	0.5 M Na_2SO_4	-0.9 V	No	------
Glassy Carbon	1.0 M KCl	-1.2 V	No	------
Glassy Carbon	1.0 N KNO_3	-1.0 V	No	------
Glassy Carbon	0.5 M Na_2SO_4	-1.4 V	No	------
Mercury	1.0 M KCl	-0.7 V	No	------
Mercury	1.0 M KNO_3	-1.4 V	No	------
Mercury	0.5 M Na_2SO_4	-1.9 V	Yes	-1.7 V

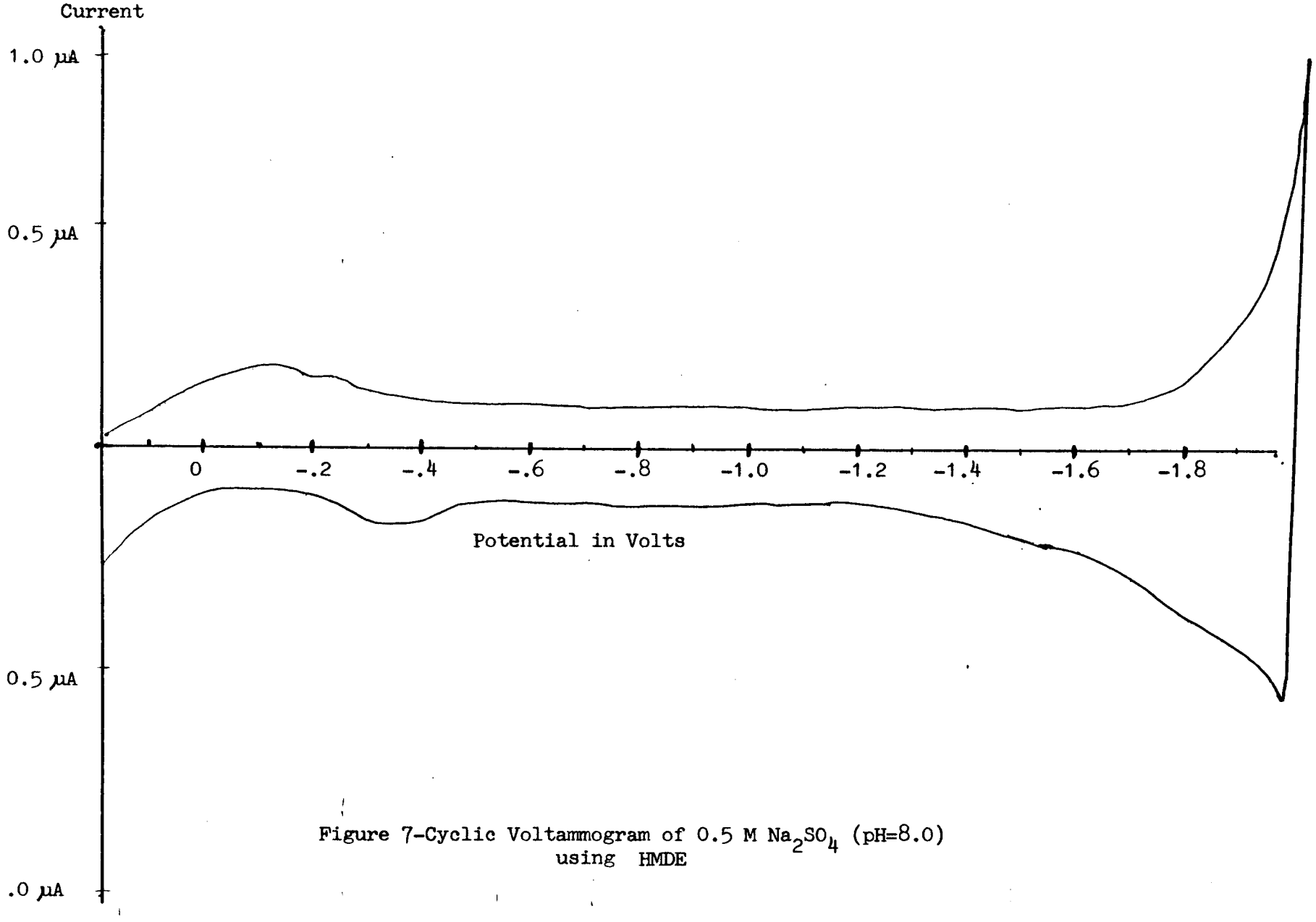


Figure 7-Cyclic Voltammogram of 0.5 M Na_2SO_4 (pH=8.0) using HMDE

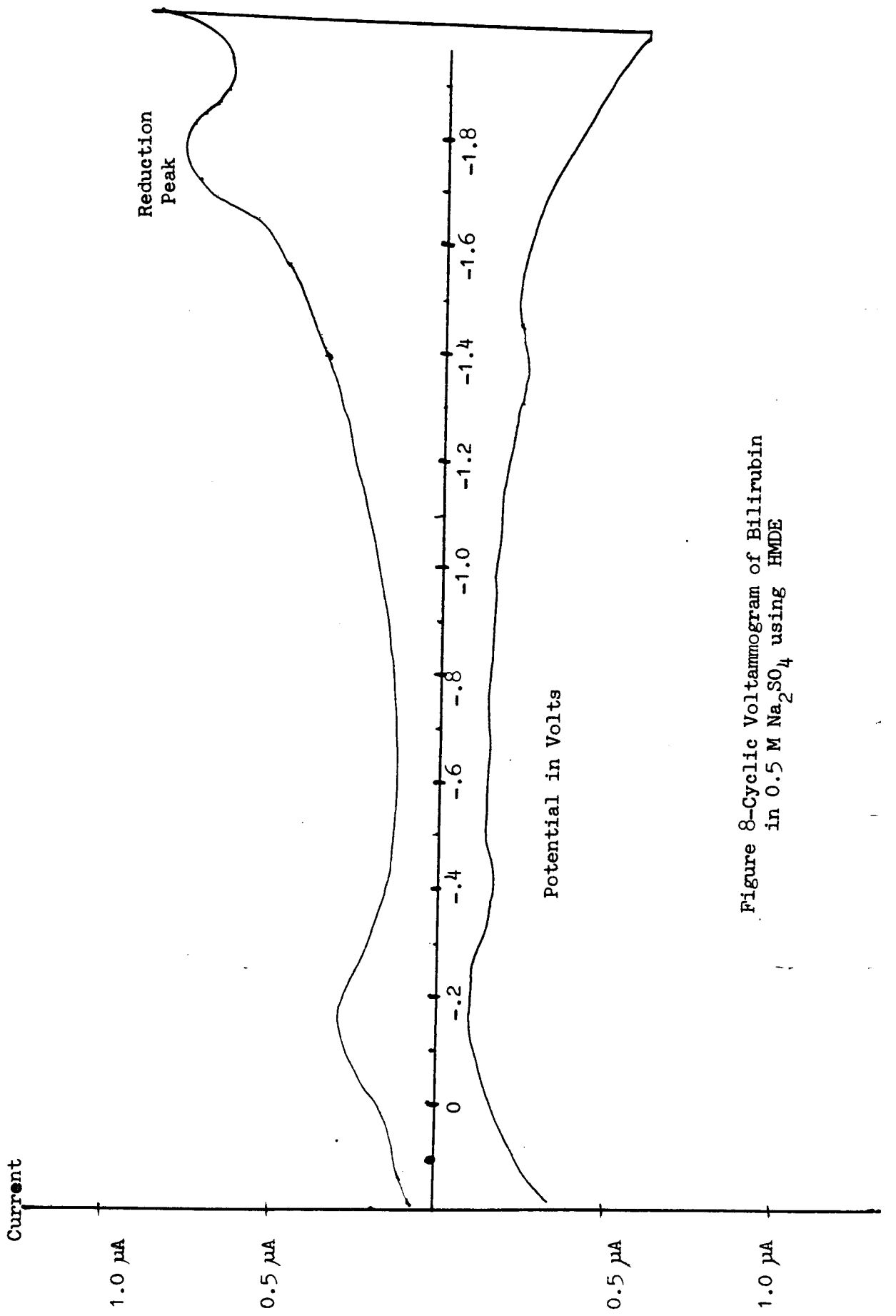


Figure 8-Cyclic Voltammogram of Bilirubin
in 0.5 M Na_2SO_4 using HMDE

potentiostatted to -1.75 V. The reaction was allowed to continue for 5 minutes. It was observed that when the solution was removed the deep orange color of bilirubin had disappeared and the solution was now colorless. A spectrum was determined on this sample immediately to prevent any appreciable oxidation. The results are shown in Figures 10 and 11.

The same procedure was then conducted with a flowing system in the electrosynthesis cell. The cell was potentiostatted to -1.75 V and the flow initiated, the rate of flow being approximately 0.5 mL/minute. The initial bilirubin solution (1 mg/100 mL) gave the identical spectrum as before, however, the spectrum given by the urobilinogen solution was slightly different. The spectrum showed a small peak at 450 nm, indicating that 100 % conversion did not take place. The spectrum is shown in Figure 12. Attempts to obtain better conversion by slowing the flow rate proved ineffective.

In order to positively identify the product of the electro-synthesis, a standard procedure was used to prepare urobilinogen so that comparison could be made.

The chemical reduction of bilirubin was accomplished by using 3 % sodium amalgam. This was prepared by reacting 3 g of sodium pellets with 75 g of mercury.[31] Due to the extremely violent nature of this reaction, it was performed under a fume hood. To minimize the rate of reaction, the sodium pellets were added one at a time, with subsequent additions only after the previous pellet had reacted. The reaction mixture solidified upon cooling, after which it was heated until molten, and again allowed to cool for at least one hour. The product was then pulverized and rinsed with deionized water. Next, it was washed with

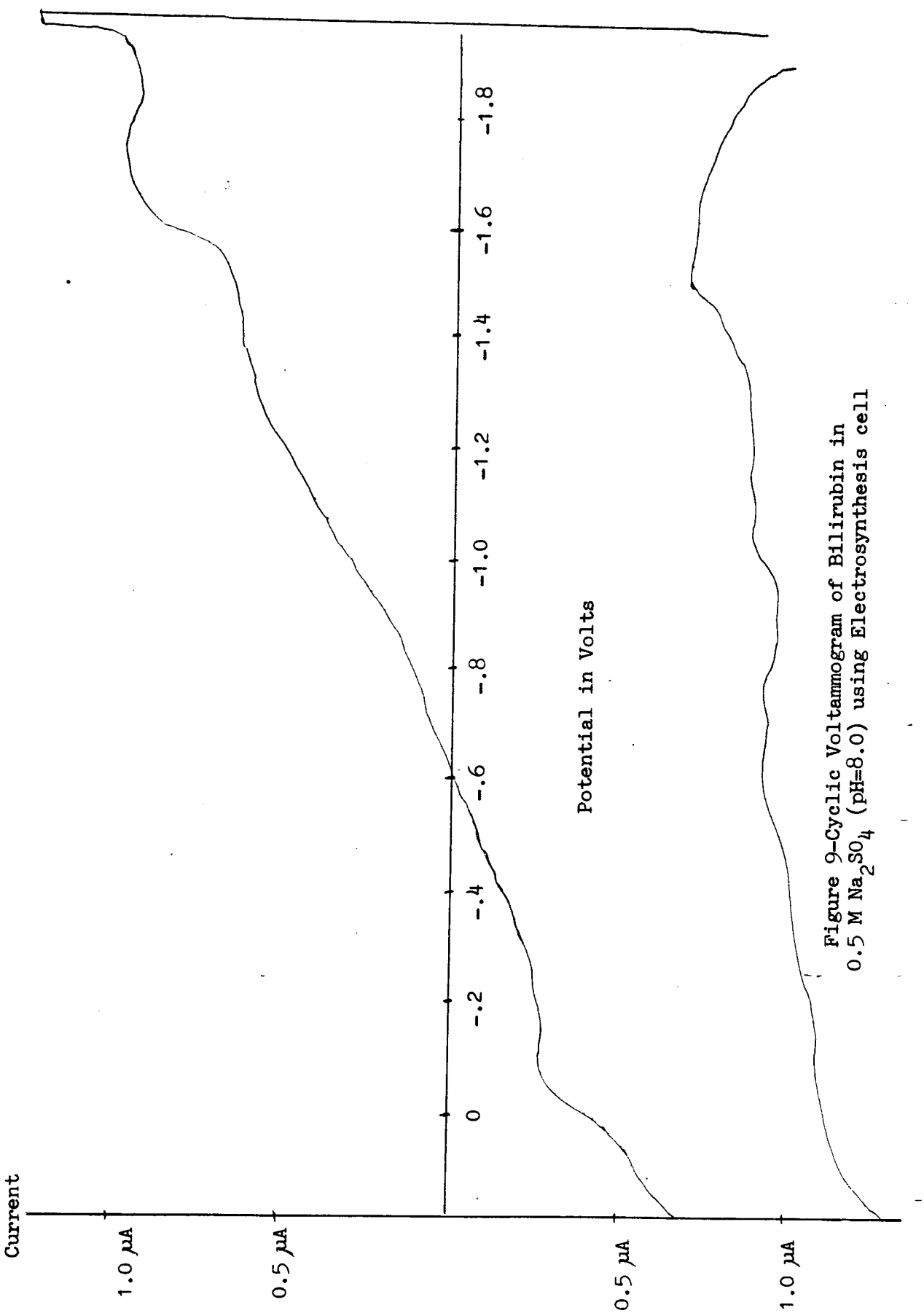


Figure 9-Cyclic Voltammogram of Bilirubin in 0.5 M Na_2SO_4 (pH=8.0) using Electrosynthesis cell

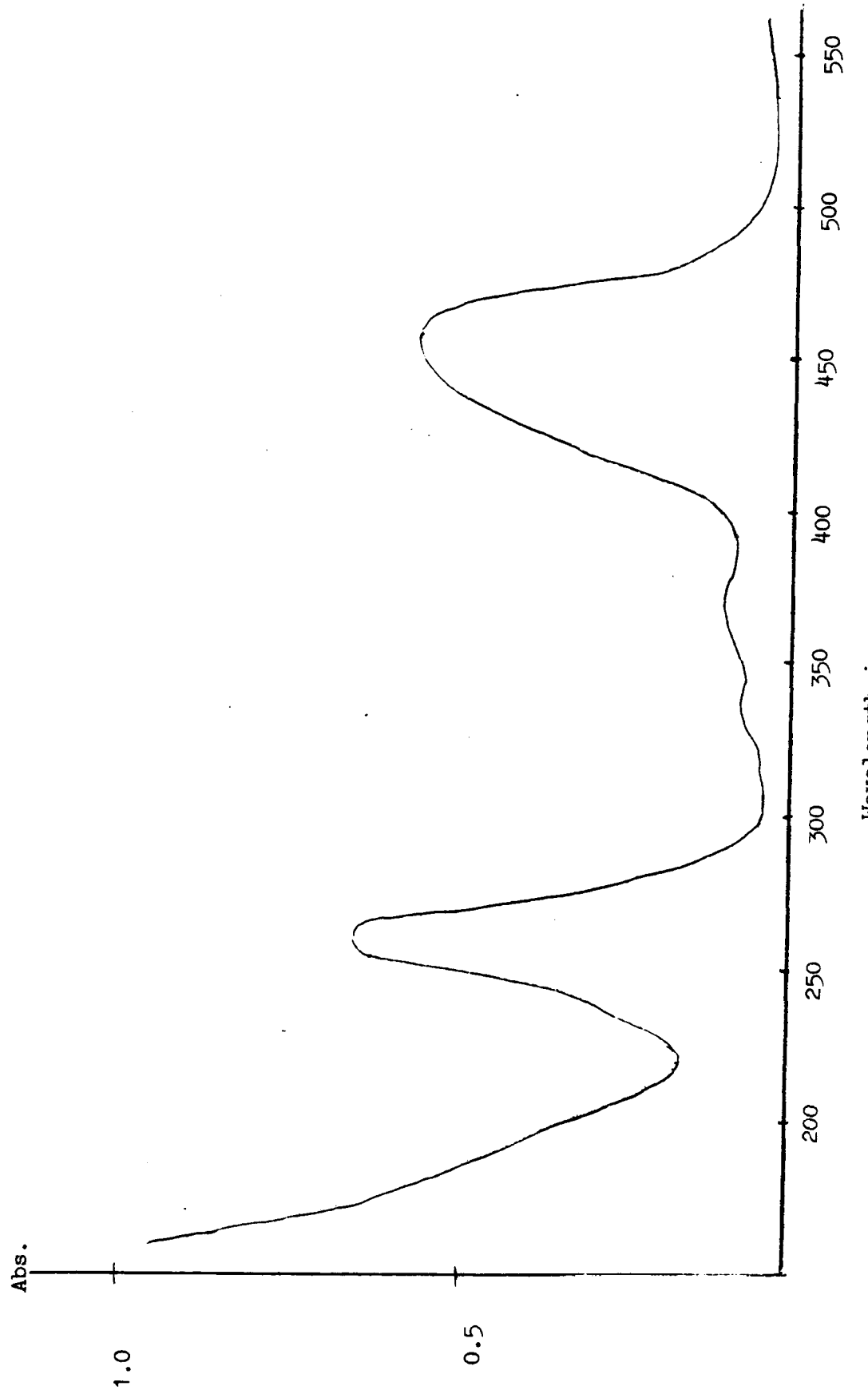


Figure 10-Absorption Spectrum of Bilirubin in 0.5 M Na_2SO_4 (pH=8.0)

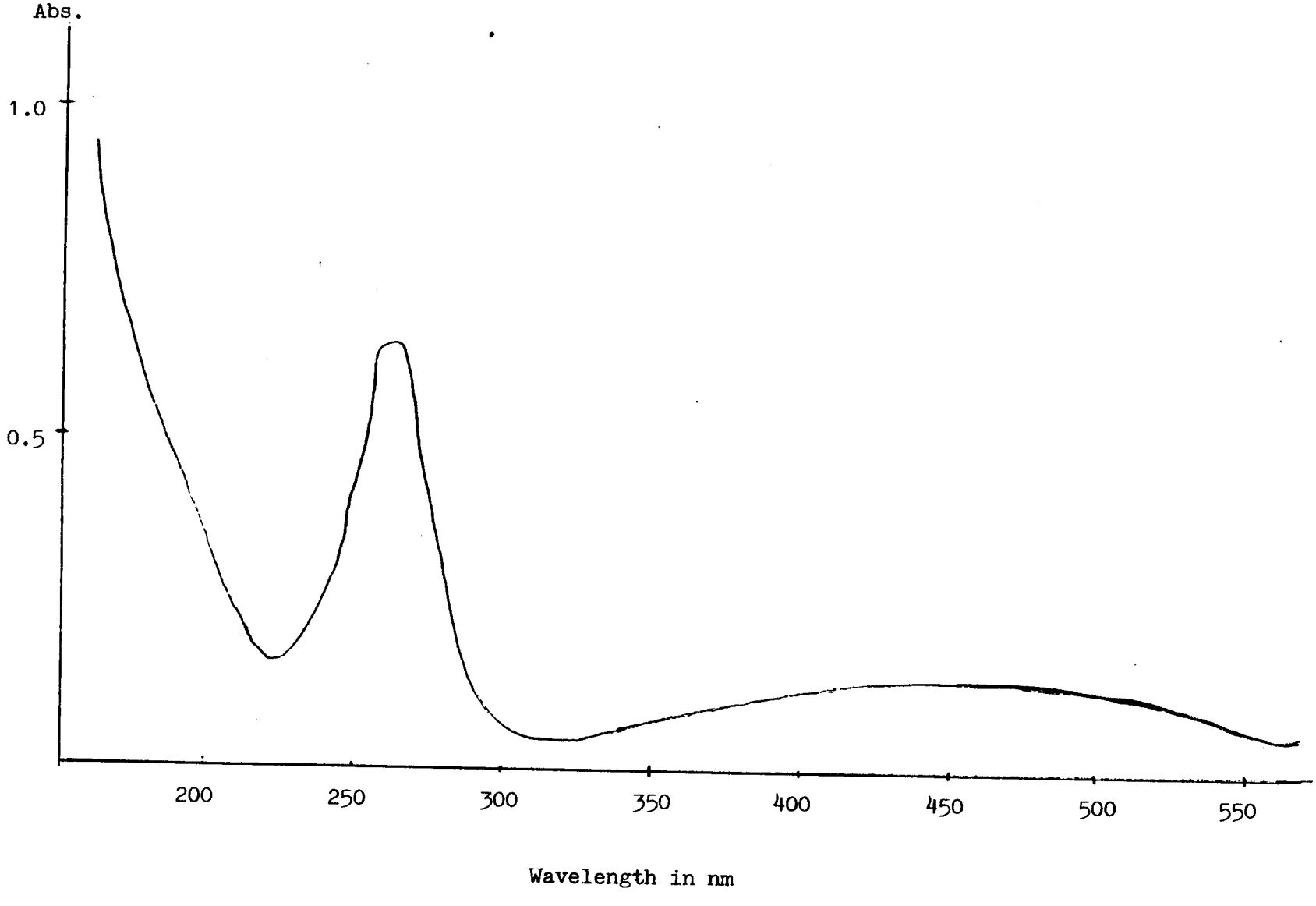


Figure 11-Absorption Spectrum of Urobilinogen in 0.5 M Na_2SO_4 (pH=8.0)

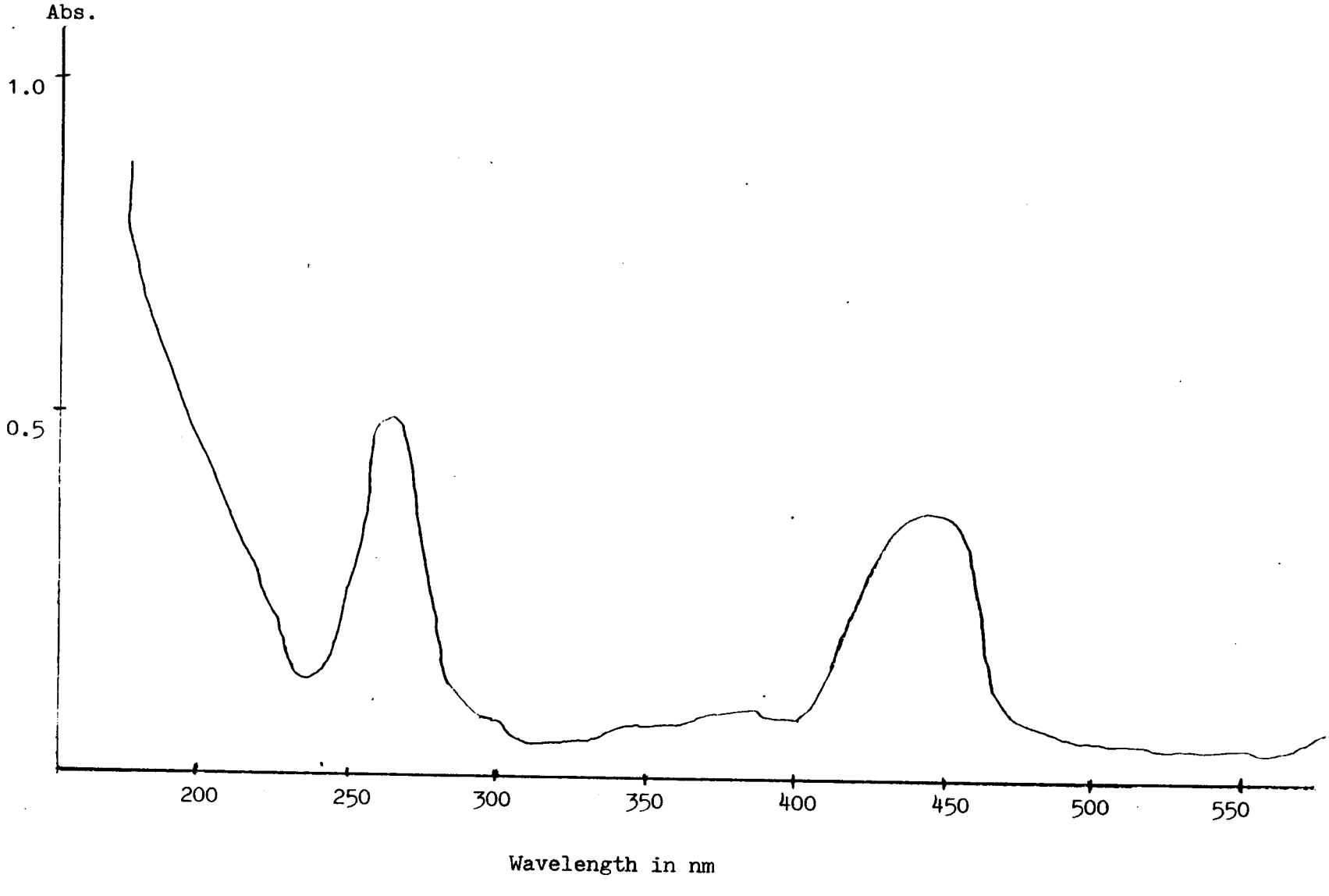


Figure 12-Absorption Spectrum of Urobilinogen/Bilirubin mixture in 0.5 M Na_2SO_4 (pH=8.0)

two 25 mL aliquots of ethanol followed by two 25 mL portions of ether. The product was then dried overnight under vacuum in a dessicator and was thereafter stored in the dessicator.

A solution containing 50 mg of bilirubin dissolved in 8 mL of H_2O and 2 mL of 0.1 M NaOH was prepared. 10 g of 3 % sodium amalgam was added to the solution, which then was mixed using a magnetic stirrer. The reaction progressed under nitrogen for two hours.

After the reaction was complete, the deep orange color of bilirubin had been replaced by colorless urobilinogen. A spectrum of this solution was then obtained and was found to be identical to the spectrum of electrochemically produced urobilinogen.

Electrochemical Detection

The first method of detection that was considered was electrochemical detection. Since the electrosynthesis cell did not give 100 % conversion of bilirubin to urobilinogen, it had become necessary to develop a method that could be used to accurately detect both species.

Again, cyclic voltammetry was used to determine the feasability of an eleotrochemical process. While some latitude existed in the choice of the working electrode material, the choice of supporting electrolyte had already been dictated by the electrosynthesis cell. Since electrochemical detection can make use of either oxidation or reduction reactions both were explored for possible application.

To determine if reduction reactions were feasible, cyclics were run on urobilinogen since the reduction potential for bilirubin had been previously determined. A solution of urobilinogen was prepared

by placing 2 mg/100 mL solution of bilirubin in 0.5 M Na_2SO_4 (pH=8.0) into the electrosynthesis cell, which was potentiostatted at -1.75 V. The reaction was allowed to progress for approximately tweleve minutes. The colorless product was removed from the cell and transferred to the cyclic voltammetry apparatus. A cyclic voltammogram was obtained using HMDE working, platinum auxiliary and Ag/AgCl reference electrodes. No reduction wave was obtained.

Next, the possible use of an oxidation reaction as a method of detection was explored. Platinum and glassy carbon were chosen as possible working electrodes. Cyclic voltammograms were obtained for both materials in 0.5 M Na_2SO_4 to locate the anodic limit and identify background current.

A cyclic voltammogram was obtained using platinum working, platinum auxiliary and Ag/AgCl reference electrodes on 0.5 M Na_2SO_4 (pH=8.0). There were no background currents observed and the anodic limit was located at +1.8 V. A 1 mg/100 mL solution bilirubin in 0.5 M Na_2SO_4 (pH=8.0) was placed in the electrosynthesis cell which was then potentiostatted at -1.75 V. The reaction was allowed to progress for approximately five minutes. The colorless product was removed from the cell and immediately transferred to the cyclic voltammetry apparatus. The results showed the oxidation of urobilinogen at +0.4 V. The cyclic was allowed to continue for several minutes. The solution changed from colorless to yellow indicating that urobilinogen was being oxidized to urobilin. The cyclic voltammogram is showen in Figure 13.

The oxidation of bilirubin to biliverdin was attempted for both working electrode materials. A 1 mg/100 mL solution of bilirubin in

0.5 M Na_2SO_4 (pH=8.0) was prepared and cyclic voltammograms run. No oxidation wave was obtained for either platinum or glassy carbon electrodes. These findings are consistent with previous work done by Humans.[32]

Fluorometry

The second method of detection that was examined was fluorometry. It was noted from the literature that several urobilinoids form a zinc complex that is highly fluorescent.[33] However, no work could be located to whether urobilinogen or bilirubin reacted to form a fluorescent compound in aqueous solution. A series of activation and emission spectra were constructed to determine the feasibility of this method.

Since the presence of dissolved oxygen can reduce the intensity of light emitted by a fluorescent compound[34], all solution were deoxygenated with nitrogen before use.

Urobilinogen samples were prepared by the electrosynthesis cell from 1 mg/100 mL solutions of bilirubin in 0.5 M Na_2SO_4 (pH=8.0). The complex of urobilinogen with zinc is formed in an alcoholic zinc acetate solution, which was made from 1 g of zinc acetate dissolved in one liter of 30 % ethanol-70 % water (at this stage, 30 $.ethanol-70 % H_2O was thought to be the most probable mobile phase).

A one milliliter aliquot of the electrochemically prepared urobilinogen solution was added to nine milliliters of the alcoholic zinc acetate solution and allowed to stand for five minutes. The excitation spectrum was then run from 300 nm to 600 nm. Several spectra were run at various scan rates to obtain the best results. It was thereby determined that the fluorescence is activated by a broad band of light between 375 and 495 nm with a peak at 440 nm. This activation curve is shown in Figure 14.

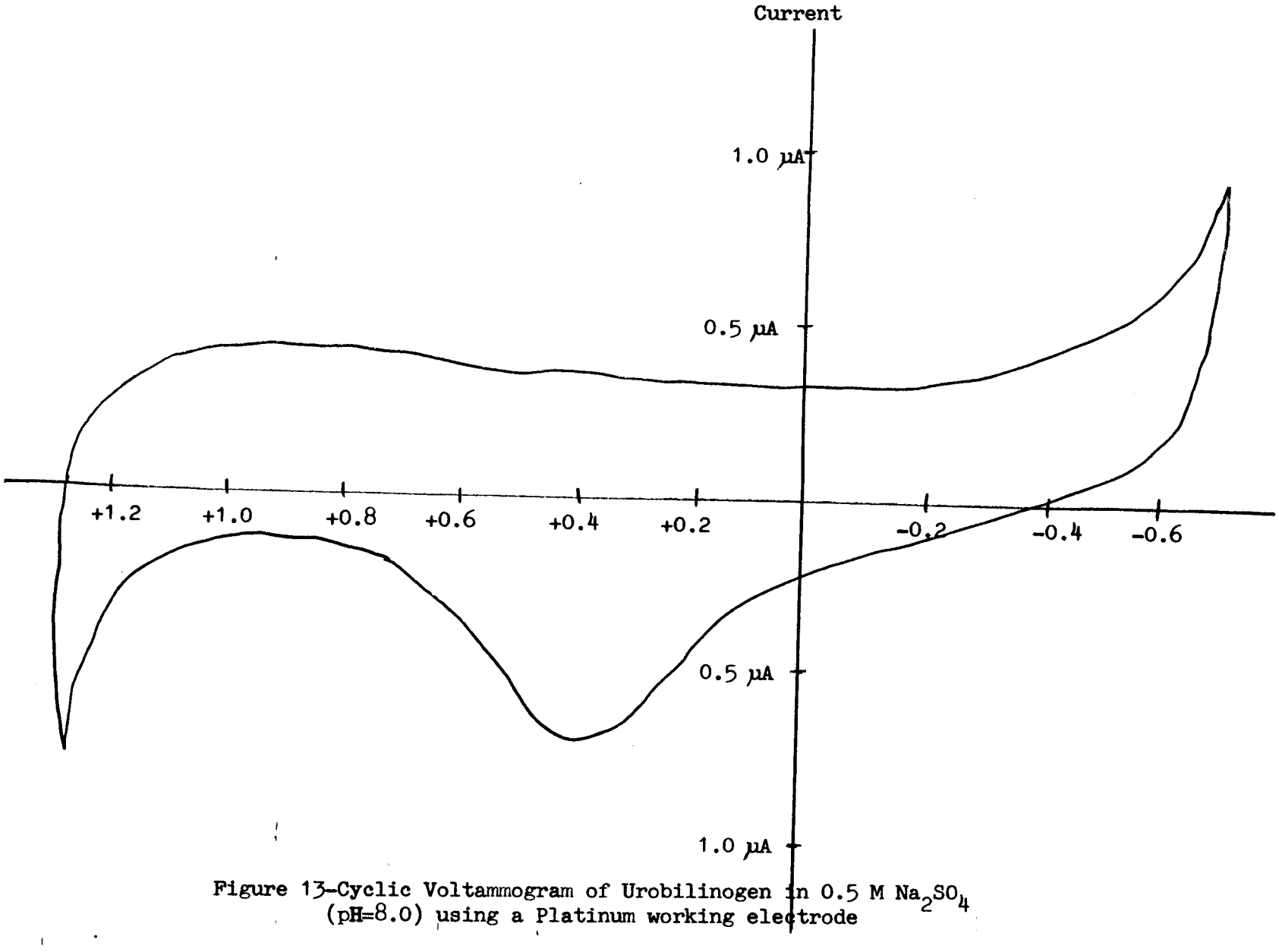


Figure 13-Cyclic Voltammogram of Urobilinogen in 0.5 M Na_2SO_4 (pH=8.0) using a Platinum working electrode

The same solution of urobilinogen and alcoholic zinc acetate was used to obtain an emission spectrum scanned from 300 to 600 nm. It was learned that the fluorescence occurs from 400 to 510 nm with a peak at 460 nm as shown in Figure 15.

It was noted that these solution had an extremely limited lifetime. After about 15 minutes the colorless solution began to turn yellow and the fluorescence decreased significantly. This is probably due to the oxidation of urobilinogen to urobilin.

A 0.5 M Na_2SO_4-alcoholic zinc acetate solution was tested for background spectra. No peaks were observed.

To determine the fluorescent ability of bilirubin a 1 mg/100 mL solution in 0.5 M Na_2SO_4 was prepared. One milliliter of this solution was added to nine milliliters of the alcoholic zinc acetate solution and allowed to stand five minutes. Several spectra were obtained by varying both the emission and excitation wavelengths between 300 and 600 nm. No fluorescence, however, was detected. Varying the concentrations of both zinc acetate and bilirubin failed to produce any significant fluorescence.

Since it is known that solvent polarity and pH have important influences on fluorescence, both of these parameters were varied in an attempt to cause bilirubin to fluoresce.

An increase in polarity of a solvent system often serves to enhance fluorescence. Therefore, the polarity of the solvent was increased by replacing 30 % ethanol and water with 10 % ethanol and water. All other parameters remained the same. No bilirubin fluorescence was observed.

Fluorescence is often pH dependent. The initial pH of the

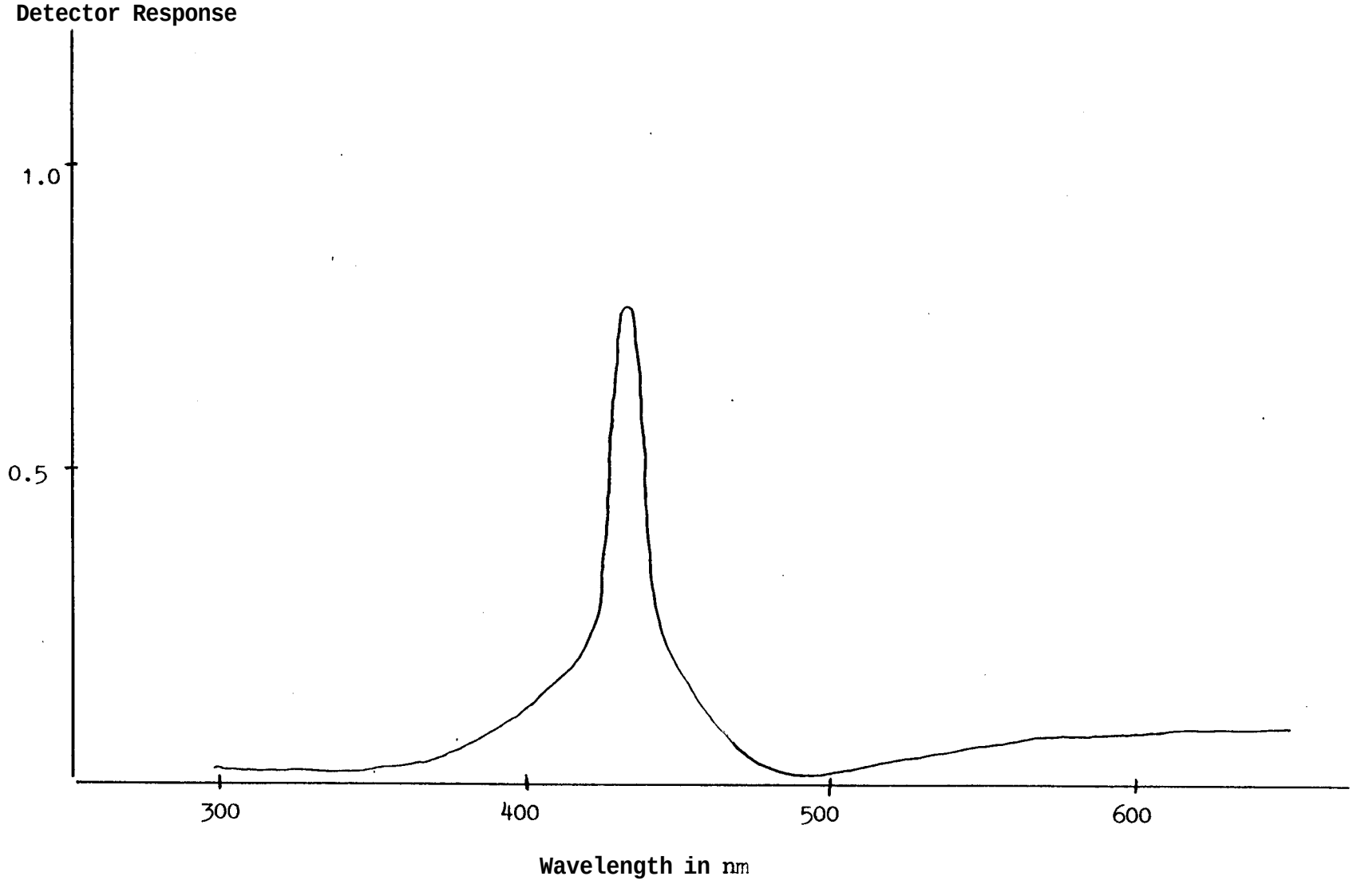


Figure 14-Activation Curve of Urobilinogen/0.5 M Na_2SO_4 in Alcoholic Zinc Acetate Solution

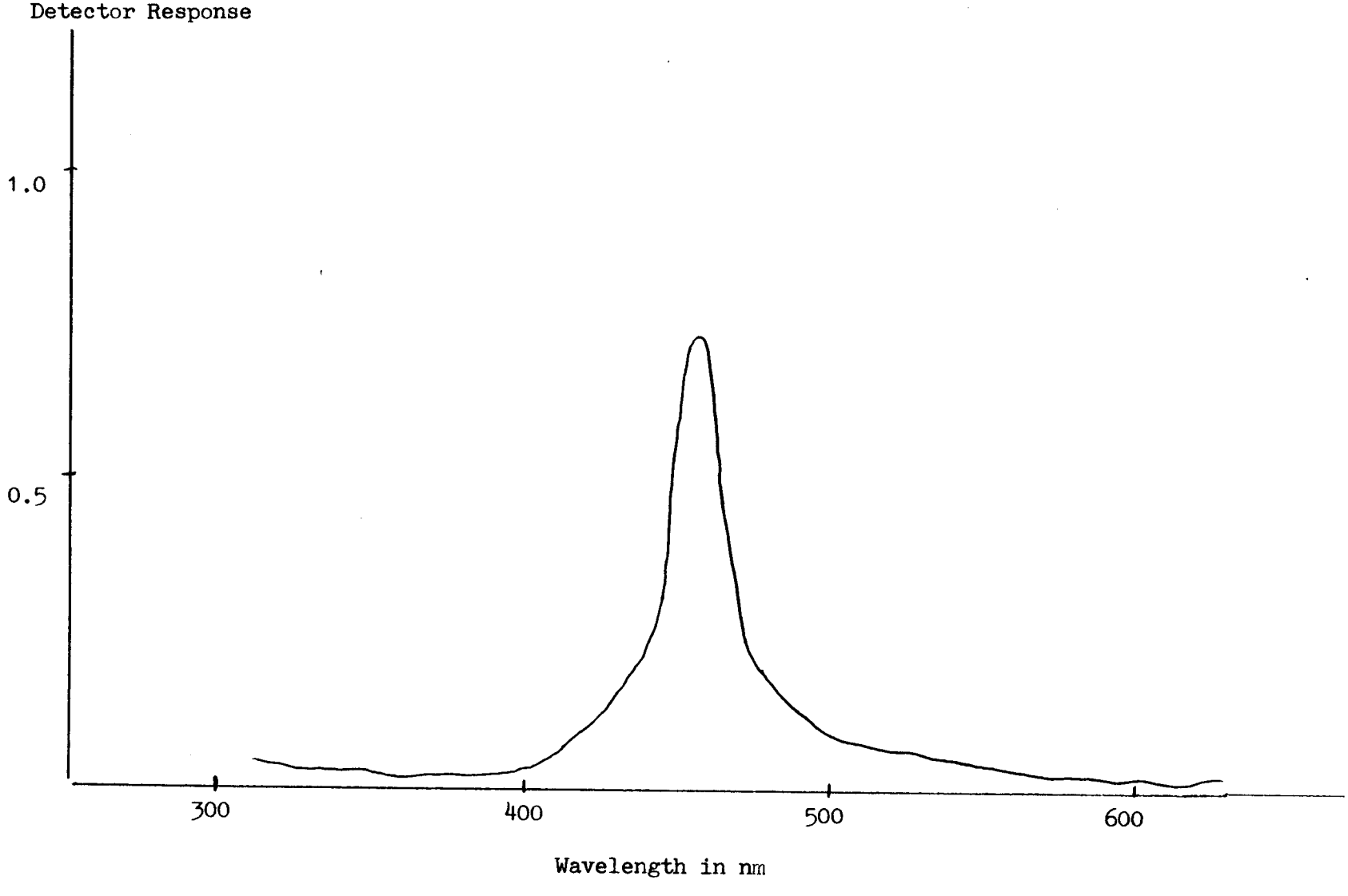


Figure 15-Emission Curve of Urobilinogen/0.5 M Na_2SO_4 in Alcoholic Zinc Acetate Solution

alcoholic zinc acetate solution (30 % ethanol-70 % water) was 6.3. By adding small amounts of NaOH the pH was raised to 8 and 10. The procedure was carried out as before, and as before bilirubin produced no fluorescence in either the pH 8 or pH 10 alcoholic zinc acetate solution.

UV Spectrometry

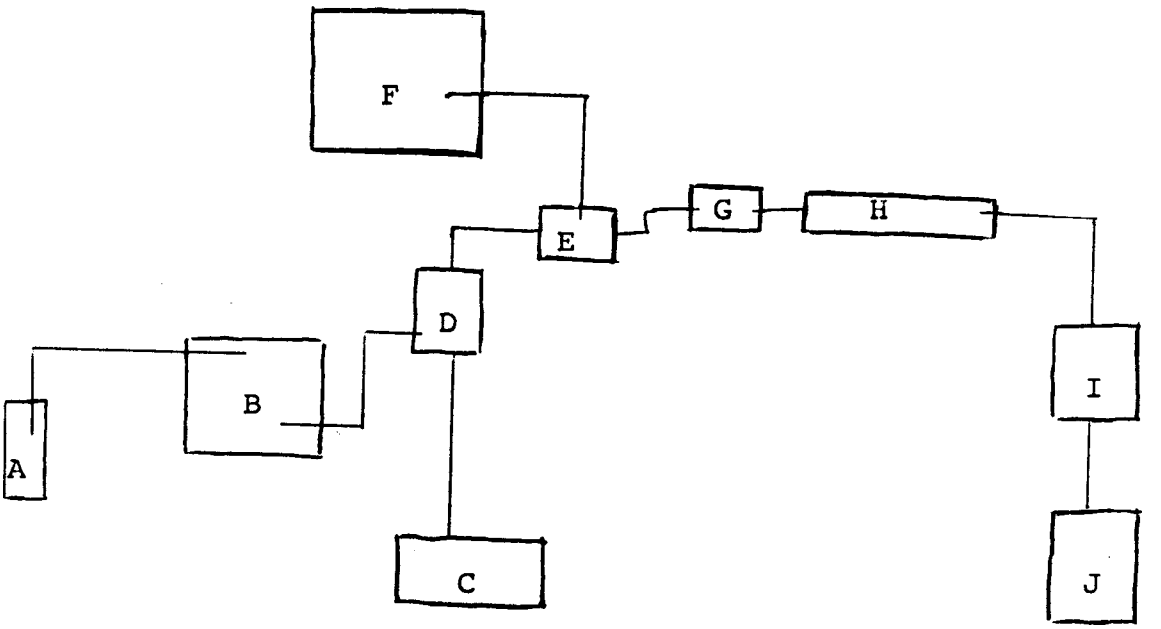
The final method of detection that was investigated was UV spectrometry. From the spectra shown in Figures 10 and 11 it can be seen that both bilirubin and urobilinogen absorb at 260 nm. Since UV absorption provided the only method able to detect both species it was chosen as the detection method.

Because such a large number of substances absorb in the ultraviolet reagion, all mobile phases considered had to be tested for possible interference.

Mobile Phases for HPLC

With all other parameters determined, the choice of the mobile phase needed to provide for the separation of urobilinogen from bilirubin was sought. The set-up was constructed (shown in Figure 16) using the electrosynthesis cell to produce the urobilinogen and a UV spectrometer as the detector.

Although several different mobile phases were employed, the remainder of the set-up was unchanged unless otherwise specified. The flow rate used in all attempts was 1.0 mL/minute and solvents and samples were prepared fresh prior to use. When injecting urobilinogen (in 20 µl aliquots), the electrosynthesis cell was potentiostatted at -1.75 V with a continuous flow rate of approximately 0.6 mL/minute.



A. Bilirubin solution in 0.5 M Na_2SO_4
B. Proportioning Pump
C. Potentiostat
D. Electrosynthesis Cell
E. Injector
F. HPLC Pump
G. Guard Precolumn
H. Reverse-phase Column
I. UV detector
J. Recorder

Figure 16-Experimental Set-up

40 % Methanol/Water

The detector was set at 260 nm and 0.02 absorbance units full scale. A 20 µl portion of 1 mg/100 mL bilirubin in 0.5 M Na_2SO_4 (pH=8.0) was injected. This gave an extremely broad peak coupled with a marked rise in the pressure on the column. After several minutes, the absorbance returned to baseline.

The electrosynthesis cell was then used to provide a 20 µl injection of urobilinogen. Again a very broad peak appeared. To determine if this peak was due to urobilinogen or uncoverted bilirubin the wavelength was changed to 450 nm. At this wavelength urobilinogen would give no response. Another 20 µl urobilinogen sample was injected. The broad peak was observed, indicating that it was unconverted bilirubin adhering to the column and that urobilinogen either came off with the solvent front or its effect could not be seen due to the intensity of the bilirubin peak. Results are shown in Figure 17.

50 % Methanol/H_2O

A slightly less polar solvent was used under the same experimental conditions. An injection of 20 µl of bilirubin gave a peak at 3.3 minutes. Using the electrosynthesis cell, 20 µl of urobilinogen-bilirubin solution was then injected. This produced two peaks at approximately 2.8 minutes that ran together. In order to determine the identity of the two peaks the wavelength was switched to 450 nm and another injection made. This showed bilirubin was retained the longest. The results are shown in Figure 18.

An attempt was made to obtain better separation by decreasing the flow rate to 0.7 mL/minute. An injection of 20 µl of urobilinogen-

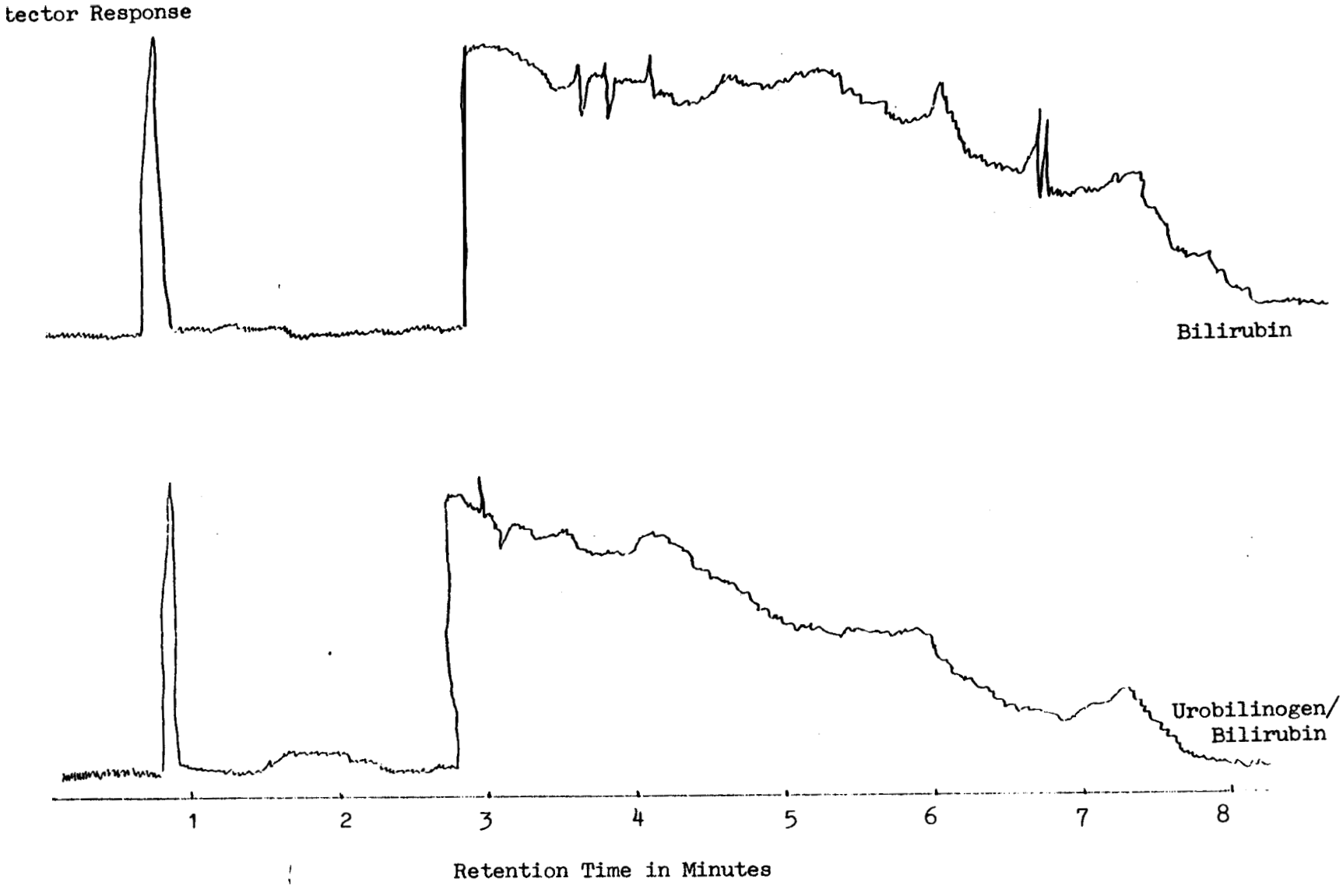


Figure 17-Chromatograms of Bilirubin and Urobilinogen/Bilirubin solutions in 40 % Methanol

bilirubin solution was made. There was no significant change in peak shape although there was a slight increase in retention time.

A buffer solution was added in an attempt to increase the separation and stabilize the compounds. The buffer was prepared by mixing 2.8 mL of 0.5 M KH_2PO_4 and 32.4 of 0.5 M Na_2HPO_4 and diluting to one liter with deionized water. This solution was used in place of pure water for the 50 % methanol mobile phase. Repeated injections provided no enhancement of peak separation. A stronger buffer was made with an ionic strength of 0.1 and a pH of 7.60 (11.5 mL of 0.5 M KH_2PO_4 and 62.8 mL of 0.5 M Na_2HPO_4 diluted to one liter). This also failed to significantly improve the separation.

60 % Methanol/Water

The mobile phase was then changed in an attempt to obtain better separation. A 20 µl injection of bilirubin was made and a peak appeared after approximately minutes.

A urobilinogen-bilirubin solution (generated by the electro-synthesis cell) was then injected. A small peak resulted after 2.5 minutes. To identify the peak the wavelength was switched to 450 nm and another injection made. This yielded a peak at 2.5 minutes. No urobilinogen peak was located using this mobile phase.

55 % Methanol/Water

The results from this mobile phase matched those given by 60 % Methanol/Water.

45 % Methanol/Water

An attempt was made to improve the results obtained with the 50 % methanol solution while avoiding the problems generated with

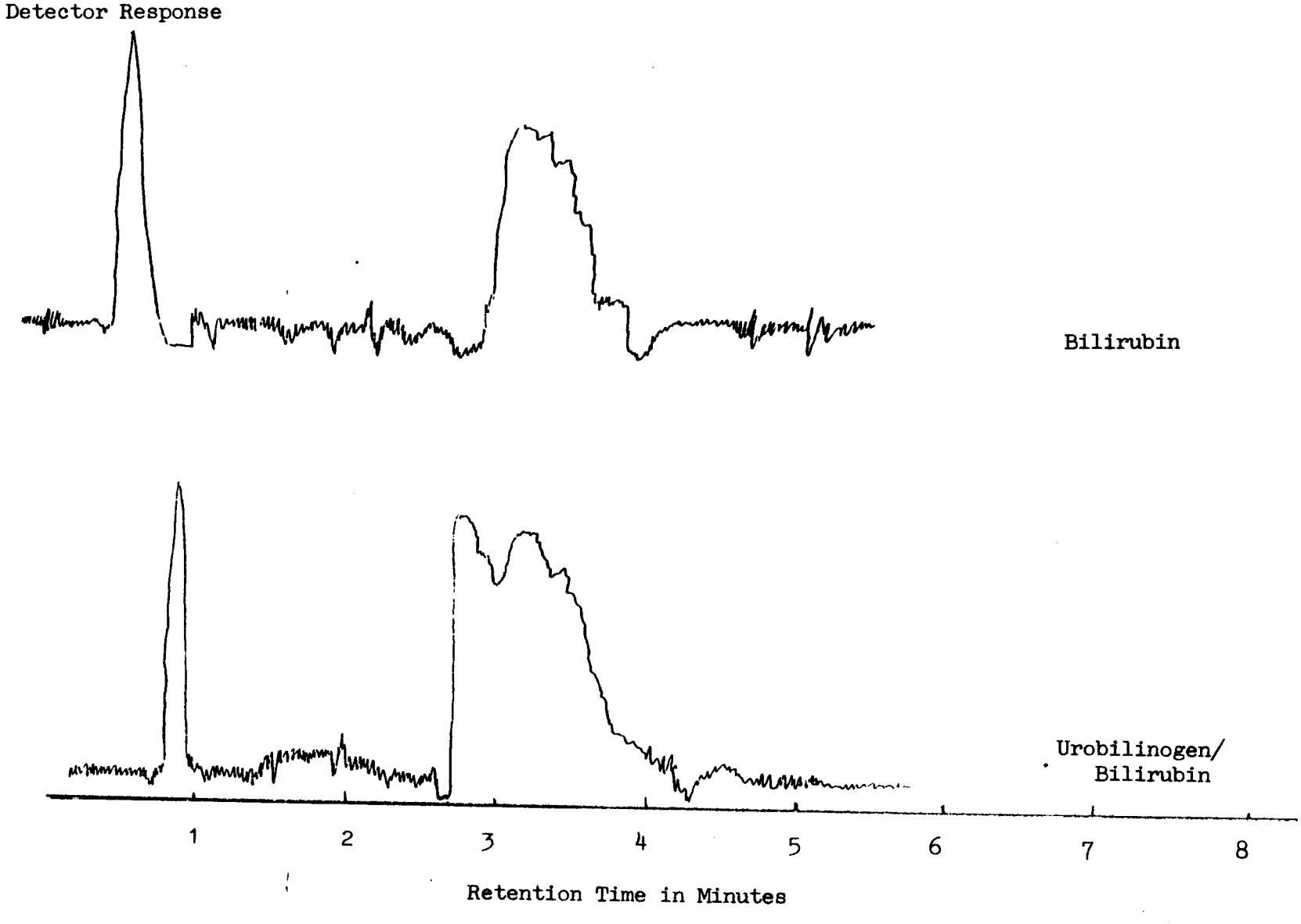


Figure 18-Chromatograms of Bilirubin and Urobilinogen/Bilirubin solutions in 50 % Methanol

the 40 % methanol and water mobile phase.

An injection of 20 µl of 1 mg/100 mL of bilirubin in 0.5 M Na_2SO_4 (pH=8.0) with 45 % methanol and water as the mobile phase gave results almost identical to those given with 40 % methanol and water.

To determine if an excess amount of bilirubin was responsible for the loss of column efficiency the injection volume was reduced to 5 µl. The resulting graph showed the same type of peak broading and increase in column pressure as before. The amount of bilirubin being injected was further reduced by diluting the solution with 0.5 M Na_2SO_4. Several different concentrations were prepared and injected. When the concentration reached 10 % of the original solution (0.1 mg/100 mL bilirubin in 0.5 M Na_2SO_4) the only peak detectable was that of the solvent, 0.5 M Na_2SO_4.

Acetonitrile-DMSO-Water

Another mobile phase that was tried was acetonitrile-DMSO-Water. The instrumentation was the same as employed in previous studies except for a decrease in the sensitivity of the UV detector. Since this solvent system gave much greater background noise, the sensitivity was changed to 0.1 absorbance units full scale.

30 % Acetonitrile-30 % DMSO-40 % H_2O

An injection of 20 µl of 1 mg/100 mL solution of bilirubin in 0.5 M Na_2SO_4 (pH=8.0) gave a peak after 4.0 minutes

Next a urobilinogen-bilirubin solution, generated by the electrochemical cell, produced a much larger peak at approximately 4 minutes. To positively identify this peak the wavelength was shifted to 450 nm and another injection made. This yielded a much smaller

peak. The much larger peak was therefore due in part to urobilinogen, which has a much greater molar absorptivity at 260 nm than does bilirubin. The resulting chromatogram is shown in Figure 19.

Other mobile phases attempted where 20 % acetonitrile-20 % DMSO-60 % H_2O and 25 % acetonitrile-25 % DMSO-50 % H_2O. Both systems failed to produce narrow peaks, but produced instead extremely broad peaks that lasted several minutes. This, coupled with a rise in pressure, indicated that at least one species was adhering to the column.

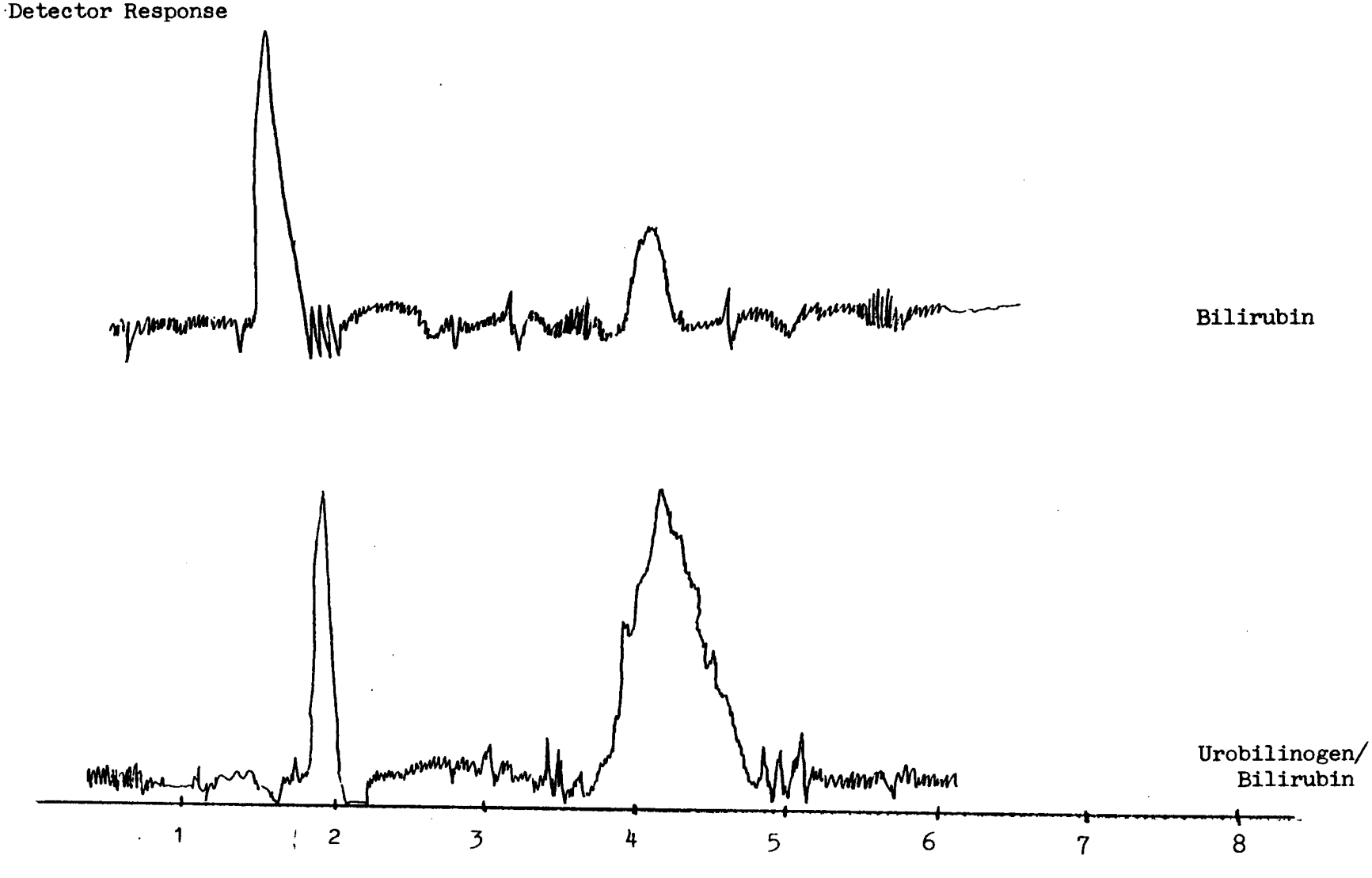


Figure 19-Chromatograms of Bilirubin and Urobilinogen/Bilirubin solutions in 30 % Acetonitrile - 30 % DMSO - 40 % Water

CHAPTER VI

Experimental Results and Discussion

Electrosynthesis of Urobilinogen

An electrochemical cell which reduces bilirubin to urobilinogen was successfully constructed. The experimental results and other factors that contributed to the design and function of this cell will be discussed in this section.

Cyclic Voltammetry

The results of cyclic voltammetry studies were used to provide the key parameters necessary to construct and operate the electrosynthesis cell. From the data generated by this method, the supporting electrolyte, working electrode material, and operating potential were suggested.

Three common supporting electrolytes were selected as possible choices. These were 1.0 M KCl, 1.0 M KNO_3, and 0.5 M Na_2SO_4. Both the 1.0 M KC1 and the 1.0 M KNO_3 proved to be unsuitable since hydrogen overvoltage occured well before the reduction of bilirubin when glassy carbon or platinum electrodes were used. When a mercury working electrode was used, highly negative potentials resulted in the reduction of K^+ ions in aqueous solutions and the formation of an amalgam.[35] This in turn was followed by an irreversible, hydrogen-yielding reaction due to the amalgam dissolution reaction of the alkali metal with water. The evolution of hydrogen, along with the current flow needed to reduce K^+ ions, prevented the system from reaching a potential high enough to

reduce bilirubin. For these reasons, no formation of urobilinogen could be observed for 1.0 M KCl and 1.0 M KNO_3 supporting electrolytes with the mercury working electrode.

The remaining electrolyte, 0.5 M Na_2SO_4, allowed for a potential high enough to reduce bilirubin only when using the mercury electrode. When employed with either the glassy carbon or the platinum working electrode, hydrogen evolution occurred prior to the reduction of bilirubin.

Three materials were considered for the working electrode: platinum, glassy carbon, and mercury. The consideration of platinum and glassy carbon electrodes reflected the desire for a solid working electrode.

Although it was known that platinum has a small overpotential for hydrogen, it was chosen for its inert nature, ease of machining and fabricating, and durability. However, platinum could not be used to attain a sufficient potential with any supporting electrolyte and could not therefore, be used for the reduction of bilirubin.

Glassy carbon was also examined. Although it could be used to attain a more negative potential than platinum, it could not be used to reduce bilirubin with any of the tested supporting electrolytes.

Mercury was chosen specifically for its ability to avoid hydrogen evolution.[36] When tested with 1.0 M KCl and 1.0 M KNO_3, no reduction of bilirubin was observed (for reasons previously cited). However, when tested with 0.5 M Na_2SO_4 as the supporting electrolyte, a negative potential high enough to reduce bilirubin was attained.

Cyclic voltammetry results not only indicated the supporting electrolyte and working electrode material appropriate for the electrosynthesis of urobilinogen, but also were used to determine the potential at which synthesis occurs. Using a mercury working electrode (HMDE)

and 0.5 M Na_2SO_4 as the supporting electrolyte the reduction potential for the conversion of bilirubin to urobilinogen was determined to be -1.7 V versus Ag/AgCl. (Figure 8)

Electrosynthesis Cell

The cyclic voltammogram obtained utilizing the electrosynthesis cell developed for this study as the three-electrode system provided evidence that urobilinogen was being produced. The cyclic voltammogram closely resembles those run with conventional electrode systems on bilirubin. The reduction peak at -1.7 V is easily detected. A comparison of Figures 9 and 10 shows that while the shapes are very similar, the voltammogram produced by the electrochemical cell has a much greater slope. This is due to greater resistance caused by the non-ideal configuration of the electrodes.

To aquire further evidence that urobilinogen was being produced, absorption spectra were obtained on solutions of bilirubin, two electrochemically produced urobilinogen samples and chemically generated urobilinogen. Bilirubin solutions gave strong absorption at 260 nm and 450 nm. The urobilinogen produced in a hydrostatic system (non-flowing) showed absorption at 260 nm. The urobilinogen produced in the cell with a flowing system gave a strong peak at 260 nm and a much weaker one at 450 nm, indicating that 100 % reduction of bilirubin to urobilinogen had not taken place. The chemically produced urobilinogen gave a large peak at 260 nm and a small peak at 450 nm.

The results indicated that urobilinogen was being generated by the electrosynthesis cell, and could be used as a workable standard with the cell constructed and its operating parameter developed, the first phase of the project had been completed. The results obtained

up to this point were used to direct the remainder of the project.

High Performance Liquid Chromatography

Since the electrochemical cell did not yield 100 % conversion, it was necessary to separate urobilinogen and bilirubin before detection. The separation was required since the only wavelength at which urobilinogen can be detected (260 nm) is also strongly absorbed by bilirubin. Although the amount of urobilinogen formed could also be determined by measuring the decrease in absorbance at 450 nm, this method would be unsuitable for quantifying samples containing unknown amounts of urobilinogen. Thus, separation proved to be the best method of detecting urobilinogen.

A known concentration of bilirubin would be injected onto the HPLC with the electrochemical cell turned off; the detector response would then be quantified. Next, the same bilirubin solution would be injected with the cell turned on, and the HPLC separating the unconverted bilirubin from the urobilinogen, would yield two separate detector responses. The amount of bilirubin that has not undergone reduction can then be determined. From this, the amount of bilirubin that was reduced can also be determined; this is the concentration of urobilinogen. The detector response can then be quantified for urobilinogen. When unknown samples were injected (with the cell off), the HPLC would separate urobilinogen from interfering substances, and the resulting detector response would yield the concentration of urobilinogen. Thus, a workable standard for urobilinogen can be produced.

Electrochemical Detection

The possibility of developing an electrochemical cell capable

of detecting urobilinogen and bilirubin was explored. Both reduction and oxidation reactions were tested for possible use in detection.

For the attempted reduction of urobilinogen, mercury was used as the working electrode material since it had already been successfully employed in the reduction of bilirubin. Although several different concentrations and scan rates were used, no reduction of urobilinogen could be produced.

For the oxidation reactions two working electrodes were tested for possible use with 0.5 M Na_2SO_4 (pH=8.0) as the supporting electrolyte. Sodium sulfate was chosen primarily for the convenience of using the same supporting electrolyte as the electrosynthesis cell. Also, it had been noted from earlier cyclic voltammograms (conducted during electrosynthesis trials) that Na_2SO_4 had no serious oxidation wave.

A platinum working electrode was employed in cyclic voltammograms with urobilinogen and bilirubin in 0.5 M Na_2SO_4. The anodic limit of platinum in 0.5 M Na_2SO_4 with a pH=8.0 was +1.8 V. The pH was then lowered to 7.5 to increase the anodic limit; this increased the range to +2.0 V. The oxidation of urobilinogen was located at +0.4 V at either pH, but no oxidation of bilirubin could be produced, although several differing concentrations and scan rates were attempted.

A glassy carbon electrode was also tested for possible usage. The anodic limit in 0.5 M Na_2SO_4 (pH=8.0) was +1.2 V. Increasing the pH to 10 had no discernible effect. The oxidation wave for urobilinogen was located at +0.4 V for both pH values of 0.5 M Na_2SO_4. No oxidation of bilirubin was observed.

The lack of an oxidation wave for bilirubin or a reduction wave for urobilinogen makes electrochemical detection impractical for

this project. Since it would not be possible to detect both species, no further work in this area was pursued.

Fluorometry

The fluorescent characteristics of urobilinogen in an alcoholic zinc acetate solution were determined using activation and emission curves (Fig. 14). The activation curve showed the peak activation at 440 nm. The emission curve showed 460 nm as the maximum emission wavelength. The overlap of the activation and emission curves is unusual, although the relative closeness of the activation and emission peaks is common.

Next, the fluorescent nature of bilirubin in alcoholic zinc acetate was examined. The conditions under which urobilinogen fluoresced failed to produce detectable results for bilirubin. In an attempt to induce the fluorescence of bilirubin, the polarity of the solvent was increased. Both emission and activation curves were taken, but no appreciable change was shown. Next, the pH was increased to 8 and then to 10; this also failed to produce any significant change in the spectrum.

Although bilirubin is known to fluoresce in the absence of zinc[37], the results show that the formation of the bilirubin-zinc complex[3*] prevents its fluorescence in aqueous solution. However, urobilinogen does not fluoresce unless zinc is present.[39] Since the detection requirements include measurement of both urobilinogen and bilirubin simultaneously, further investigation into this method was abandoned.

UV Spectrometry

It had been previously determined that both urobilinogen and

bilirubin have strong absorption peaks at 260 nm (Figures 11 and 12). This method was not immediately embraced because of the large number of interfering substances thatabsorb in the UV region. This method, however, provides a simple and reliable way to detect both species. Therefore, it was the method of detection employed for the remainder of the project.

Mobile Phases for HPLC

Mobile phases were selected that had been used in earlier research projects to separate chemical species similar to urobilinogen and bilirubin.[40,41] Care was exercised to use mobile phases that were compatable with C_{18} columns and would not interfere with UV detection.

Polar solvents were explored since the polarity of the mobile phase often strongly affects the tendency of compounds to be retained on the column. It has been reported[b2] that careful adjustment of solvent polarity allows for the separation of closely related bile pigments by reverse-phase HPLC. The exact mechanism of separation is uncertain, but is thought to involve varying degrees of adsorption and partitioning on the column.

40 % Methanol and Water

Because most of the knowledge in this field is empirical; the choice of solvent polarity for a given separation must be made on a trial-and-error basis, The selection of 40 % methanol and water was purely arbitrary.

The results indicate that this mobile phase was too polar and bilirubin was strongly associating with the column, due either to absorption on free silanol sites or to a strong partitioning of hydrophobic

bilirubin into the hydrophobic bonded phase. The binding onto the column was shown by increasing pressure and broad peaks lasting several minutes. (Fig, 17)

When samples containing bilirubin and urobilinogen were injected no clear conclusions could be drawn since no urobilinogen peak could be located. This could have been due to urobilinogen coming off either with the solvent front or at the same time as the bilirubin peak.

50 % Methanol and Water

The polarity of the solvent was reduced using 50 % methanol in water in an attempt to prevent bilirubin from strongly adhering to the column. Injections of bilirubin yielded a good peak and showed no increase in column pressure. Next, an injection of bilirubin-urobilinogen solution was made in an attempt to achieve separation. The results showed some separation, but it was insufficient for a quantitative measurement. (Fig. 18)

In order to gain better separation, the flow rate was decreased to 0.7 mL/minute. The retention time increased slightly for both species, but no enhancement of separation was evident.

The addition of a buffer to the aqueous portion of the mobile phase provided a constant pH. It was thought that perhaps bilirubin and urobilinogen were remaining with the solvent (0.5 M Na_2SO_4) because they preferred its basic pH to the slightly acidic mobile phase. This preference for the solvent would have prevented proper retention and separation.

Two different ionic strength, pH 8.0, buffers were used. Several injections of bilirubin-urobilinogen solutions failed to

produce any improvement in separation or increase in retention time.

60 % Methanol and Water

The polarity of the mobile phase was decreased to 60 % methanol in water in an attempt to allow urobilinogen to elute more quickly, but minimize the effect on bilirubin retention times. The results show that while bilirubin was retained on the column, urobilinogen was not, and came off in the solvent front.

55 % Methanol and Water

The polarity was increased slightly in an attempt to separate urobilinogen from the solvent. The results showed no improvement over the 60 % Methanol and water mobile phase. That is, urobilinogen was not separated from the solvent front.

45 % Methanol and Water

Since separating urobilinogen from bilirubin could not be accomplished by reducing the polarity, separation was tried by obtaining a polarity between that of 50 % methanol and 40 $ methanol solutions. Also, to ensure that an excess amount of bilirubin was not the cause of the loss of column efficiency, several extremely low bilirubin concentrations were used.

The results were the same as those obtained with 40 % methanol and water. That is, bilirubin absorbed strongly to the column. This was the case with all concentrations until the concentration fell below the detectable limit.

Acetonitrile-DMSO-Water

Another mobile phase examined was acetonitrile-DMSO-water. This system was chosen because it has been successfully employed in the separation of bile pigments.[43] The first ratio of components used was employed with encouraging results.

30 % Acetonitrile-30 % DMSO-40 % H_2O

Using a mobile phase of 30 % acetonitrile-30 % DMSO- 40 % H_2O, an injection of bilirubin yielded a fairly sharp peak in about 4.0 minutes. An injection of bilirubin-urobilinogen solution gave one very large peak at approximately the same location. To determine if the peak was due in part to urobilinogen, the wavelength was changed to 450 nm. The resulting peak was noticably smaller, indicating that it was at least in part due to urobilinogen (the absorption coefficient of bilirubin is roughly the same at 450 nm and 260 nm).

Other mobile phases with higher polarity were also tried (20 % acetonitrile-20 % DMSO-60 % H_2O and 25 % acetonitrile- 25 % DMSO-50 % water) in other attempts to separate urobilinogen and bilirubin. The increased polarity produced the same loss of column efficiency as was observed previously. This indicates that separating urobilinogen and bilirubin may require a more sophisticated approach.

CHAPTER VII

Conclusions

In this investigation, cyclic voltammetry was used to examine both electrochemical processes, oxidation and reduction, for bilirubin and urobilinogen. The oxidation wave for urobilinogen was located at +0.4 V. No oxidation wave could be located for bilirubin. For the reductive process, bilirubin was found to undergo reduction at -1.75 V while urobilinogen gave no reduction wave.

The electrosynthesis cell designed and constructed during this research project was used to reduce bilirubin to urobilinogen in a flowing system. This provides, for the first time, a quick and reliable method of generating urobilinogen in sufficient quantities for in-depth study.

In this project, electrochemical detection was examined as a method of measuring bilirubin and urobilinogen. It was found that electrochemical detection could be used to detect urobilinogen if utilizing the oxidation process (+0.4 V) or bilirubin if utilizing the reductive process (-1.75 V). This, however, proved unsatisfactory since both species must be measured simultaneously.

Fluorometry was also examined for possible use. It was determined that urobilinogen reacted with zinc to produce a fluorescent complex. This complex could be measured using an activation wavelength of 440 nm and an emission wavelength of 460 nm. The bilirubin-zinc complex, however, proved not to be fluorescent. Therefore, fluorometry was not

used since detection of both urobilinogen and bilirubin was not possible.

UV spectrometry proved to be a suitable method of detection for urobilinogen and bilirubin. Both species gave a strong absorbance peak at 260 nm, this allowed for simultaneous detection of both species.

Samples of urobilinogen and bilirubin injected onto the HPLC did not provide sufficient separation for a quantitative measurement. This separation is needed to provide a workable urobilinogen standard. Therefore, more sophisticated HPLC systems (stepwise elution, paired-ion-chromatography, linear gradient elution, etc.) not available during this project would be worthy of further study.

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