Development of a Spectroelectrochemical Assay for Bilirubin: Preliminary Spectral and Voltammetric Studies

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A Thesis

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By:

Anessa Janine Lewis

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III. ABSTRACT

The spectral and voltammetric properties of bilirubin ditaurate conjugate in aqueous solution were determined by employing the indicated analytical techniques. The wavelength of maximum absorbance was found to be 450nm. The molar absorptivity was determined from plots of absorbance at 450nm versus concentration to be 49,900 cm-1 M-1 and was found to decrease significantly over a time period of 22 days. A bilirubin oxidation wave was observed at a peak potential of 0.58V (vs. AgCl/Ag). This wave exhibited the expected scan rate and concentration dependances at a glassy carbon working electrode. Results indicated that the oxidation of aqueous bilirubin conjugate is a simple, diffusion-controlled electrode reaction.

IV. INTRODUCTION

A. BILIRUBIN

Blood is typically composed of three (3) major components: red blood cells, also known as erythrocytes, which are functional in oxygen and carbon dioxide transport to and from body tissue; white blood cells, commonly known as leukocytes, which are instrumental in immune response; and platelets, or thrombocytes, which help to clot the blood. (see Figure 1) Erythrocytes are of particular importance in the investigation of hemoglobin and, in turn, bilirubin. Erythrocytes make up nearly up nearly 45% the volume of whole blood. (see Figure 2) They are biconcave to provide optimal surface area for gas exchange, they are enucleated in their mature form and their major component is hemoglobin. Hemoglobin makes up nearly 95% of the dry weight of an erythrocyte. Each erythrocyte is packed with nearly 280 million molecules of the oxygen transporting respiratory pigment, hemoglobin. Hemoglobin is a globular protein of somewhat spherical shape due to folding of the protein chains upon themselves. Each hemoglobin molecule contains four iron containing heme groups and four globin polypeptide chains, each composed of several hundred amino acids. (see Figure 3a) The red color of blood is due to the presence of oxygenated hemoglobin in the erythrocytes.

TYPES OF BLOOD CELLS

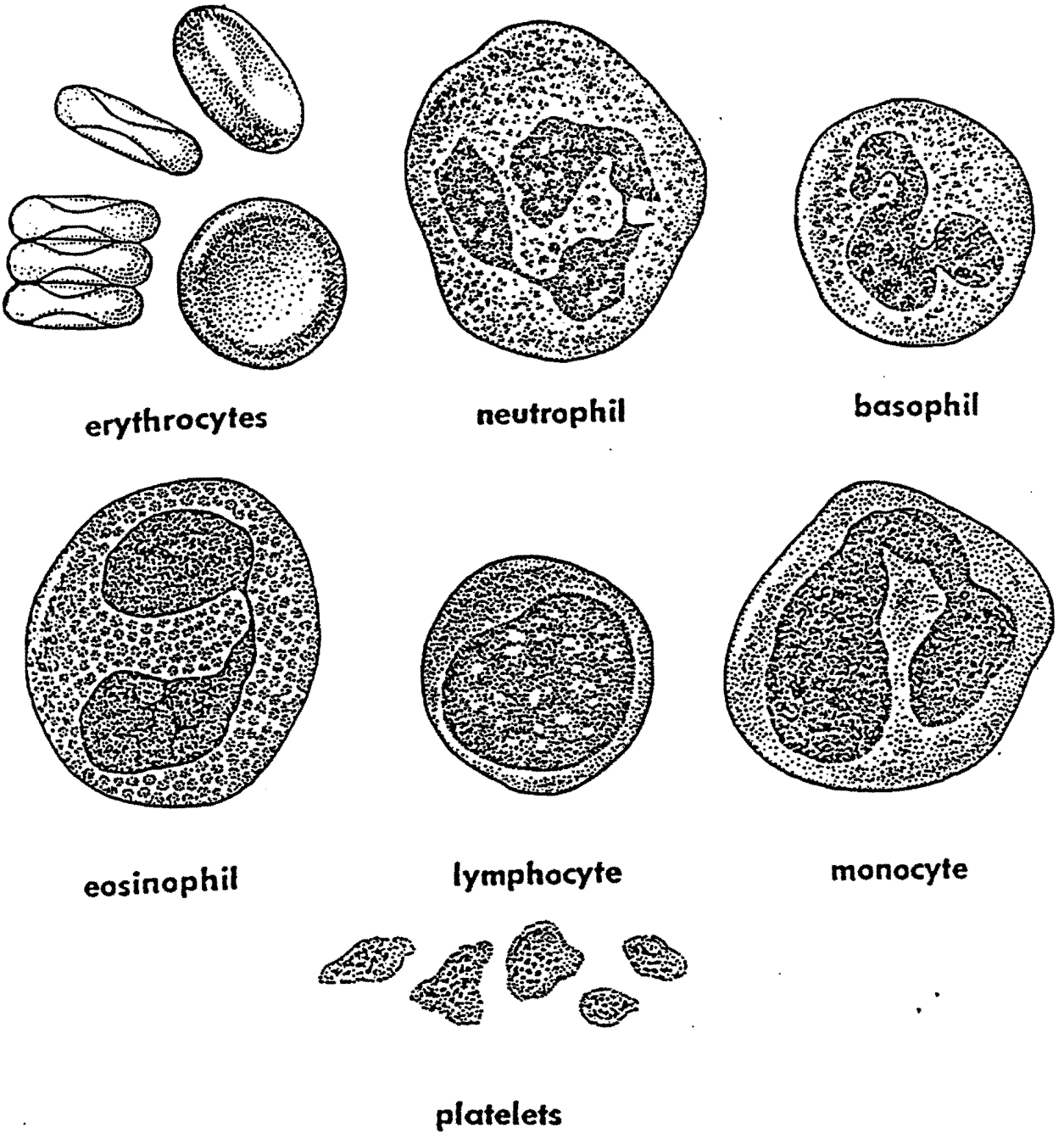


Figure 1. **Types of Blood Cells**
There are three components of blood: erythrocytes; neutrophils, basophils, eosinophils, lymphocytes and monocytes, collectively known as leukocytes; and platelets.

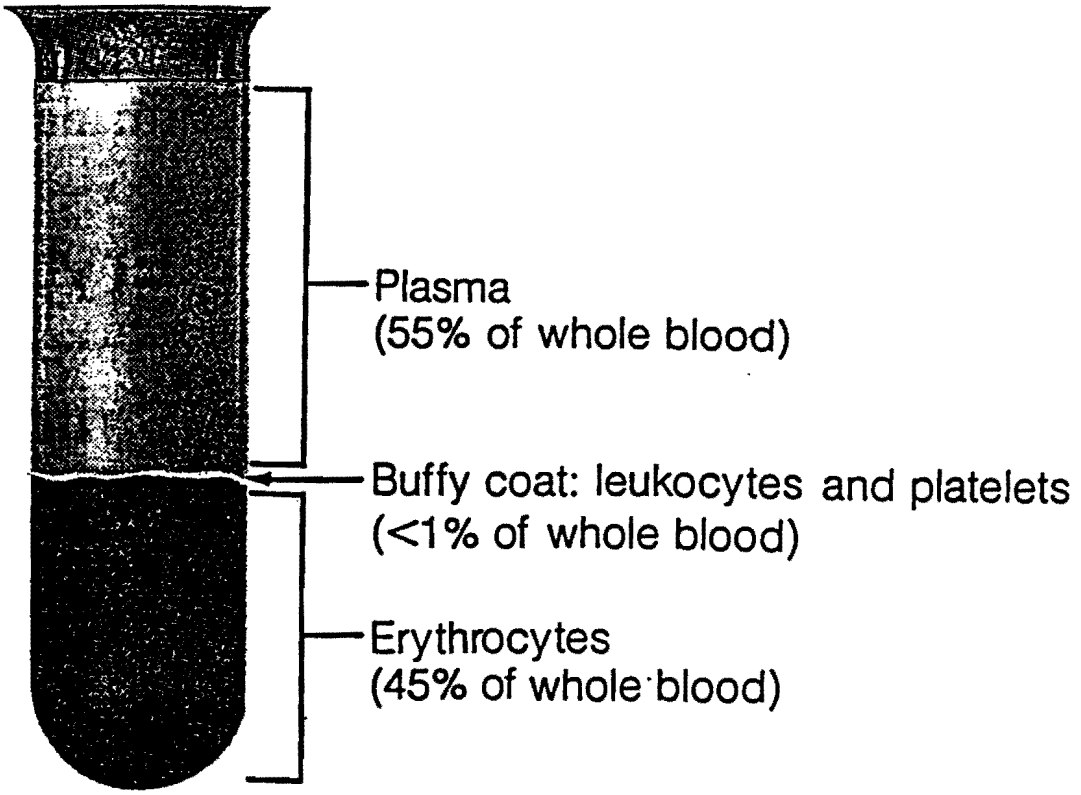
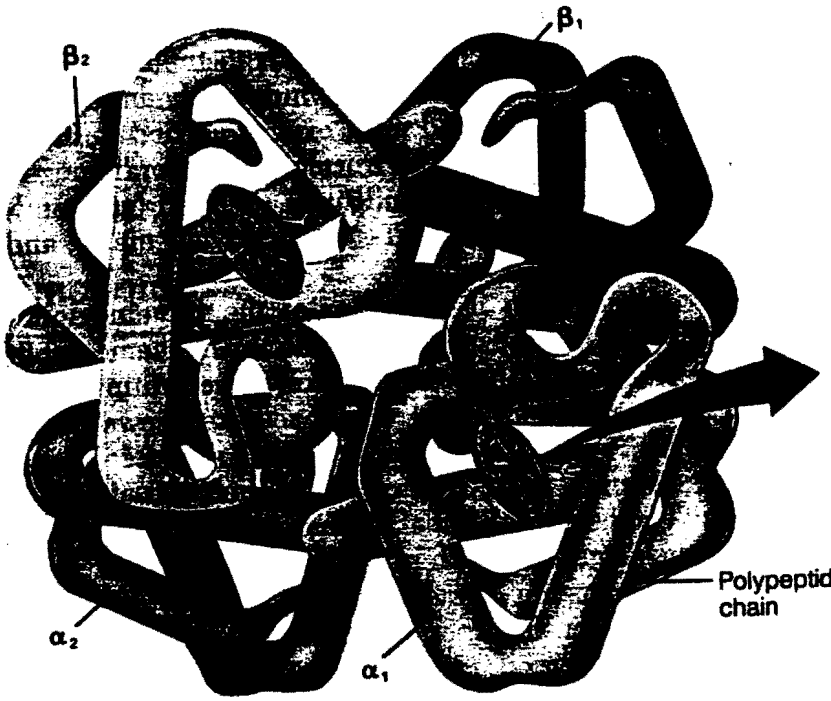
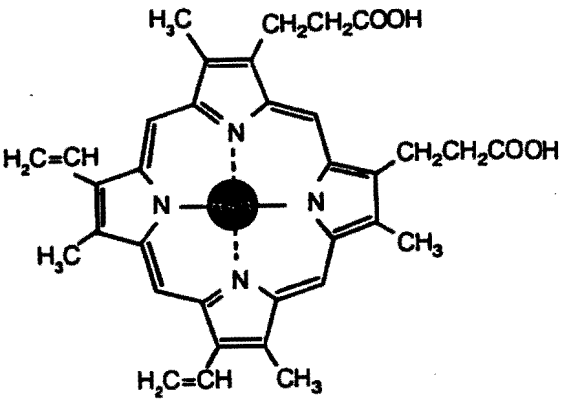


Figure 2. Blood Composition
Erythrocytes normally constitute about 45% of the total volume of a whole blood sample, a percentage known as the hematocrit.



(a) Hemoglobin



(b) Iron-containing heme group

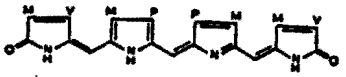
Figure 3. Structure of Hemoglobin
(a)The intact hemoglobin molecule is composed of the protein globin bound to the iron-containing heme pigments. Each globin molecule has four polypeptide chains: two alpha and two beta chains. Each chain is complexed with a heme group, shown here as a disk. (b) Structure of a single heme group.

At the end of the average four month life span of an erythrocyte, it is estimated that it has traveled nearly 700 miles through the human vascular channels. Nearly ten million erythrocytes are produced and another ten million are destroyed every second in the human body. Most of these erythrocytes are destroyed in the reticuloendothelial cells of the liver or spleen. The hemoglobin is liberated and subsequently broken down into its heme and globin components. The fate of the globin is not clear, but much of the heme, which is basically an iron in combination with a porphyrin ring system (see Figure 3b), is either returned to the bone marrow to be reused or is converted to a bile pigment called bilirubin.

Bilirubin is not, however, the immediate product of hemoglobin degradation. Breakdown of hemoglobin begins with the denaturation and subsequent removal of the protein globin. The oxidation and opening of the tetrapyrole ring followed by the removal of the iron forms the primary product of hemoglobin catabolism, biliverdin. Biliverdin is then reduced in the presence of hydrogen to form bilirubin. (see Figure 4) Nearly 300 milligrams of bilirubin are formed daily as a result of the catabolism of hemoglobin. The structure of bilirubin in one of it's forms can be seen in Figure 5. This process of erythrocyte degradation occurs in the reticuloendothilial cell of the liver or spleen as

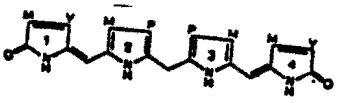
Hemoglobin

CATABOLISM



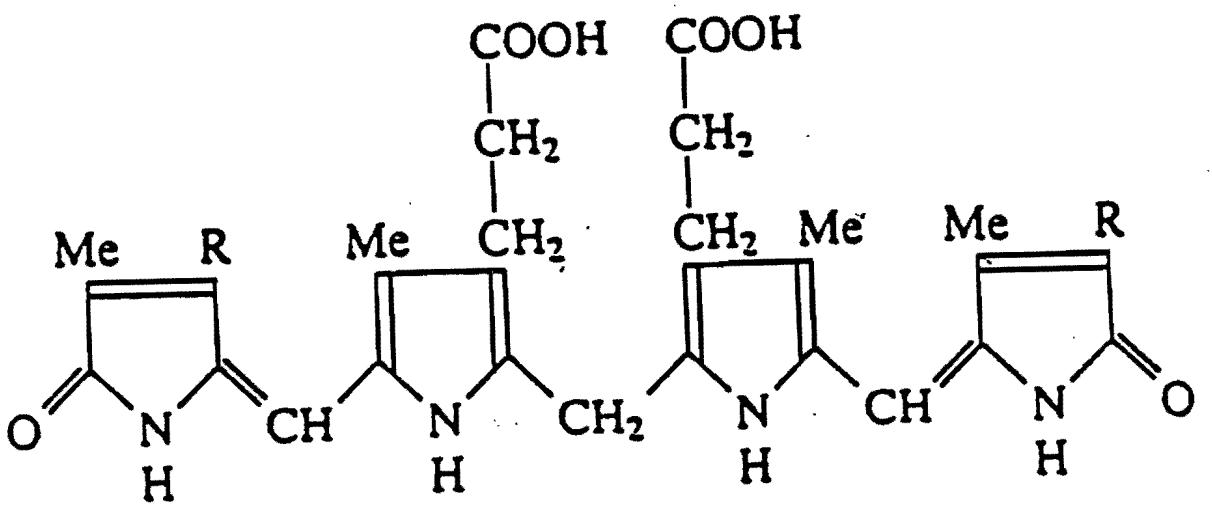
Biliverdin (C_{33} H_{34} O_6 N_4)

REDUCTION OF A METHANE BRII



Bilirubin (C_{33} H_{36} O_6 N_4)

Figure 4. Catabolism of Hemoglobin
The heme portion of hemoglobin loses it's iron. It is then oxidized and there is an opening of the tetrapyrole ring to form a more linear intermediate: Bilverdin. Biliverdin is then reduced to form bilirubin.



Bilirubin

Figure 5. **Structure of Bilirubin**
The structure above is a representation of one of the possible forms of bilirubin. This form is not water soluble due to its low polarity. In physiological conditions the liver converts bilirubin into a water soluble form which can be excreted.

previously mentioned. Under normal circumstances bilirubin is passed into the bile where it gives stool its characteristic color.

Sometimes, however, this elaborate scheme is disturbed by certain pathological conditions. Many disorders can result in the increase of bilirubin in the blood, (see Table 1), and if an inability to dispose of this bilirubin occurs, a common condition known as jaundice may ensue. Jaundice is a condition in which bilirubin becomes accumulated in the blood and body tissue and causes a yellowish tinge in the skin and sclera. When conjugated bilirubin levels in the blood exceed the normal amount, it begins to be filtered at the glomerulus and appears in the urine where it is normally present only in trace amounts, if at all. This situation is typically indicative of a number of liver or biliary malfunctions ranging from prolonged fasting to cirrhosis of the liver (see Table 1). Patients with jaundice typically notice a darkening of the urine and tears, but their stool becomes lighter. Jaundice is often seen in newborn infants but is not, however, the result of some devastating disease. In newborns and premature infants, the liver is often immature and bilirubin may deposit in the fatty tissue, and the babies subsequently become jaundiced. Many babies are born with this condition and lead long and healthy lives

TABLE 1

CAUSES OF HIGH SERUM BILIRUBIN
PATHOLOGICAL CONDITIONS
- Congestive Heart Disease - Pulmonary Embolism - Dubin-Sprinz Syndrome - Hepatitis - Cirrhosis - Neoplasia - Alcoholism - Biliary Obstruction - Infectious Mononucleosis - Gilbert's Disease - Dubin-Johnson Syndrome - Anorexia - Prolonged Fasting - Bleeding with Hematoma - Pernicious Anemia - Hemolytic Anemia - Erythroblastosis Fetalis - Crigler-Najjar's Disease - Rotor's Disease
MEDICATIONS THAT INCREASE BILIRUBIN BY IN VIVO ACTION
- Phenothiazides - Certain Antibiotics - Sulfonylureas - Oral Contraceptives - Anabolic-Androgenic Steroids - Halothane - Aminosalicylic Acid - Isoniazid - Methyldopa - Indomethacin - Pyrazinamide - other drugs which may cause hypersensitivity cholestasis

free of the multitude of causes for jaundice in the adult. Due to the developmentally immature blood-brain barrier, the brain of a baby can become jaundiced if enough bilirubin is deposited. If this condition goes untreated, neurological damage may occur. The infant may demonstrate an absence of infant reflexes such as sucking. Reduced motor tone, lethargy and a high pitched cry are also symptomatic of this condition. If the condition is untreated long enough, permanent mental retardation may occur.

There are a number of clinical tests to determine urinary and serum levels of bilirubin in an effort to diagnose a patient (See Table 2). The two most commonly employed methods for detecting bilirubin, the Malloy-Evelyn method and the Jendrassik-Grof method, measure the absorbance of a colored complex resulting from the reaction of bilirubin with some reagent. Though it is frequently used, the Malloy-Evelyn method exhibits significant hemoglobin interference. The Malloy-Evelyn method is performed in very acidic solution spectrophotometrically at a wavelength of 560nm. The Jendrassik-Grof method is much more sensitive at low bilirubin concentrations than is the Malloy-Evelyn method. It can be performed in neutral solution as well as very alkaline (pH 13) solution at a wavelength of 600nm.

The bilirubin oxidase method is very similar to the

TABLE 2

METHOD	TYPE OF ANALYSIS	USAGES
Malloy-Evelyn	kinetic, end point, with or without blank	Very frequently used
Jendrassik-Grof	kinetic, end point, with or without blank	Most commonly used
Bilirubinometer	direct spectrophotometric	Simple to perform Interference with carotinoids
High Performance Liquid Chromatography	chromatographic separation	Research use only
Bilirubin Oxidase	kinetic, endpoint, enzymatic	Newly Available
Spectral Shift	endpoint	Available on Kodak or Ektachem only

proposed spectroelectrochemical assay to be developed. This method is newly available and may become a reference method. It exhibits no hemoglobin interference. The bilirubin oxidase method is very specific because it employs an enzyme. The enzymatic oxidation of bilirubin to biliverdin and water occurs here much in an analogous manner to that of the spectroelectrochemical assay proposed. The major difference between the techniques is that an enzyme is used in the bilirubin oxidase method while an electrode will perform the oxidation in the spectroelectrochemical assay. The proposed spectroelectrochemical assay will be much quicker and simpler in terms of sample preparation, though it will of course not be as specific as the enzymatic method.

Much research and investigation into methods for the determination of bilirubin in serum has been published. Enzymatic and electrochemical techniques for determining bilirubin in serum have been tested. Of the experimental publications that have been surveyed, most have been concerned with the unconjugated forms of bilirubin which are not water soluble. The chemistry of bilirubin is not well understood due to its lack of stability and solubility in aqueous solutions, including physiological fluids.

B. SPECTROSCOPY

Spectroscopy is a powerful tool for determining qualitative and quantitative information about an analyte. Basically, UV/Visible spectroscopic techniques can yield information about the species of interest by passing electromagnetic radiation of various wavelengths through the sample and monitoring at which wavelengths the sample absorbs or transmits the radiation. A solution's wavelength of maximum absorbance can be used as an identifying property in some cases. However, typically more information must be extracted to identify the analyte. For example, data derived from a plot of absorbance versus concentration at the wavelength of maximum absorbance can yield a characteristic molar absorptivity of the analyte in solution. This relationship between absorbance and concentration is known as Beer's law (A=abc), where A is absorbance, a is molar absorptivity, b is pathlength, and c is analyte concentration.

C. VOLTAMMETRY

Voltammetry is useful in providing information about an analyte in solution by measuring the current which flows through an electrochemical cell as a

function of applied potential. Voltammetric analyses typically require the use of three electrodes: a working electrode at which the reaction of interest occurs; the reference electrode, whose potential remains constant throughout the experiment; and the auxiliary or counter electrode, which serves to conduct current from the source through the solution to the working electrode. Oxidation and/or reduction of the analyte species is induced and the current produced by this reaction at the working electrode is measured as a function of the working electrode potential.

Presented herein are the results of preliminary spectral and voltammetric studies of bilirubin in aqueous solution. These studies were performed to aid in the development of a spectroelectrochemical assay for bilirubin in urine to be used in the medical field for patient diagnoses.

V. EXPERIMENTAL

A. REAGENTS

A vial of bilirubin ditaurate, (Lot # 011091/B-850) was obtained from Porphyrin Products, P.O. Box 31 Logan, Utah 84321. This bilirubin conjugate is highly water soluble at all pH values greater than 7. Storage conditions called for the bilirubin conjugate to be stored in a cold, dark place and suggested it be stored under an inert gas such as nitrogen or argon. The bilirubin conjugate was stored in the freezer in a brown bottle and covered with aluminum foil to reduce photoreaction. Due to the fact that dry bilirubin conjugate is slightly hygroscopic and will pick up moisture from the air, the vial was sealed with wax. The formula weight of this bilirubin conjugate was listed as 844.7 grams per mole. For the spectral studies the bilirubin conjugate was dissolved in distilled/deionized water.

Tris buffer was made with Tris from Sigma Chemical Company (99.9+% purity). The formula weight of Tris was listed as 121.1 grams per mole. In order to obtain a 0.1M solution of Tris buffer, 12.1 grams of Tris were dissolved in distilled/deionized water, adjusted to a pH of 8.3 with acetic acid, and diluted to 1 liter.

The 0.01M bilirubin conjugate solution for voltammetric study was prepared by dissolving 0.0422 grams of the bilirubin conjugate in 50 mL of the Tris buffer solution.

B. INSTRUMENTATION

All spectral studies were done using a double beam spectrometer like the one seen in Figure 6. In particular, a Bausch & Lomb Spectronic 2000 was employed. The Spectronic 2000 has a tungsten filament source, a grating monochromator, one centimeter glass cuvettes, a photomultiplier tube detector and two readout devices (an LED and an X-Y recorder).

Voltammetric studies were carried out using a BAS-CV27 voltammagraph. A sample cell much like the schematic shown in Figure 7 was employed. The sample solution was purged and blanketed with nitrogen gas. The working electrode used was a glassy carbon disk, the reference electrode was a AgCl/Ag (Silver-Chloride/Silver) electrode, and the auxiliary electrode was a platinum wire.

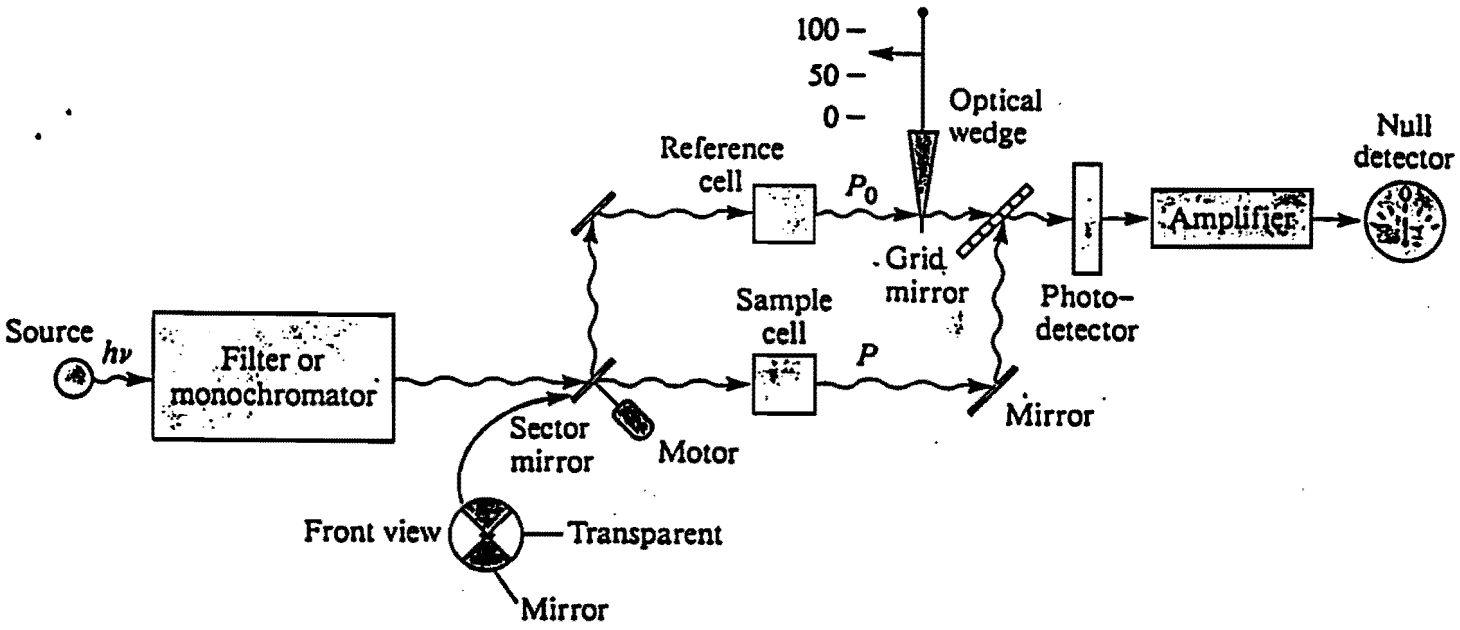


Figure 6. Schematic of a double-beam spectrometer
The components of the Spectronic 2000 used are as follows: Tungsten filament source, grating monochromator, 1 cm glass cuvette, photomultiplier detector, readout devices were an LED and an X-Y recorder.
From Skoog, et. al. "Fundamentals of Analytical Chemistry, 6th edition".

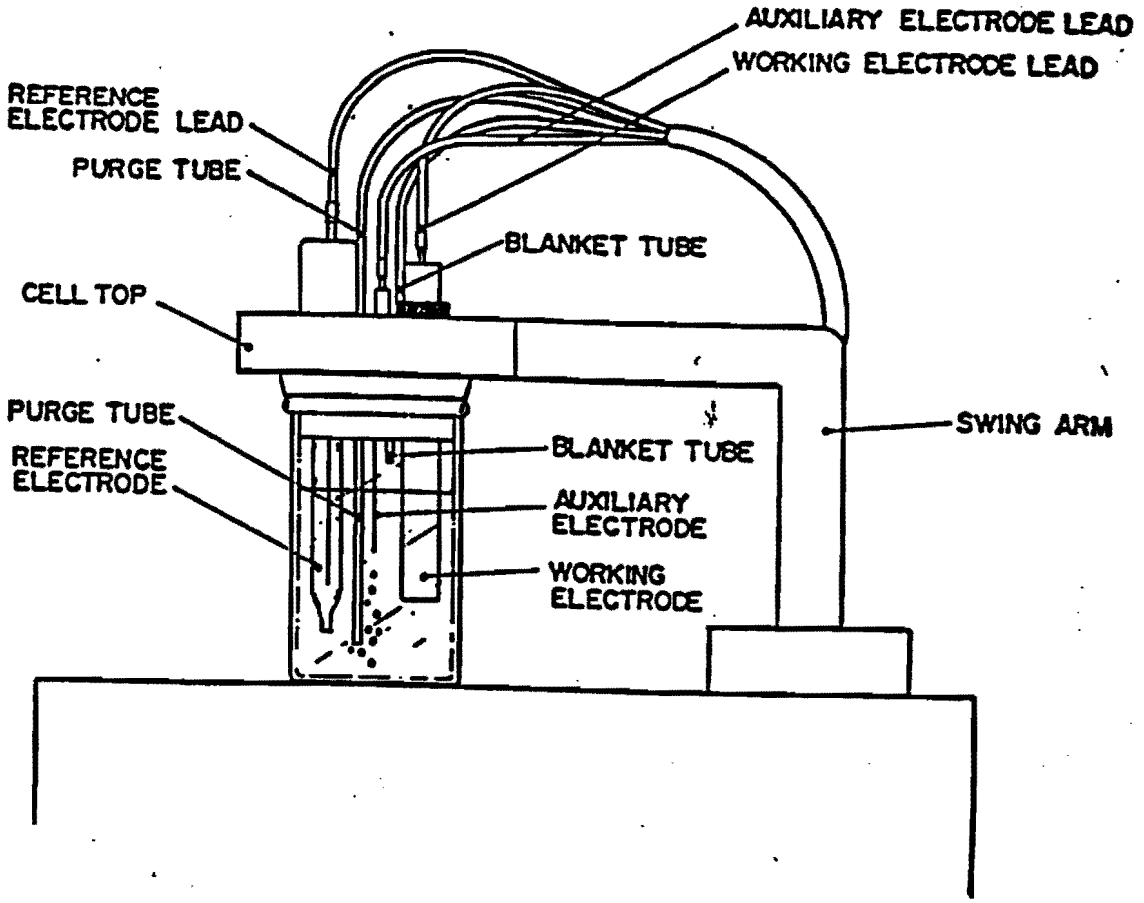


Figure 7. Schematic of a typical Voltammetric cell
Shown is an assembled cell. In particular, a AgCl/Ag reference electrode, a platinum auxiliary electrode, and a glassy carbon working electrode were employed.
From Skoog, et. al. "Fundamentals of Analytical Chemistry, 6th edition".

C. PROCEDURE

a. Spectral Studies

Aqueous bilirubin conjugate was exposed to a range of wavelengths and the wavelength of maximum absorbance was determined. The Spectronic 2000 was then set to monitor absorbance at this wavelength. Absorbance at this wavelength was determined for a number of bilirubin solutions of differing concentrations.

b. Voltammetric Studies

Solutions were placed in an electrochemical cell and degassed by bubbling nitrogen gas through the solution. Nitrogen gas was subsequently blanketed over the solution. The solution was stirred with a magnetic stir bar between scans. Stirring was stopped 1-2 minutes prior to application of potential. Voltammetric scans were performed on solutions of differing concentrations and at different scan rates.

VI. RESULTS & DISCUSSION

A. Spectral Studies

a. Wavelength of Maximum Absorbance

The exposure of the aqueous bilirubin solution to a number of wavelengths in the UV/Visible region consistently yielded of wavelength of maximum absorbance of 450nm which can be seen in the bilirubin spectrum shown in Figure 8.

b. Determination of Molar Absorptivity

Absorbances for bilirubin solutions of differing concentrations at 450nm were determined as listed in Table 3.

TABLE 3

Concentration (10^-7 M)	Mean Absorbance (au)	Standard Deviation
23.2	0.039	0.001
46.4	0.079	0.002
69.6	0.122	0.002
92.8	0.161	0.001
116	0.203	0.001

(GRAPHICAL REPRESENTATION OF THESE DATA CAN BE FOUND IN FIGURE 9)

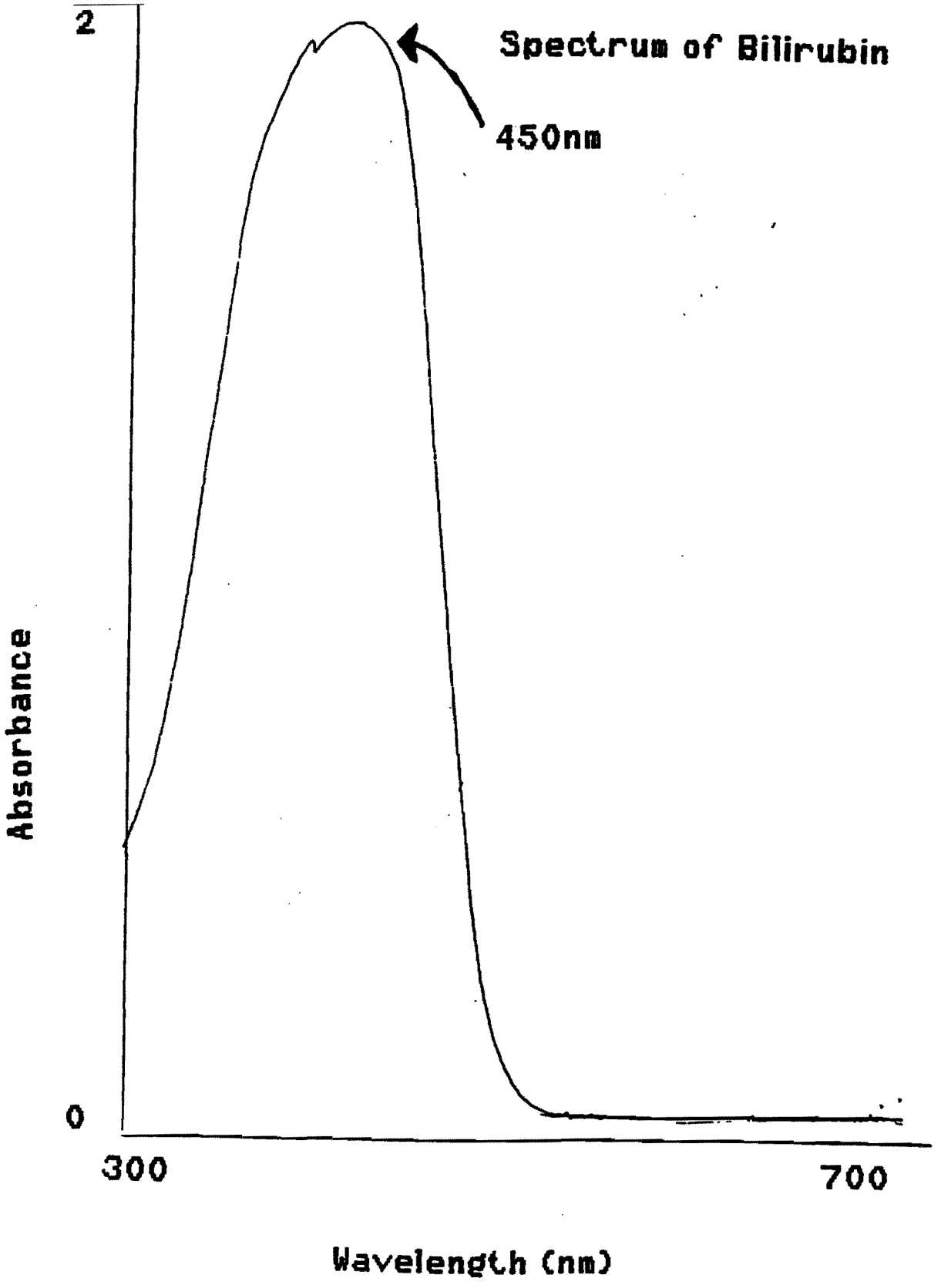


Figure 8. **Spectrum of Bilirubin**
A series of wavelengths from 700 to 300nm were scanned and 450nm was consistently the wavelength of maximum absorbance A cell of optical pathlength 1cm was used. Bilirubin concentration was 1.16 X 10^-4M.

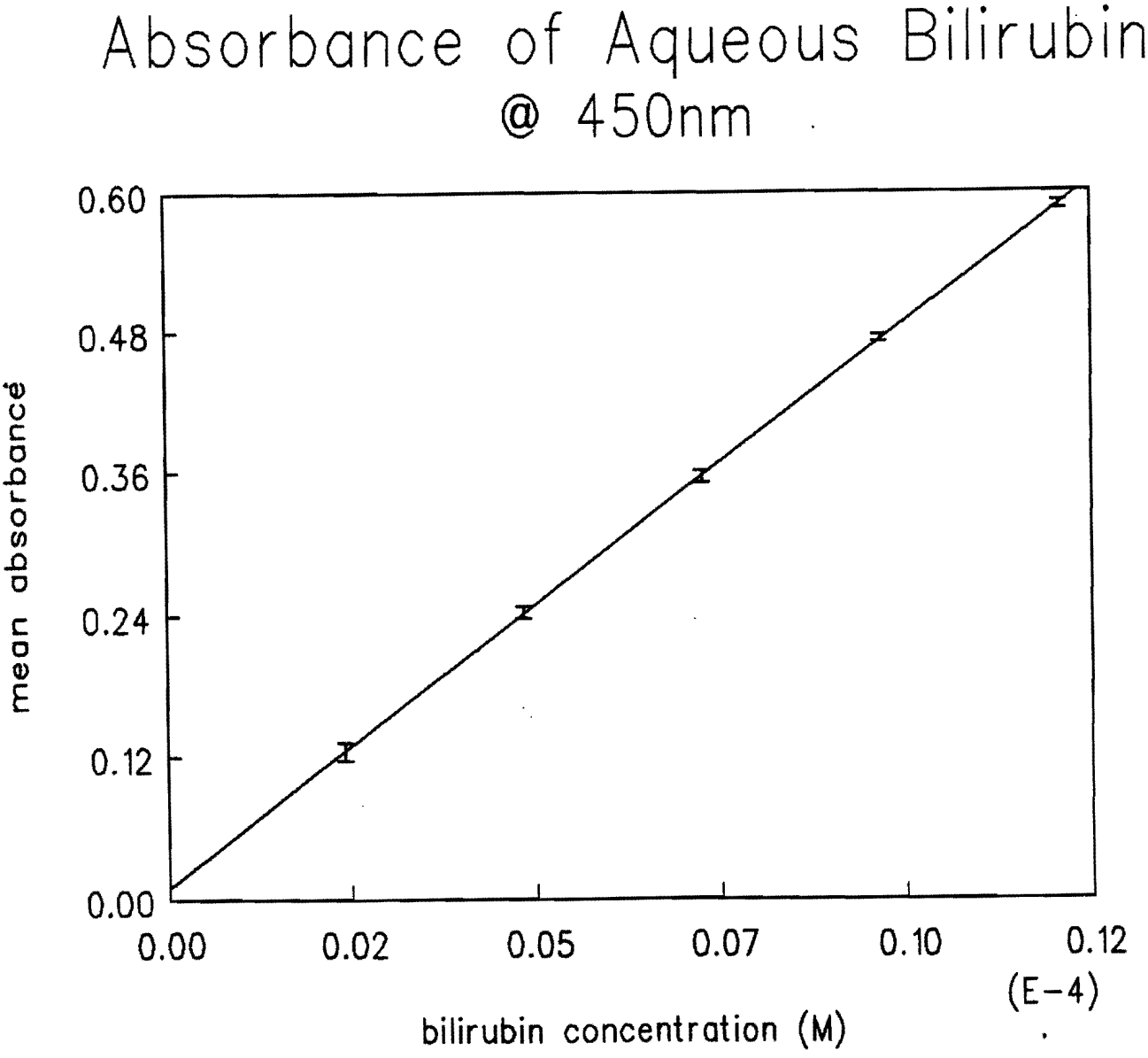


Figure 9. Absorption of Bilirubin @ 450nm: A plot of Absorbance vs. Concentration
The measurement of absorbance at 450nm for a number of bilirubin solutions varying in concentration indicated that the absorbance is directly proportional to the concentration of aqueous bilirubin. (Cell pathlength was equal to 1 cm)

A plot of absorbance versus concentration for these data was then constructed. The concentration was known, and the optical pathlength was measured with Vernier calipers allowing the employment of Beer's law to determine the molar absorptivity of the aqueous bilirubin solution.

Beer's Law $A=abc$

$$a = \frac{\text{slope of Absorance vs. Concentration Plot}}{b}$$

b = pathlength of cell

Measured with Vernier caliper's to be 1.00cm

molar absorptivity (a) = 49,900 cm-1 M-1

The expected molar absorptivity derived from information provided by Porphyrin Products was: 54000 cm-1 M-1. When compared to the value experimentally determined, a 7.59% deviation from the expected value is observed.

$$\% \text{ Difference} = \frac{54{,}000 - 49{,}900}{54{,}000} \times 100\% = 7.59\%$$

Some impurity of the sample was therefore indicated.

c. Degradation of Bilirubin

The degradation of aqueous bilirubin was also of interest and warranted a study. Therefore, the stock solution from which the previous dilutions were made was saved and the same study was conducted twice more on different days to determine the extent of bilirubin degradation in an aqueous solution. Due to the air and photosensitivity of these solutions, they were stored in a cool, dark place without significant exposure to light of air.

TABLE 4

Days after solution preparation	Calculated Molar Absorptivity (cm-1 M-1)
1	49,900
6	39,900
22	17,640

(GRAPHICAL REPRESENTATION OF THESE DATA CAN BE FOUND IN FIGURE 10)

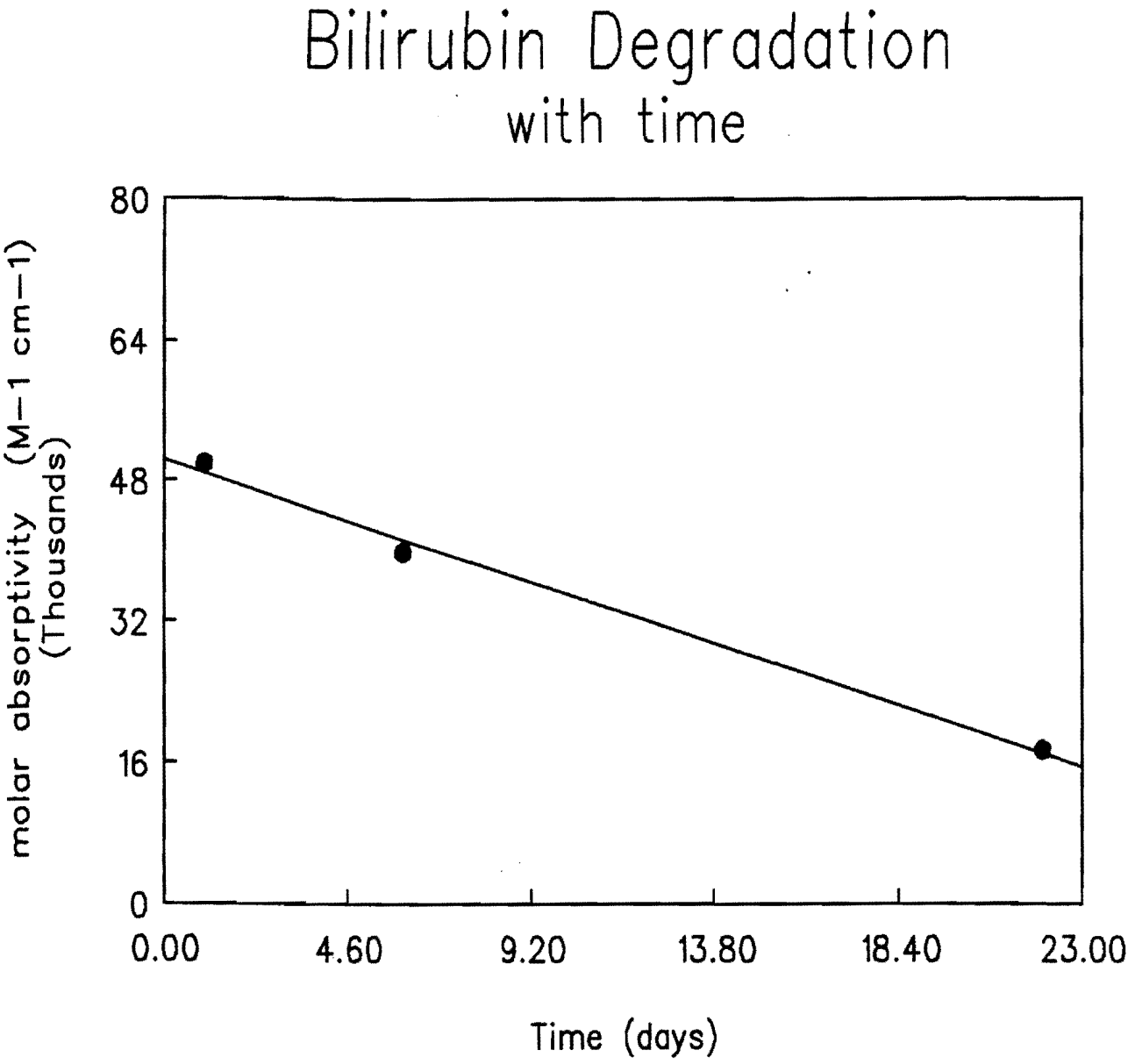


Figure 10. Bilirubin Degradation Plot
The molar absorptivity of aqueous bilirubin was determined for several days after its preparation. The results indicate that immediate determination of bilirubin is imperative for accurate results.

B. VOLTAMMETRIC STUDIES

Since this thesis involves work preliminary to studies of spectroelectrochemistry of bilirubin, the next step was to determine the characteristic electrochemistry of the water soluble form of bilirubin in solution.

a. Ferrocyanide (Scan Rate Study)

There are many factors which can affect the observed current in a voltammetric experiment. For a simple diffusion-controlled electrode reaction, it is expected that the observed current should be proportional to the square root of the scan rate and should exhibit a zero intercept. In order to exemplify this expectation, a preliminary voltammetric study was performed on a system of known electrochemistry. It is known that the voltammetry of ferrocyanide involves a simple diffusion-controlled process. A scan rate study was carried out. Shown in Table 5 and Figure 11 are the results.

TABLE 5

Scan Rate (mV/s)	Square Root of Scan Rate (mV/s)	Peak Current (micro amps)
20	4.472	6.5
50	7.071	10.5
100	10.000	14.0
200	14.142	19.5
300	17.321	23.0
400	20.000	26.0
500	22.361	29.0

(GRAPHICAL REPRESENTATION OF THESE DATA CAN BE FOUND IN FIGURE 11)

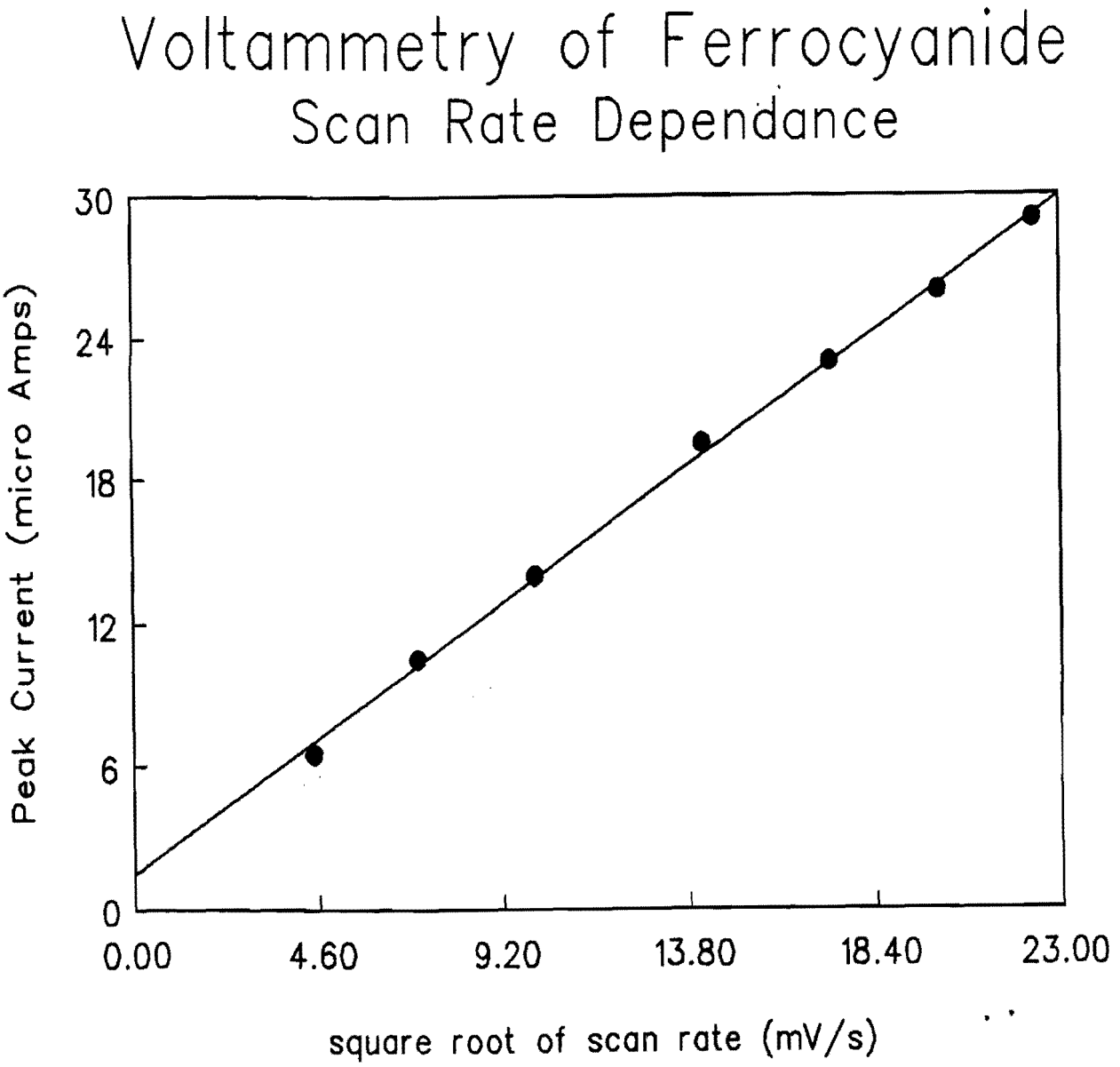


Figure 11. Voltammetric Plot of Ferrocyanide
A scan rate study of ferrocyanide exemplifies the expected dependance for a simple diffusion-controlled electrode reaction. Voltammetry was performed in 1.15mM aqueous ferrocyanide solution at a glassy carbon electrode. The reference electrode was AgCl/Ag and the auxiliary electrode was a platinum wire.

b. **Bilirubin**

The bilirubin conjugate in Tris buffer, was used in all the voltammetric studies. The first step was to determine the solvent limits within which to work. The solvent was placed in the electrochemical cell alone and the potential scan was extended until a noticeable current was observed. After the solvent limits had been determined, a cyclic voltammagram of the Tris buffer within these limits was obtained.
The bilirubin solution was then placed in the electrochemical cell and a cyclic voltammagram of was obtained within the limits of the solvent to look for any noticeable feature. An oxidation wave was noticed and the voltammagraph limit settings were narrowed to scan between 0.00 and 0.87V, the region where this wave was observed. At this time, a voltammagram of the Tris buffer in this region was performed to provide information about the type of background that could be expected from the use of Tris buffer as a solvent (see Figure 12a). It is assumed that anything other than that seen in the Tris buffer voltammagram is the result of bilirubin in the solution. The bilirubin conjugate solution was subsequently scanned at this more narrow potential range (see Figure 12b). The feature believed to

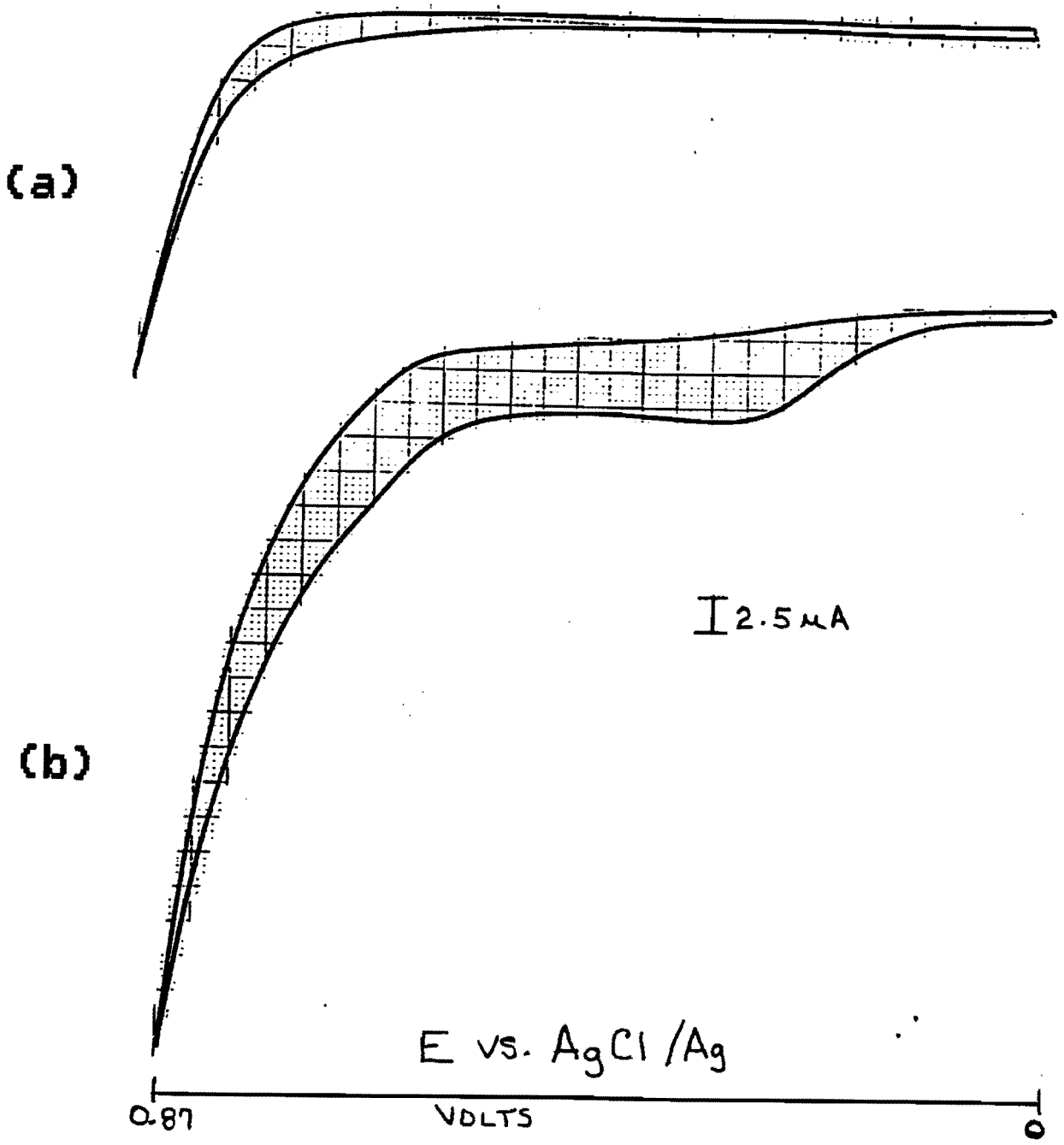


Figure 12. Comparison Voltammetric scans of Tris Buffer and Bilirubin in Tris. (a) scan of Tris buffer at a glassy carbon working electrode between 0.00V and 0.87V. (b) scan of bilirubin dissolved in Tris buffer under the same conditions (vs. AgCl/Ag reference electrode). The auxiliary electrode was a platinum wire. Scan rate was 50 mV/s. The bilirubin solution was 10^-3M.

be a bilirubin oxidation wave was again present at a peak potential of 0.58V (vs. AgCl/Ag).

i. Scan Rate Study

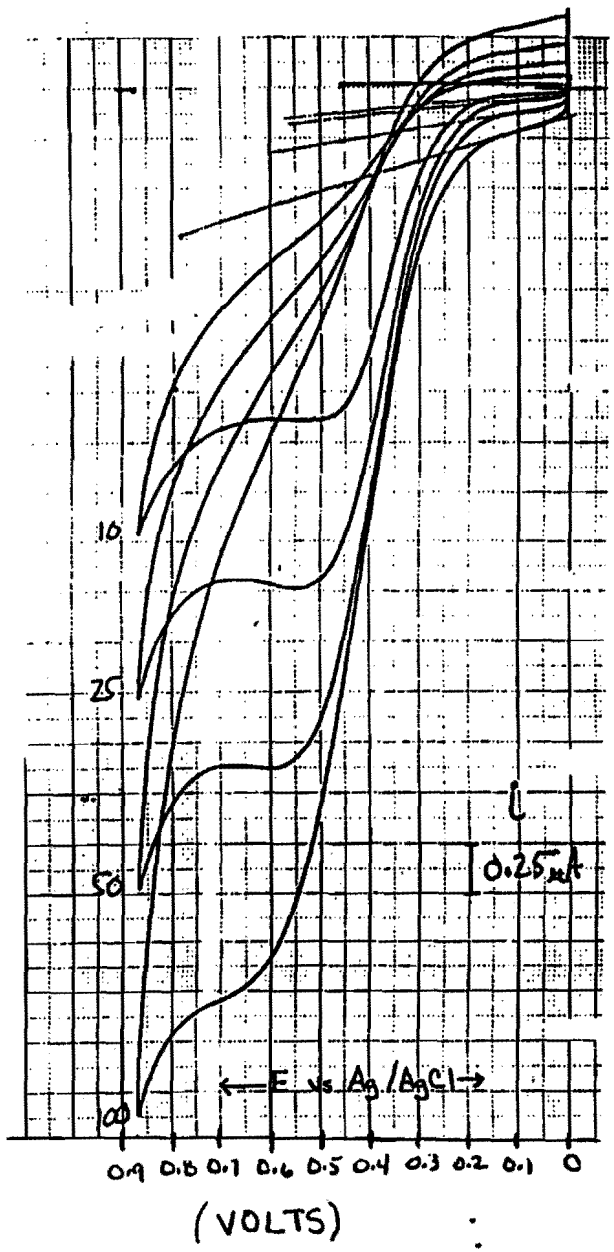
To ensure that what was being seen was the result of bilirubin oxidation, a scan rate study, like the one performed earlier for ferrocyanide, was carried out.

TABLE 6

Scan Rate (mV/s)	Square Root of Scan Rate (mV/s)	Peak Current (micro amps)
10	3.1	1.5
25	5.0	2.3
50	7.1	3.1
100	10.0	3.9

(SCANS AND GRAPHICAL REPRESENTATION OF THESE DATA CAN BE FOUND IN FIGURES 13)

The graphical representation was nicely linear as expected, though there was some deviation from the zero intercept. This is curious and may be the result of adsorption of bilirubin, though this phenomenon has not been further examined.



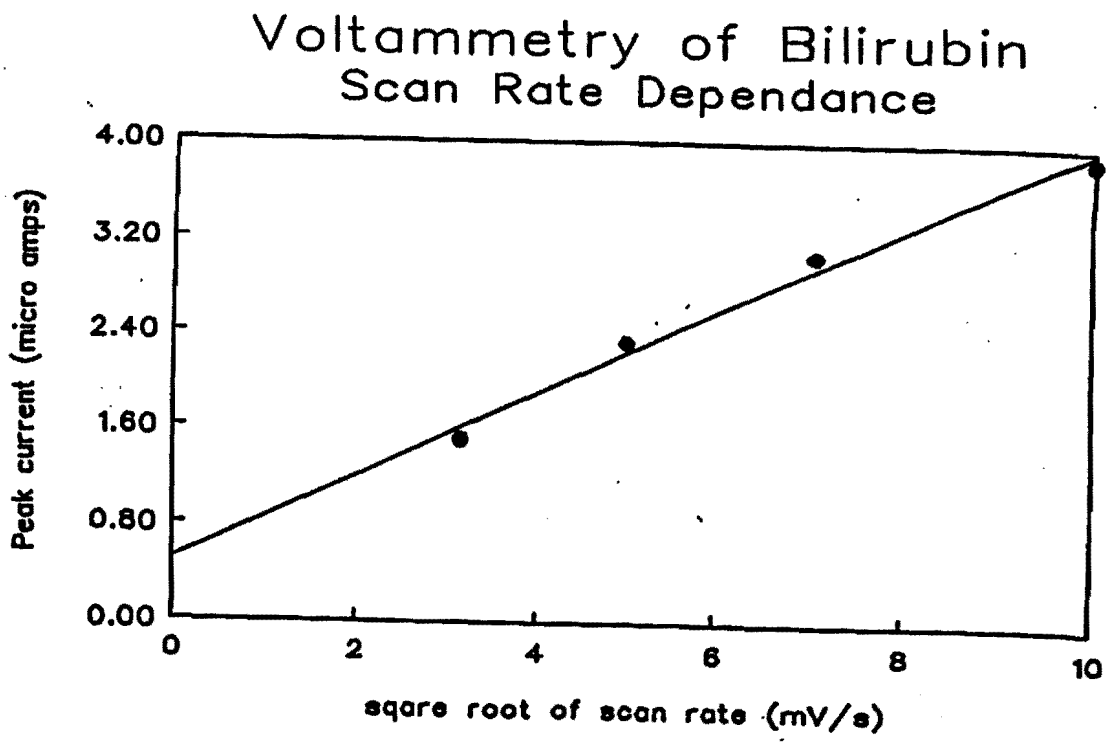


Figure 13. Voltammetry of Bilirubin: Scan Rate Dependance Each scan was conducted from 0.00V to 0.87V for a 10^-3M bilirubin conjugate solution. The working electrode was a glassy carbon disk. The auxiliary electrode was a platinum wire. The oxidation wave was present at 0.58V (vs. AgCl/ Ag reference electrode).

ii. Concentration Dependance Study

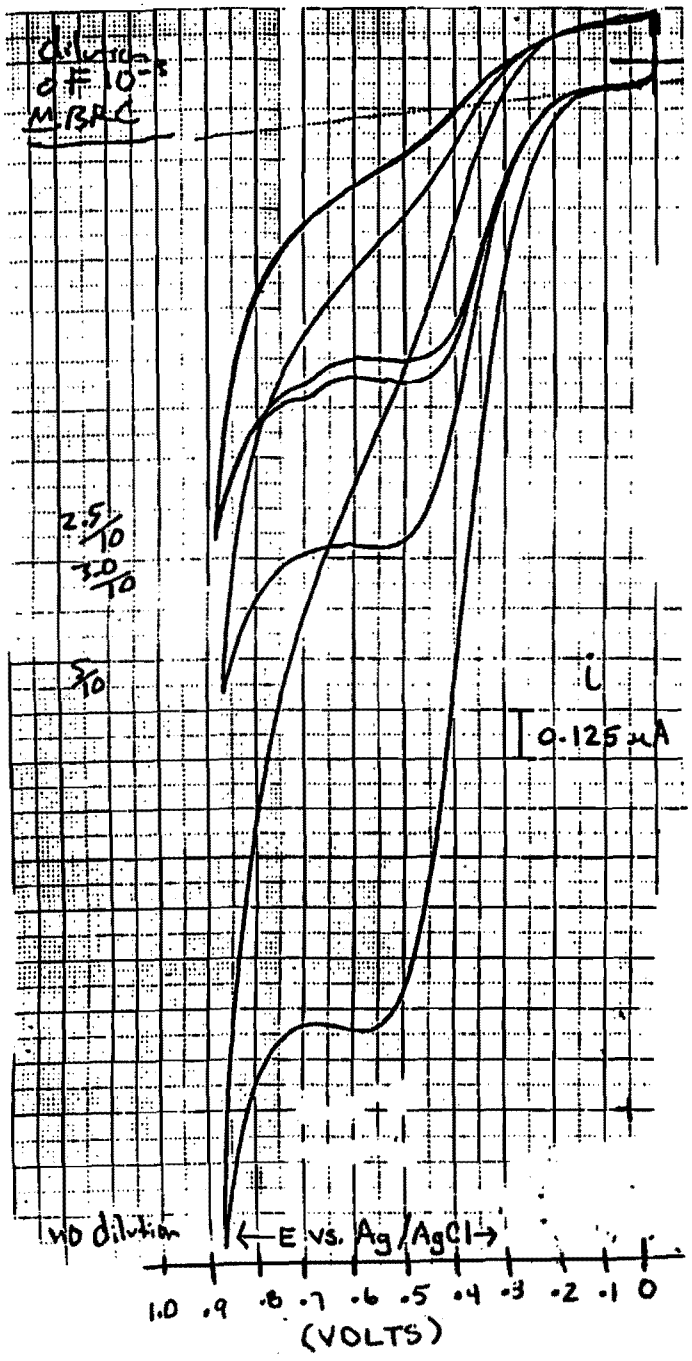
A concentration dependance study followed in which bilirubin solutions of differing concentration were scanned over the same potential range as a means of confirming the idea that the wave seen was the result of bilirubin oxidation. A linear increase of current proportional to the increase in concentration was expected.

TABLE 7

Concentration (10^-3 M)	Peak Current (micro amps)
1.00	2.30
0.50	1.10
0.30	0.69
0.25	0.64

(SCANS AND GRAPHICAL REPRESENTATION OF THESE DATA CAN BE FOUND IN FIGURE 14)

A plot of these data was linear with a virtually zero intercept as expected. This leads to the conclusion that the observed voltammetric feature is due to oxidation of bilirubin.



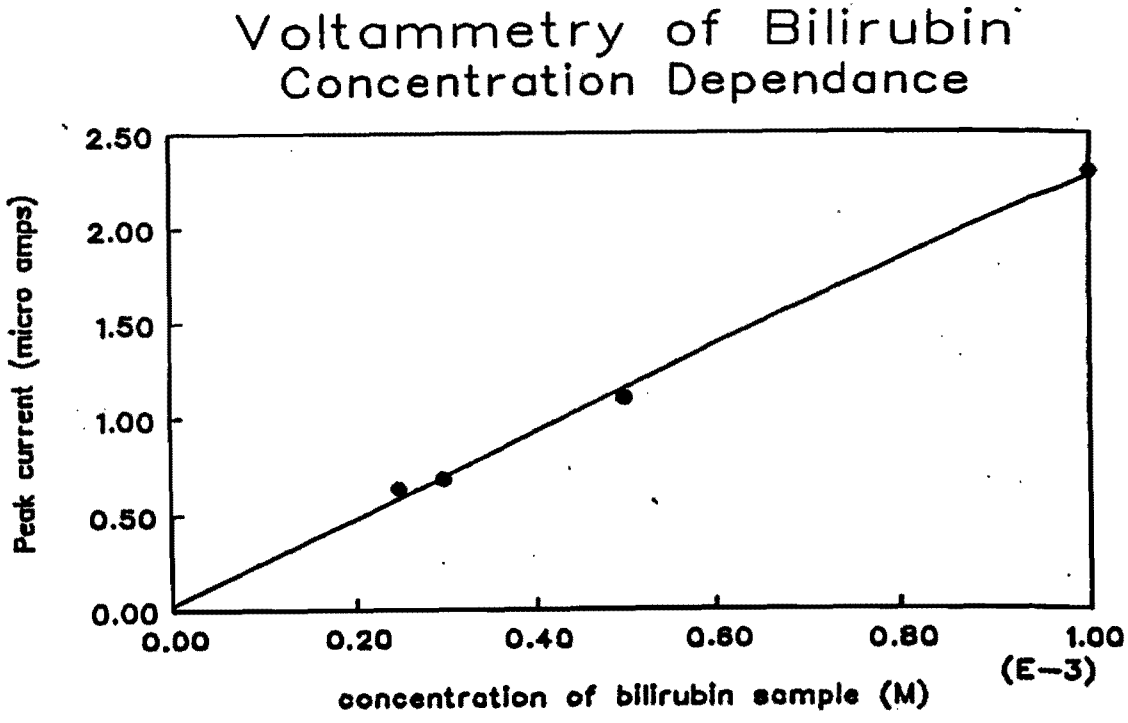


Figure 14. Voltammetry of Bilirubin: Concentration Dependance Each scan was performed from 0.00V to 0.87V at glassy carbon electrode and a scan rate of 100 mV/s. The oxidation wave appeared at 0.58V (vs.AgCl/Ag reference electrode). A platinum wire acted as the auxiliary electrode.

VII. FUTURE WORK

As the name implies, spectroelectrochemistry involves the simultaneous application of the two analytical techniques previously discussed; spectroscopy and electrochemistry (or, more specifically in this case, voltammetry). The combination of these studies requires a specialized cell similar to both those described earlier. A schematic of such a specialized cell is seen in Figure 15. Research towards the development of this spectroelectrochemical assay for bilirubin in body fluids, in particular urine, is scheduled to continue with subsequent publication of the results anticipated.

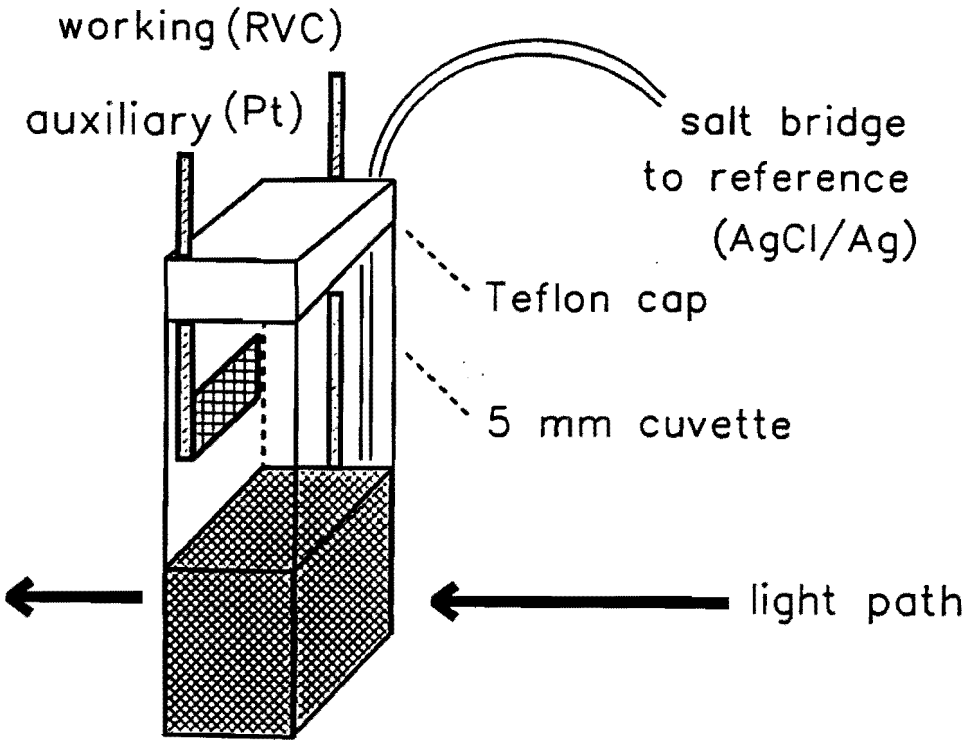


Figure 15. Schematic of a Spectroelectrochemical Cell The spectroelectrochemical studies will be performed in this cell and will utilize a platinum auxiliary electrode, a AgCl/Ag reference electrode and reticulated vitreous carbon as the working electrode. The optical pathlength for this cell is 5mm.

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IX. BIBLIOGRPHY

Brady, J.E. and J.R. Holum, Fundamentals of Chemistry, Third Edition 1988. John Wiley & Sons, Inc., New York. pp. 934, 1037.

Coleman, J.T., In Vivo Analysis of Bilirubin for the Early Detection of Erythroblastosis Fetalis.

Considine, D.M., Van Nostrand's Scientific Encyclopedia Seventh Edition 1989. Van Nostrand Reinhold Publishing, New York. pp. 354-355.

Doumas, B.T., B. Perry, B. Jendrzejczak, and L.Davis, Measurement of Direct Bilirubin by Use of Bilirubin Oxidase. Clinical Chemistry. 33/8, 1349-1353 (1987).

Fessenden, R. and J. Fessenden, Organic Chemistry, Fourth Edition 1990. Brooks/Cole Publishing Co, Pacific Grove, CA. pp. 951, 984, 987-988.

Hickman, C.P., L.S. Roberts, and F. M. Hickman, Biology of Animals, Fourth Edition 1986. Times Mirror/Mosby College Publishing, St. Louis. pp. 82, 177, 193.

Ihara, H., H. Nakamura, M. Yoshida, T.W. Wu, Effect of Bilirubin Covalently Attached to Albumin on Measurement of Serum Creatnine. Clinical Chemistry. 38/2, 321-322 (1992).

Koch, T.R. and Olumide O. Akingbe, Feasibility of Measuring Free and Total Bilirubin Electrochemically in Serum. Clinical Chemistry, 27/7, 1295-1299 (1981).

Krupp, M.A., L.M. Tierney, E. Jawetz, R.L. Roe, and C.A. Camargo., Physician's Handbook Twenty-First Edition 1985. Lange Medical Publications, Los Altos, CA. pp. 126, 230-232

Marieb, Elaine N., Human Anatomy and Physiology 1989. Benjamin/Cummings Publishing Co., New York. pp. 138, 402, 570-572, 790, 877.

McGraw-Hill Encyclopedia of Science and Technology Seventh Edition 1992. McGraw-Hill, Inc., New York. Vol. 2 p. 554.

Moussa, F., Gaoussou Kanoute, Christine Herrenknecht, Pierre Levillain and Francois Trivin, Electrochemical Oxidation of Bilirubin and Biliverdin in Dimethyl Sulfoxide. Analytical Chemistry. 60/11, 1179-1185 (1988).

Pradko, J.R., S. Pons, J.F. McAleer and A.S. Hinman, A Spectroelectrochemical Investigation of the Oxidation of Bilirubin IX-a in Dimethylformamide. Bioelectrochemistry and Bioenergetics. 13, 267-285 (1984).

Skoog, D.A., D.M. West, and F.J. Holler, Fundamentals of Analytical Chemistry, Sixth Edition 1992. Saunders College Publishing.

Slifstein, CH. and M. Ariel, pH Effects on the Reduction of Bilirubin in Aqueous Media. Journal of Electroanalyical Chemistry. 75, 551-564 (1977).

Slifstein, CH. and M. Ariel, The Electrochemistry of Bilirubin in Dimethylsulfoxide. Electroanalytical Chemistry and Interfacial Electrochemistry. 48, 447-463 (1973).

Van Norman, J.D., Electroanalytical and Spectral Study of Bilirubin in Dimethyl Formamide. Analytical Chemistry. 45/1, 173-175 (1973).

Van Norman, J.D., and Robert Szentirmay, Chemistry of Bilirubin and Biliverdin in N,N-Dimethylformamide. Analytical Chemistry. 46/11, 1456-1464 (1974).

Van Norman, J.D., and Michelle M. Humans, Bilirubin-Metal Ion Complexes. Analytical Chemistry, 46/7, 926-929 (1974).