ELECTROCHEMICAL STUDIES OF BILIRUBIN AND RELATED COMPOUNDS IN AQUEOUS SOLUTIONS

by

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Submitted in Partial Fulfillment of the Requirements
for the Degree of
Master of Science
in the
Chemistry
Program

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YOUNGSTOWN STATE UNIVERSITY
June, 1973

ABSTRACT

ELECTROCHEMICAL STUDIES OF BILIRUBIN
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This investigation is concerned with the electrochemical behavior of the bile pigments-bilirubin and biliverdin. Both oxidation with solid platinum electrodes and reduction with a hanging drop mercury electrode were attempted for bilirubin in buffer systems ranging from pH 4.0 to pH 12.0. Oxidation proved unsuccessful, however, reduction of bilirubin was accomplished at a reduction potential of approximately -1.40 volts vs. S.C.E. in a physiological buffer of pH 7.8. Since the process was found to be irreversible, diffusion constants were calculated using the Randles-Sevcik equation for both reversible and irreversible systems.

Metal ion-bilirubin complexes and their electrochemistry were investigated. Metal ions used were Zinc (II), Copper (II), Nickel (II), Cobalt (II), Cadmium (II), Mercury (II), and Iron (II). Shifts of the reduction potential used to calculate stability constants were obtained successfully with Zinc (II), Cadmium (II), and Iron (II).

Reduction of biliverdin was also accomplished using the hanging drop mercury electrode. An irreversible reduction of biliverdin in a phosphate buffer of pH 8.0 was accomplished at a reduction potential of approximately -0.40 volts vs. S.C.E. Diffusion coefficients were calculated using the Randles-Sevcik equation for both reversible and irreversible systems.

ACKNOWLEDGEMENTS

I wish to thank Dr. John D. Van Norman, my thesis advisor for his suggestion of the thesis project and especially for his guidance and limitless cooperation throughout the research. I would also thank the Chemistry Department faculty and especially Dr. Leon Rand, Dr. Thomas N. Dobbelstein, and Dr. Leonard B. Spiegel for their suggestions and advice. I wish to thank my parents for their vast understanding and constant support during this time and always.

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CHAPTER I

INTRODUCTION

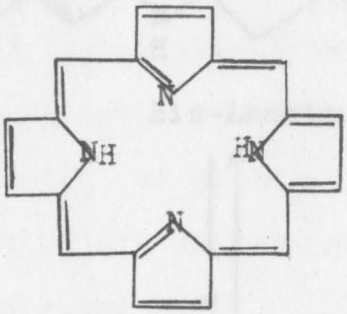
A. Nature of Bilirubin

Bilirubin is a bile pigment and a linear tetrapyrrole. Linear tetrapyrroles are porphyrin derivatives and naturally occuring porphyrins are derivatives of the fundamental substance Porphin. Porphin ($C_{20}H_{14}N_4$) contains four pyrrole-like rings linked by =CH- groups in a ring system as is shown in Figure 1.

The porphyrin structure contains a central sixteen-membered ring formed from twelve carbon atoms and four nitrogen atoms. Figure 1 also shows the structural formula of the porphyrin structure with the pyrrole rings represented by a pentagon where the hydrogens and carbons are omitted. Each of the numbered carbons can be a substituted position.

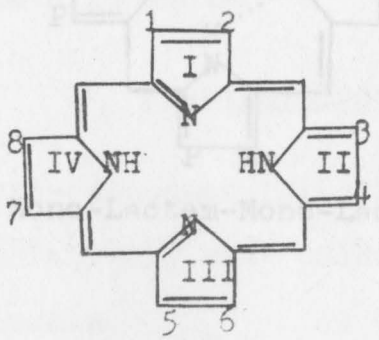
The empirical formula of bilirubin is $C_{33}H_{36}O_6N_4$ and some structural formulas of bilirubin are represented in Figure 2. The molecular weight of bilirubin is 584.68 and it is a reddish-brown solid.

There are two forms of bilirubin in the body. The first type is free bilirubin which is a water insoluble linear tetrapyrrole. The second type is conjugated bilirubin which is conjugated with small molecules such as glucuronic acid forming bilirubin glucuronides which are water soluble.



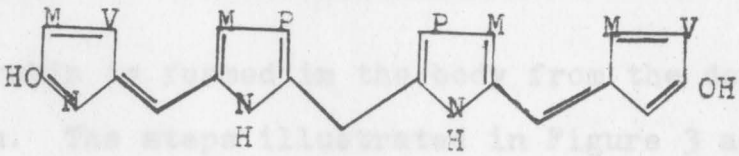
Porphin

Pyrrole

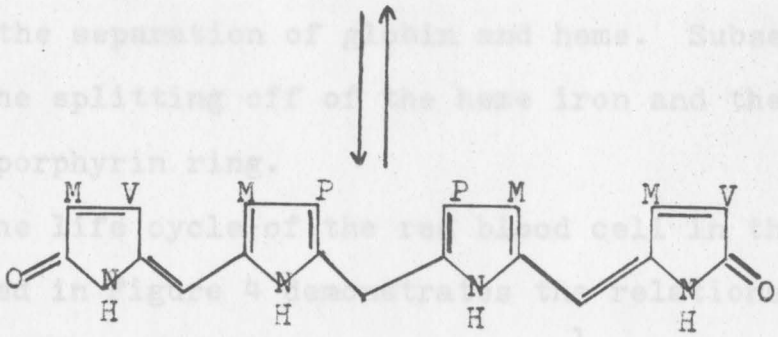


Porphyrin

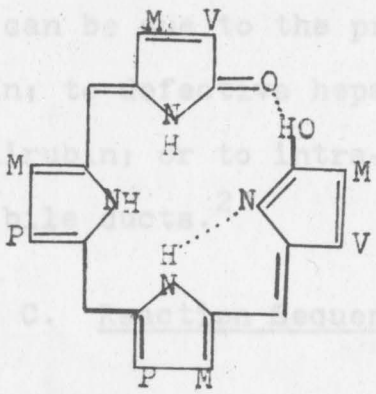
Fig.1-Porphin, Pyrrole and Porphyrin



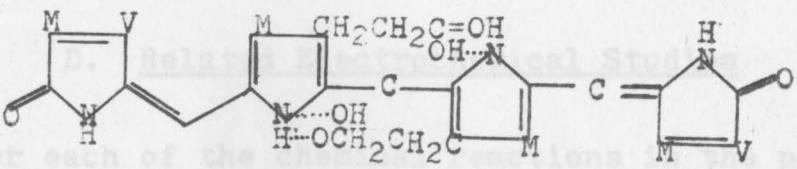
Bis-Lactim



Bis-Lactam



Mono-Lactam-Mono-Lactim



Intramolecular Hydrogen bonded Bis-Lactam

Fig.2-Bilirubin Structures

B. Biological Importance

Bilirubin is formed in the body from the degradation of hemoglobin. The steps illustrated in Figure 3 account for the formation of bilirubin from hemoglobin. The first step involves the separation of globin and heme. Subsequent steps involve the splitting off of the heme iron and the opening of the protoporphyrin ring.

The life cycle of the red blood cell in the human body illustrated in Figure 4 demonstrates the relationship of bilirubin to body processes and organs.[1]

The yellowness of the skin, scleras, and mucous membranes known as jaundice (icterus) is usually detectable when the total plasma bilirubin content is greater than 2mg/100ml. Hyperbilirubinemia can be due to the production of excessive amounts of bilirubin; to defective hepatic uptake, conjugation, or secretion of bilirubin; or to intra- or extra-hepatic obstruction of the bile ducts.[2]

C. Reaction Sequence

The reaction sequence illustrated in Figure 5 shows both strong and relatively weak oxidation and reduction reactions of bilirubin.

D. Related Electrochemical Studies

For each of the chemical reactions in the previous section there is a possible electrochemical analogue. Very

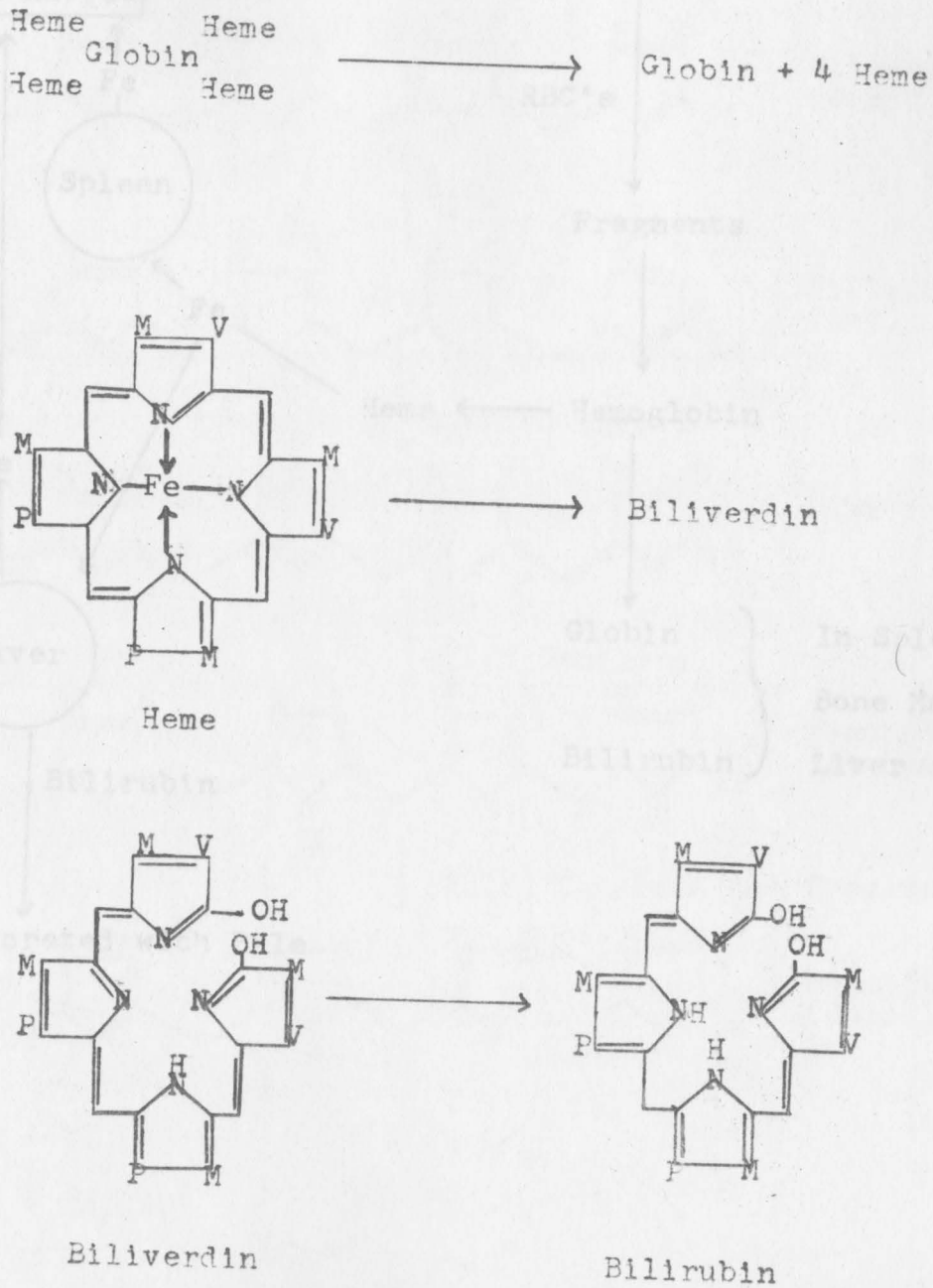


Fig.3-Hemoglobin Degradation

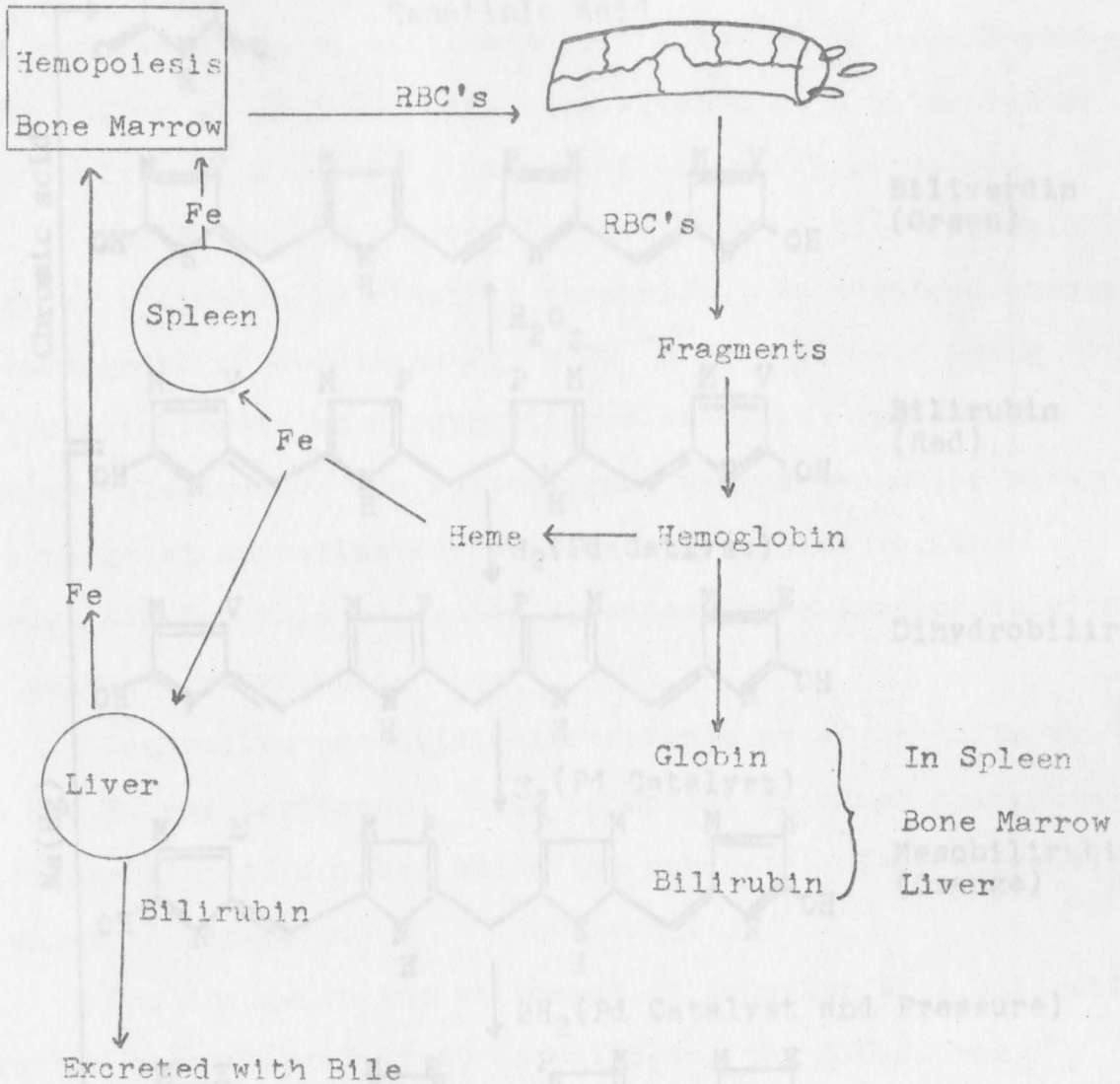
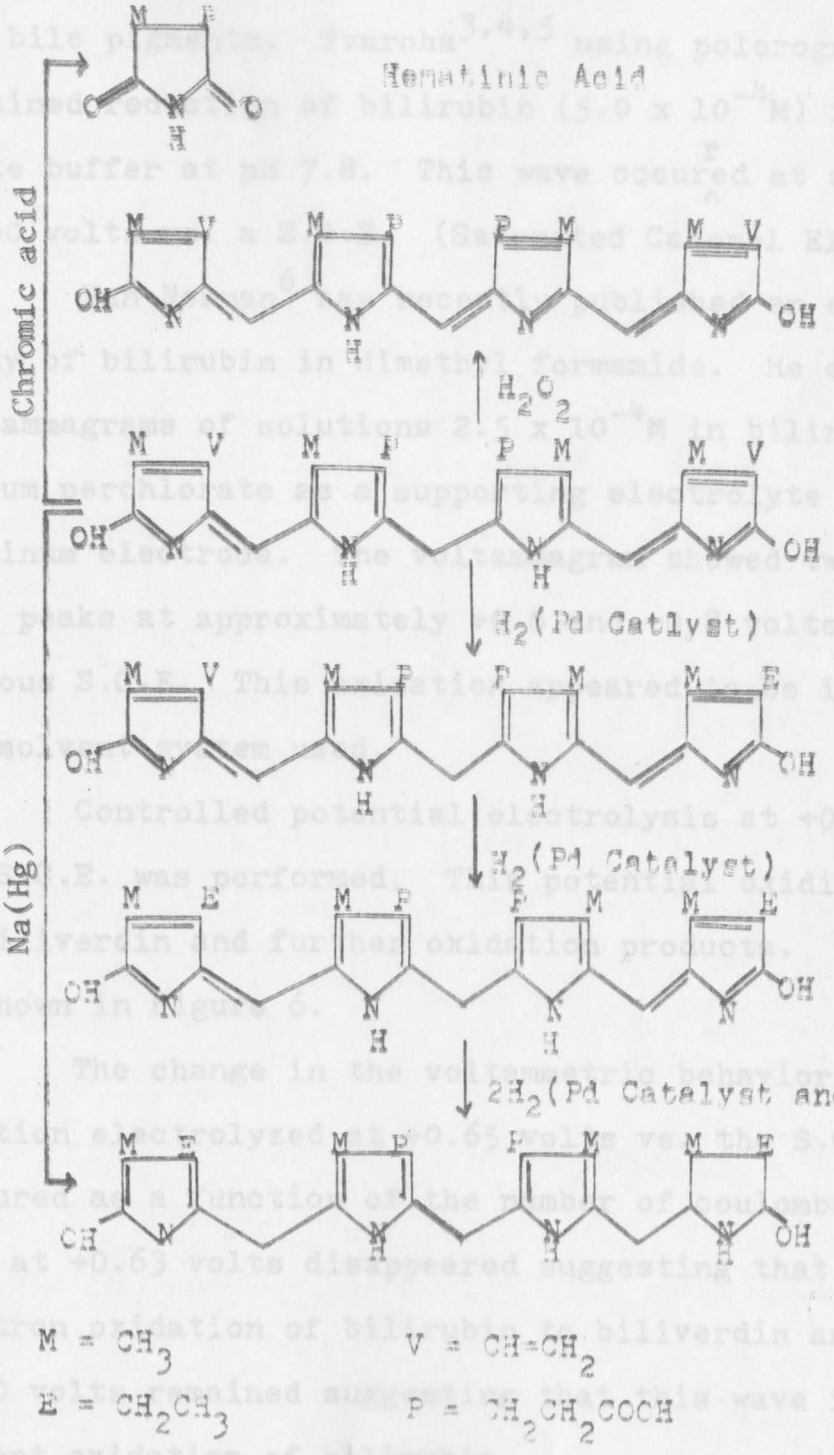


Fig. 4-Life History of Red Blood Cell



M = CH_3 V = $CH=CH_2$

E = CH_2CH_3 P = CH_2CH_2COOH

Fig.5-Reaction Sequence

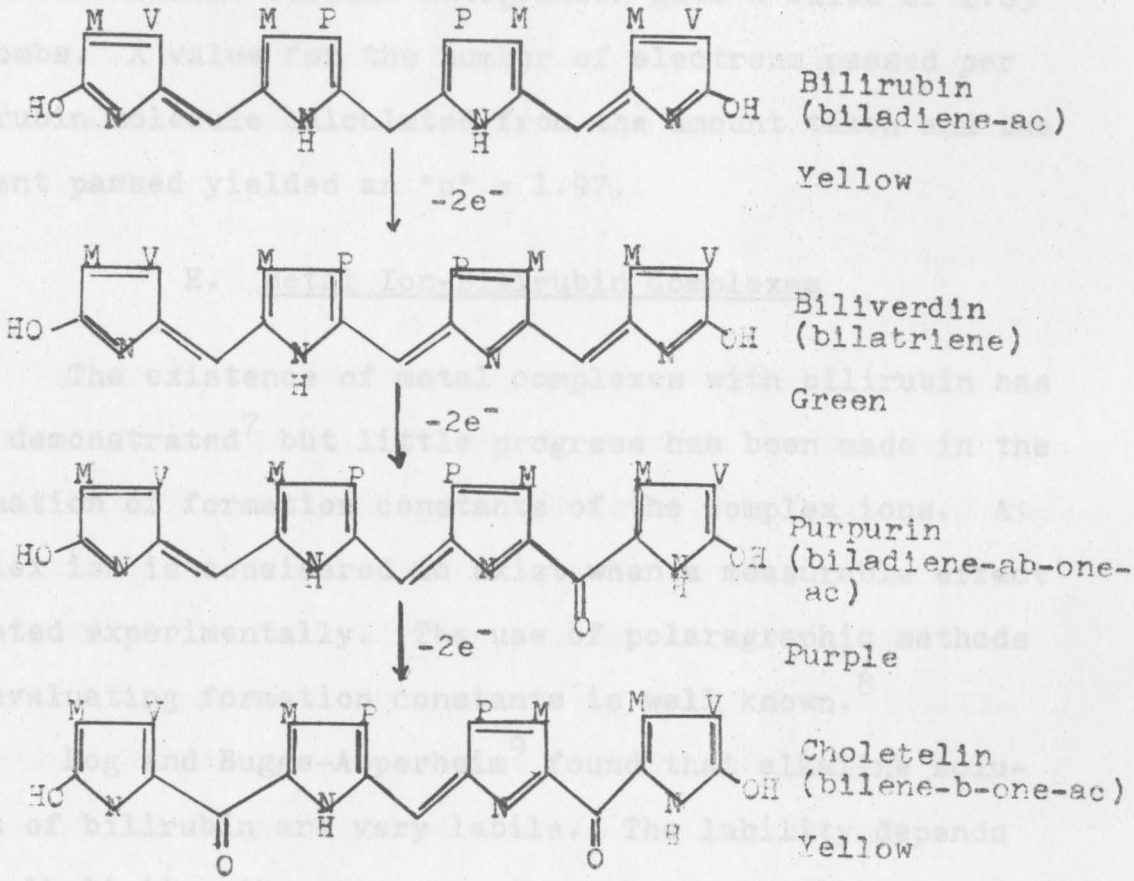
little electrochemical work has been done on bilirubin and the bile pigments. Tvaroha[3,4,5] using polorography at a DME, obtained reduction of bilirubin (5.0×10^{-4}M) in a 0.1M phosphate buffer at pH 7.8. This wave occured at a potential of -1.40 volts vs. a S.C.E. (Saturated Calomel Electrode).

Van Norman[6] has recently published an electrochemical study of bilirubin in dimethyl formamide. He obtained anodic voltammagrams of solutions 2.5×10^{-4}M in bilirubin using sodium perchlorate as a supporting electrolyte and a solid platinum electrode. The voltammagram showed two major waves with peaks at approximately +0.6 and +0.8 volts vs. the aqueous S.C.E. This oxidation appeared to be irreversible in the solvent system used.

Controlled potential electrolysis at +0.85 volts vs. the S.C.E. was performed. This potential oxidized bilirubin to biliverdin and further oxidation products. The sequence is shown in Figure 6.

The change in the voltammetric behavior of a bilirubin solution electrolyzed at +0.65 volts vs. the S.C.E. was measured as a function of the number of coulombs passed. The wave at +0.63 volts disappeared suggesting that this is a two-electron oxidation of bilirubin to biliverdin and the wave at +0.80 volts remained suggesting that this wave is due to subsequent oxidation of bilirubin.

Bilirubin solutions were electrolyzed at a controlled potential of +0.65 volts with current integration while both the voltammetric behavior of the solution was monitored and



M = $-CH_3$ P = $-CH_2CH_2COOH$

V = $-CH=CH_2$

Fig.6-Oxidation Sequence

the absorbance of dilute samples was measured. Extrapolation of absorbance vs. coulombs gave a value of 1.85 coulombs and a plot of peak height vs. coulombs gave a value of 1.84 coulombs. Direct current integration gave a value of 1.83 coulombs. A value for the number of electrons passed per bilirubin molecule calculated from the amount taken and the current passed yielded an "n" = 1.97.

E. Metal Ion-Bilirubin Complexes

The existence of metal complexes with bilirubin has been demonstrated[7] but little progress has been made in the evaluation of formation constants of the complex ions. A complex ion is considered to exist when a measurable effect is noted experimentally. The use of polaragraphic methods for evaluating formation constants is well known.[8]

Fog and Bugge-Asperheim[9] found that alkaline solutions of bilirubin are very labile. The lability depends upon alkalinity, the presence of metal ions and light. The intramolecular hydrogen bonds are broken (see Figure 2) and the central methylene bridge is adapted to dehydrogenation either by isomerization or oxidation. This process is catalyzed by such metal ions as zinc or by metal-complexing reagents such as EDTA (ethylenediamine tetracetic acid).

Carra[10] has shown that both bilirubin and mesobilirubin form non-fluorescent complexes with zinc ions. Velapoldi and Menis[7] have shown the existence of bilirubin-metal complexes with transition and rare earth elements such

as Zinc (II), Copper (II), Nickel (II), Cobalt (II), Iron (II) and (III) and many others. Spectrophotometric methods were used to study the spectral shifts upon metal-ion addition to bilirubin. Molar absorptivities were calculated for several of the complexes. The stability of bilirubin in several solvents and under various experimental conditions was reported.

F. Statement of the Problem

Bilirubin and its biological and chemical effects upon the body are of significant importance to the field of clinical chemistry. An excellent source of clinical information on bilirubin and related compounds is With's text on bile pigments.[11] Since little work had been done electrochemically with the bile pigments in general and bilirubin in particular, the entire range of electroanalytical techniques and investigations was open to the researcher.

Polarography and particularly cyclic voltammetry offer many opportunities to study the details of an electrochemical oxidation or reduction process. This investigation is an attempt to gain further information through electrochemical methods about bilirubin, its related metal complexes and biliverdin. The electrochemical methods will be utilized to calculate diffusion coefficients for the bile pigments alone and formation constants of the metal ion complexes.

CHAPTER II

THEORY

A. Electrochemical Oxidation and Reduction

Whenever a direct current passes through an electrolytic cell, an oxidation-reduction reaction takes place. At one electrode, defined as the anode, oxidation occurs with transfer of electrons from the reduced species to the electrode; at the cathode, reduction takes place and electrons are transferred from the electrode to the oxidized species. This property of electron transfer through current passage is fundamental to electroanalytical techniques.

In these studies, phenomena which occur in an electrolysis cell will be investigated where one electrode is polarizable and one is not. Polarizabity in the electrochemical sense concerns an electrode whose potential shows a departure from the value predicted by the Nernst equation. This method is a means of studying the composition of a solution and is called voltammetry. The nonpolarized electrode is the reference electrode and is usually a S.C.E. (Saturated Calomel Electrode). The polarized electrode is usually a noble metal such as mercury or platinum. The voltage, V, applied to a polarographic cell circuit may be equated to the difference in potential of the anode, E_a, and the cathode, E_c, and the summation of all the ohmic-potential

drops in the system:

$$V=E_a-E_c+I\Sigma R \qquad (1)$$

Some circumstances require that the IR term not be neglected but a way of eliminating the effect of the resistive drop is through the use of a three-electrode cell which in these investigations consists of a working electrode, a reference electrode and an auxilary electrode.

In voltammetry, information about the solution in which the working electrode is immersed is obtained by studying voltammetric waves, a graph of current flowing through the cell against applied potential. The graph shown in Figure 7 is a voltammetric plot of current vs. potential.

When the voltage scan is applied, the current will start near the origin (A) and only residual current will flow until a potential is reached (B) where the species of interest is either oxidized or reduced. Since this process is diffusion controlled, current is proportional to concentration and since the species of interest in the diffusion layer is depleted, the current drops back toward zero (C). A diffusion controlled process is one based on diffusion of the ions to the electrode rather than a migration or convection controlled process.

The voltammetric process obeys the Nernst equation:

$$E_{we} = E_{1/2} - \frac{2.303\ RT}{nF} \log \frac{i}{i_d - i} \qquad (2)$$

where E_{we} is the potential of the working electrode for a current i, $E_{1/2}$ is the half-wave potential, that is the

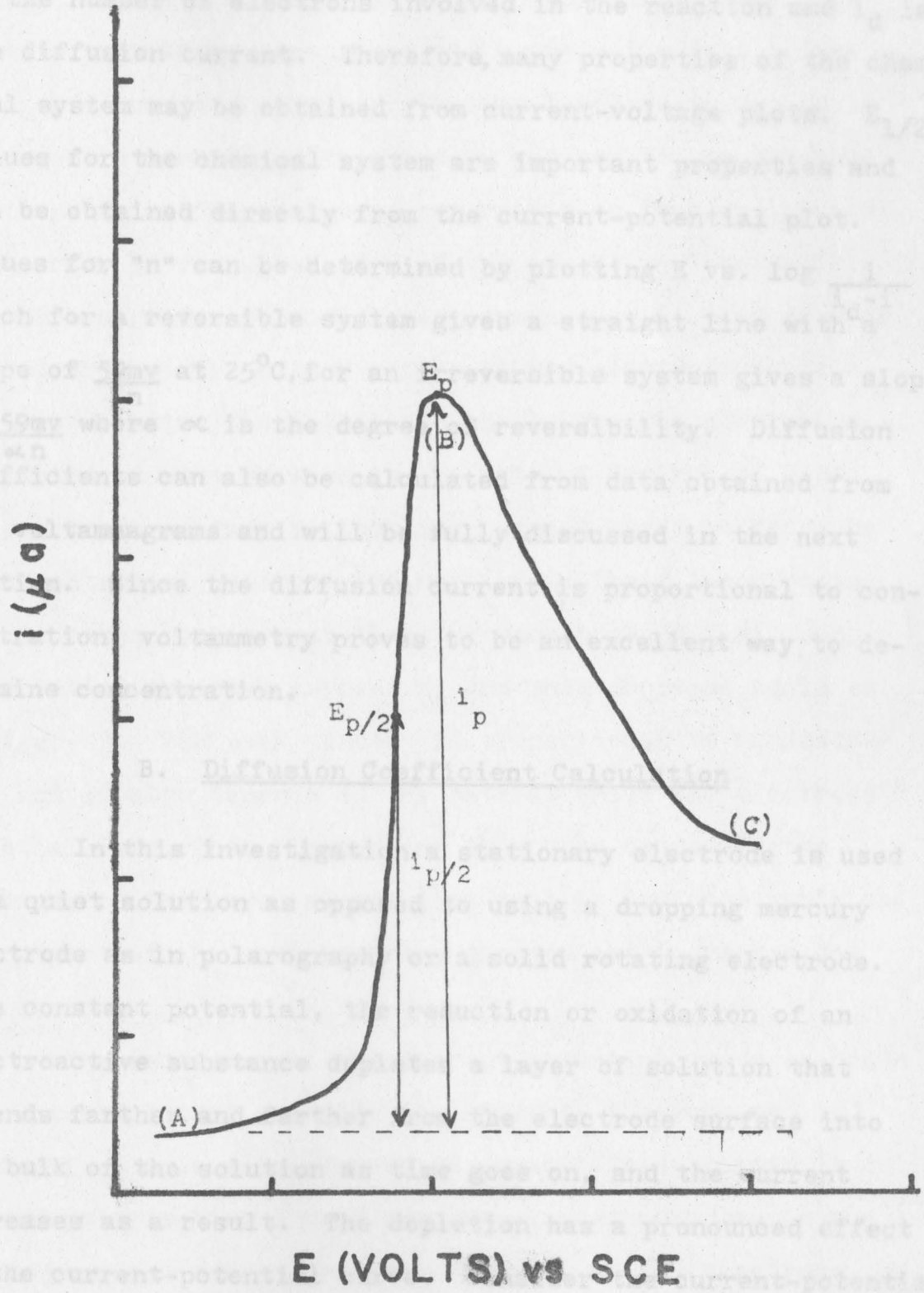


Fig.7-Plot of Current vs. Potential

potential corresponding to a position halfway up the wave, "n" is the number of electrons involved in the reaction and i_d is the diffusion current. Therefore, many properties of the chemical system may be obtained from current-voltage plots. $E_{1/2}$ values for the chemical system are important properties and can be obtained directly from the current-potential plot. Values for "n" can be determined by plotting E vs. log $\frac{i}{i_d - i}$ which for a reversible system gives a straight line with a slope of $\frac{59mv}{n}$ at 25°C, for an irreversible system gives a slope of $\frac{59mv}{\alpha n}$ where α is the degree of reversibility. Diffusion coefficients can also be calculated from data obtained from the voltammagrams and will be fully discussed in the next section. Since the diffusion current is proportional to concentration, voltammetry proves to be an excellent way to determine concentration.

B. Diffusion Coefficient Calculation

In this investigation a stationary electrode is used in a quiet solution as opposed to using a dropping mercury electrode as in polarography or a solid rotating electrode. At a constant potential, the reduction or oxidation of an electroactive substance depletes a layer of solution that extends farther and farther from the electrode surface into the bulk of the solution as time goes on, and the current decreases as a result. The depletion has a pronounced effect on the current-potential curve. Consider the current-potential curve for the reduction of an oxidized species, O, to its

reduced form, R, beginning at a potential so positive that the rate of reduction is negligible and proceeding toward more negative potentials. At first the principal effect is the increase of current resulting from an increase in the ratio of concentrations C^{o}_{R}/C^{o}_{O} required to conform to the Nernst equation if the O-R couple is reversible or from an increase in the rate of reduction of O if the couple is irreversible. When more negative potentials are reached, molecules of O are reduced as rapidly as they arrive at the electrode surface. The more negative the potential, the longer the electrolysis has lasted and the smaller is the rate of arrival of O at the electrode surface so that the current tends to decrease as the potential becomes more negative. These two opposing effects cause the current first to pass through a maximum value called the peak current i_p and then decrease again as in Figure 7. The peak current is proportional to concentration and it also depends on the rate at which the electrode potential is varied. Increasing this rate decreases the thickness of the layer of solution that is depleted while the rising part of the wave is being scanned and increases the peak current.

The recording of the current-potential curve is begun at a potential that precedes the start of the wave and is scanned from this value toward the peak. This process is called forward polarization and if the wave is cathodic, as in an electrochemical reduction, the potential of the electrode becomes more negative during the recording.

For linear diffusion to a plane electrode of area A square centimeters, the peak current obtained on forward polarization from the reversible reduction of O to a dissolved species R is described by the Randles-Sevcik equation:

$$i_p = 2.687 \times 10^5 \, n^{3/2} \, A \, D_o^{1/2} \, C_o \, v^{1/2} \qquad (3)$$

where i_p is the peak current in amperes, C_o is the bulk concentration of O in moles/cubic centimeter, v is the scan rate in volts/sec., A is the electrode area in square centimeters and D_o is the diffusion coefficient for the species O.[12]

For a totally irreversible system, the equation used to calculate the peak current is given by:

$$i_p = 2.985 \times 10^5 \, n \, (\alpha n_a)^{1/2} \, A \, D_o^{1/2} \, C_o \, v^{1/2} \qquad (4)$$

where all the symbols have the same meanings as in the Randles-Sevcik equation and αn_a can be defined as:

$$\alpha n_a = \frac{0.04771}{E_{p/2} - E_p} \qquad (5)$$

from the relationship:

$$E_{p/2} = E_p + \frac{0.04771}{\alpha n_a} \qquad (6)$$

where E_p is the peak potential and $E_{p/2}$ is the potential where the current has a value of $\frac{i}{2}p$.

C. Technique of Evaluating Formation Constants of Complex Ions

If a metal ion and its complex reduce reversibly to the amalgam, then measurements of the shift in half-wave potential or full-wave potential to more negative potentials with increase in ligand concentration can be applied to determine the overall stability constant.

When a fairly stable complex exists in the solution and the complexing agent is in large excess, the following relationship holds:

$$(E_p)_s - (E_p)_c = \frac{0.059}{n} \log \beta_p + \frac{0.059}{n} p \log C \qquad (7)$$

where $(E_p)_s$ and $(E_p)_c$ are peak potentials for the simple and complexed ions respectively, p is the number of ligands coordinated to the metal ion, β_p is the overall stability constant and C is the ligand concentration.[8]

CHAPTER III

MATERIALS AND APPARATUS

A. Materials

All chemicals used were of reagent grade quality and were used without recrystallization. Table I gives the chemicals used, the manufacturer and the grade of the reagent.

The buffer solutions used were made up according to the C.R.C. Handbook as in the following table.

TABLE II

BUFFER PREPARATIONS

pH	Reagents used
4.0	50ml 0.1M $KHC_8H_4O_4$ + 0.1ml 0.1M HCl
6.0	50ml 0.1M KH_2PO_4 + 5.7ml 0.1M NaOH
8.0	46.8ml 0.1M NaOH + 50ml 0.1M KH_2PO_4
10.0	50ml 0.05M $NaHCO_3$ + 10.7ml 0.1M NaOH
12.0	50ml 0.05M Na_2HPO_4 + 26.9ml 0.1M NaOH

The bilirubin was obtained from Sigma Chemical Company. It was 99% pure and had a molar absorbtivity in chloroform of 60,000±600. The bovine serum albumin was also obtained from Sigma Chemical. Biliverdin dihydrochloride (approximately 80% biliverdin) was obtained from Sigma Chemical. Both Argon and Nitrogen gases were used as inert atmospheres in this study. The argon was purified by passing

TABLE I

REAGENTS USED

Material	Formula	Grade Reagent	Manufacturer
Potassium Hydrogen Phthalate	$KHC_8H_4O_4$	Certified	Fisher Scientific
Sodium Bicarbonate	$NaHCO_3$	Analyzed	Fisher Scientific
Hydrochloric Acid	HCl	Analyzed	Baker Chemical
Potassium Phosphate	KH_2PO_4	Analyzed	Baker Chemical
Sodium Hydroxide	NaOH	Certified	Baker Chemical
Zinc Metal	Zn	Analyzed	Baker Chemical
Cobaltous Chloride	$CoCl_2 \cdot 6H_2O$	Analyzed	Baker Chemical
Mecuric Chloride	$HgCl_2$	Analyzed	Baker Chemical
Sodium Phosphate	Na_2HPO_4	Analyzed	Allied Chemical
Copper Metal	Cu	Analyzed	Matheson, Coleman & Bell
Nickel Metal	Ni	Analyzed	Stansi Scientific Co.
Cadmium Acetate	$Cd(C_2H_3O_2)_2 \cdot 2H_2O$	Analyzed	Allied Chemical
Iron Wire	Fe	Analyzed	Allied Chemical

over clean copper chips at 600°C.

B. Apparatus

1. N.I.L. Electrolab

All electrochemical measurements were obtained with a National Instrument Laboratory "Electrolab",[13] a multifunctional electroanalytical system, in conjunction with a Val Tech, Model 1024 X-Y Recorder.

2. PH Measurement

All pH measurements were accomplished using a Chemtrix Type 40E or a Sargent Model LS pH meter.

3. Electrodes and Electrochemical Cell

Several different types of electrodes were used in this study. For electrochemical oxidation, a cell illustrated in Figure 8 was utilized. The electrodes in this cell were two Sargent-Welch Pt indicator electrodes and a Coleman saturated calomel electrode.

The electrochemical cell for reduction was a Metrohm Model EA 874 titration vessel which had five openings in the upper portion to permit insertion of the appropriate electrodes and gas bubblers. This cell is illustrated in Figure 9. The cell consisted of a Metrohm Hanging Mercury Drop Electrode (H.M.D.E.) as the working electrode, illustrated in Figure 10 a Sargent-Welch Pt auxiliary electrode and a S.C.E.

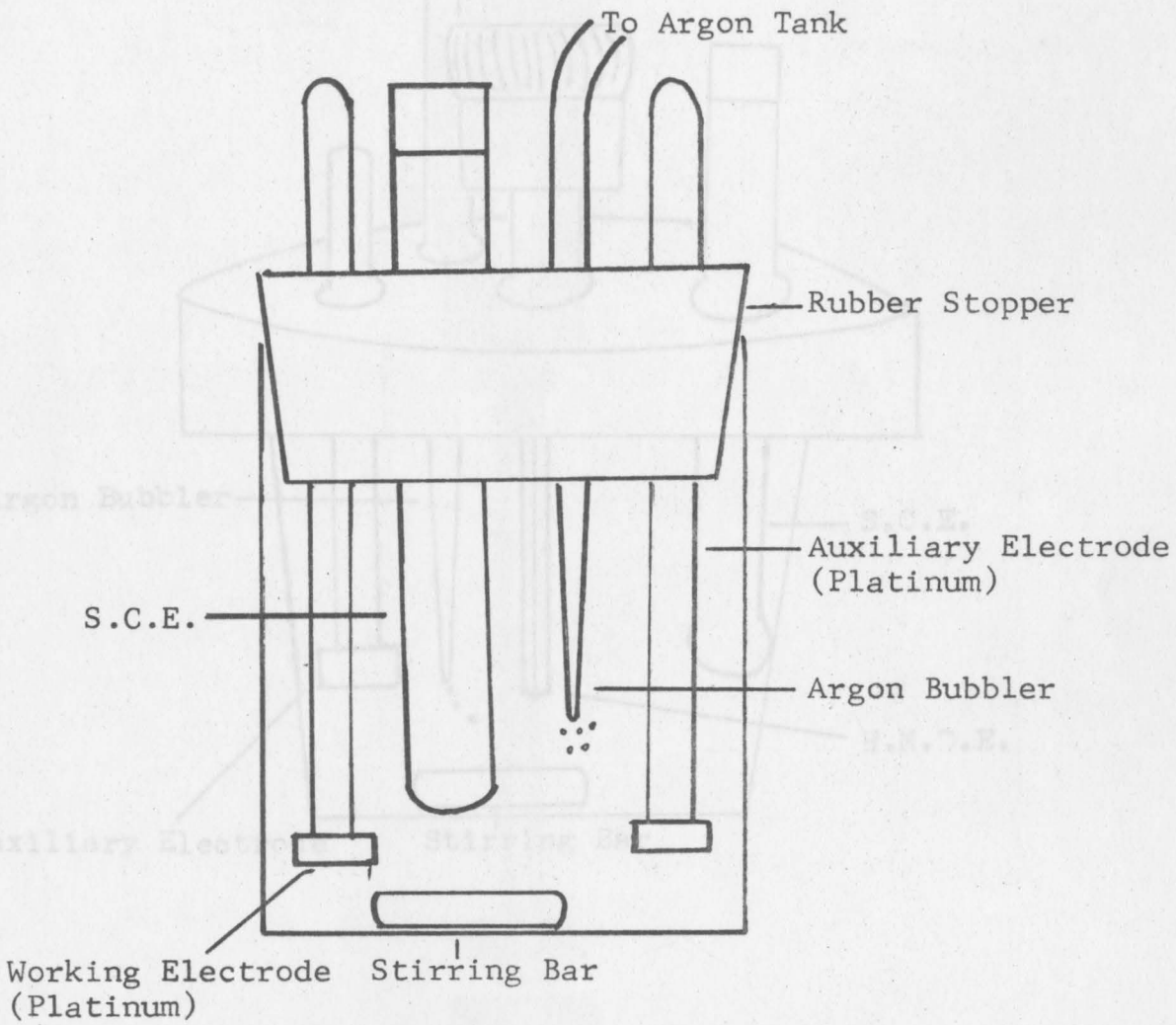


Fig.8-Cell for Electrochemical Oxidation

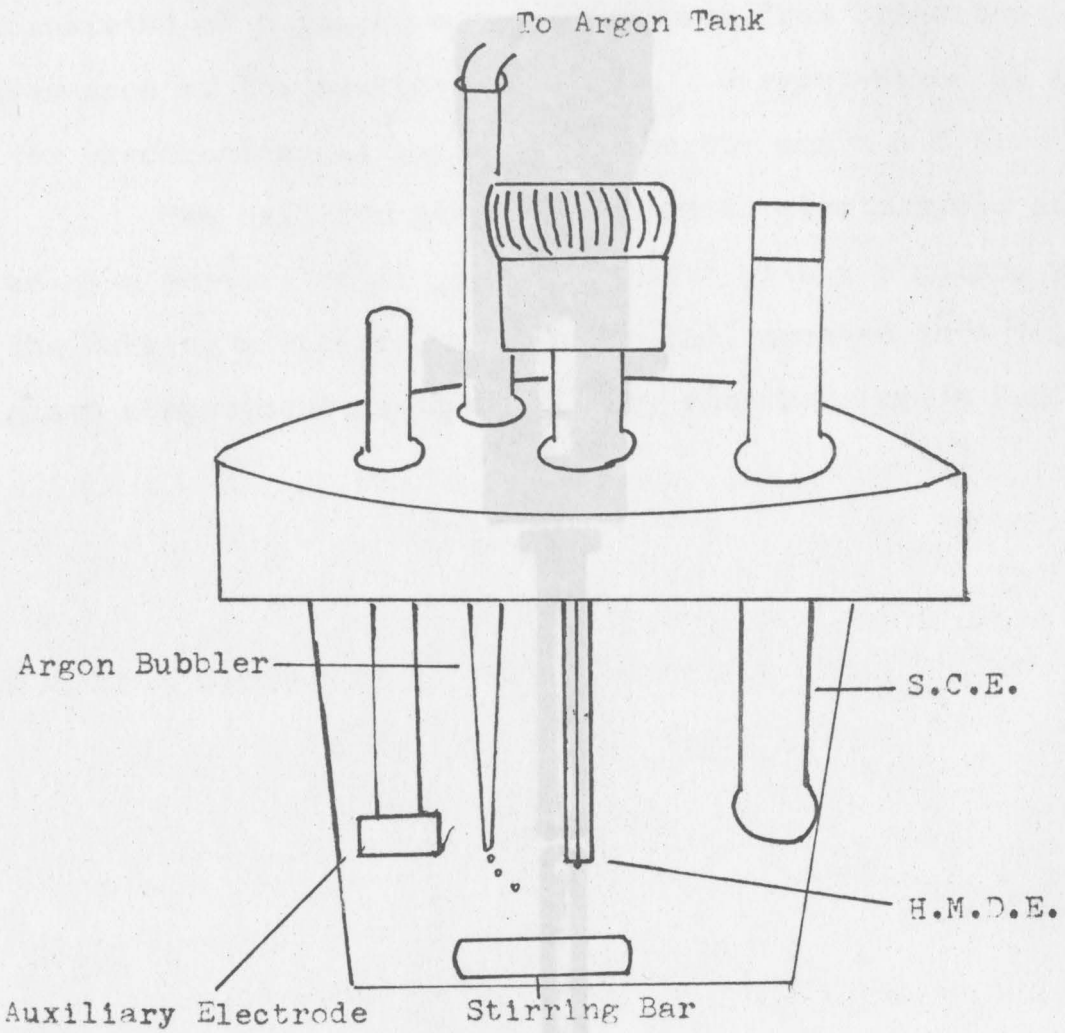


Fig.9-Cell for Electrochemical Reduction

Mercury Drop Size for all Studies is $0.0222 cm^2$

Fig.10-Hanging Mercury Drop Electrode (H.M.D.E.)

An electrochemical oxidation cell that was also used consisted of a glassy carbon electrode from Princeton Applied Research as the working electrode, a Sargent-Welch Pt indicator electrode as an auxiliary electrode and a S.C.E.

The cell for constant potential electrolysis consisted of a mercury metal pool in contact with a platinum wire as the working electrode, a platinum foil encased in a fritted glass compartment as the auxiliary electrode and a S.C.E.

CHAPTER IV

EXPERIMENTAL PROCEDURE AND RESULTS

A. Solubility of Bilirubin in Aqueous Systems

A bilirubin solution ($1x10^{-2}M$) was prepared using reagent grade bilirubin (anhydrous molecular weight 584) by dissolving .0584g of bilirubin in 10 milliliters of deoxygenated 0.1M NaOH. Buffer solutions were prepared in a range of pH 4.0 to pH 12.0. All buffer solutions were deareated with an inert atmosphere of argon gas before addition of bilirubin.

Table III summarizes the solubility of bilirubin in the various buffer systems.

TABLE III

BILIRUBIN SOLUBILITY

Bilirubin conc.=$1x10^{-4}M$	
pH	
4.0	Insoluble
6.0	Insoluble
8.0	Soluble
10.0	Soluble
12.0	Soluble

B. Electrochemical Oxidation of Bilirubin

The oxidation of bilirubin to biliverdin was attempted electrochemically using the N.I.L. Electrolab. The electrochemical cell used for this procedure consisted of two solid platinum electrodes and a S.C.E. as illustrated in Figure 8. The supporting electrolyte for this procedure was a phosphate buffer (pH 7.8) which was made up according to the directions previously described in Chapter III. One hundred milliliters of the buffer were placed in the electrolysis cell and deoxygenated with an inert atmosphere of argon gas for one half hour. The buffer solution was electrochemically oxidized using a potential scan of 0.0 to +2.0 volts and a full scale current span of 100mA as a background curve for the bilirubin oxidation. One milliliter of a 1.0×10^{-2}M bilirubin solution was then added to the 100ml of buffer making a solution that was 1.0×10^{-4}M in bilirubin. The solution was oxidized using a potential scan of 0.0 to +2.0 volts and a full scale current span of 100mA. No oxidative wave was found. Identical studies involving bilirubin dissolved in buffers of pH 8.0, 10.0, and 12.0 also provided no oxidative wave.

Investigations were also undertaken to oxidize bilirubin using a glassy carbon electrode as the working electrode, a platinum auxiliary electrode and a S.C.E. A buffer solution of pH 8.0 was used as the supporting electrolyte. The bilirubin solution oxidized was 1.0×10^{-4}M using a potential scan of 0.0 to +1.2 volts and a full scale current span of 100mA. No oxidative wave was obtained.

C. Complexation of Bilirubin with Bovine Serum Albumin

In order to make bilirubin more soluble in buffers of low pH, bovine serum albumin was introduced to complex the bilirubin. Bovine serum albumin complexes with bilirubin in a 1:1 ratio. Bovine serum albumin was dissolved in 100ml of buffer solutions of pH 3.5, 4.0, 6.0, 7.0, and 8.0 to obtain a solution that was 1.0×10^{-4}M in B.S.A. One milliter of 1.0×10^{-2}M bilirubin was added to each buffer to obtain the complex to be oxidized. Oxidations using two platinum electrodes and the S.C.E. illustrated in the cell in Figure 8 were attempted. The potential scan was 0.0 to +2.0 volts and various current settings (1mA, 10mA and 100mA) were utilized. However no oxidative wave could be obtained for the complex in all the aforementioned buffer solutions.

D. Reductive Studies of Bilirubin

1. Reductive Wave

The next target of the research was to attempt the opposite electrochemical process-reduction. For this reduction the N.I.L. Electrolab was utilized and the cell for this work is shown in Figure 9.

Buffers in the pH range 7.8 to 8.0 were deoxygenated and a background reductive scan was taken using a potential range of 0.0 to -2.0 volts and a full scale current span of either 2.5μA of 10.0μA. One milliliter of bilirubin solutions of various concentrations was added to the 100ml of buffer

and electrochemically reduced using the potential scan and current setting used for the background. Reduction of bilirubin was accomplished for several concentrations which are shown in Table IV. The first reductive wave was found at a potential of -1.40 volts vs. S.C.E. using 2.5μA current span and a potential scan of 0.0 to -2.0 volts for a bilirubin solution 1.0×10^{-4}M. This wave is represented with various potential scan rates in Figure 11.

2. Calculation of the Diffusion Coefficient

Using the data obtained from the reductive wave, a diffusion coefficient was calculated using the Randles-Sevcik equation:

$$D_o = \left(\frac{i_p}{Kn^{3/2} A C_o v^{1/2}} \right)^2 \qquad (8)$$

where K is the Randles-Sevcik equation constant

n is the number of electrons

A is the area of the electrode in cm^2

C_o is the concentration in moles/cm^3

v is the scan rate in volts/sec.$^{1/2}$

i_p is the peak current in amperes

Table V is a representation of the D_o values obtained for a 1.0×10^{-4}M bilirubin solution. Figure 12 illustrates the reductive waves used for the D_o calculations.

TABLE IV

REDUCTION POTENTIALS FOR BILIRUBIN

Concentration (Moles/liter)	pH	Scan Rate (Volts/ min.)	Red. Potential (Volts)	Current Span (μA)
1.0×10^{-4}	7.8	2.5	-1.40	2.5
2.5×10^{-5}	8.0	2.5	-1.50	10.0
5.0×10^{-5}	8.0	2.5	-1.52	2.5
"	"	1.0	-1.48	"
"	"	5.0	-1.54	"
6.0×10^{-5}	"	1.0	-1.51	10.0
"	"	2.5	-1.53	"
"	"	5.0	-1.56	"
7.5×10^{-5}	"	2.5	-1.53	2.5
1.0×10^{-4}	"	1.0	-1.52	10.0
"	"	2.5	-1.55	"
"	"	5.0	-1.58	"
2.2×10^{-4}	"	.5	-1.50	"
"	"	1.0	-1.52	"
"	"	2.5	-1.55	"
"	"	5.0	-1.58	"

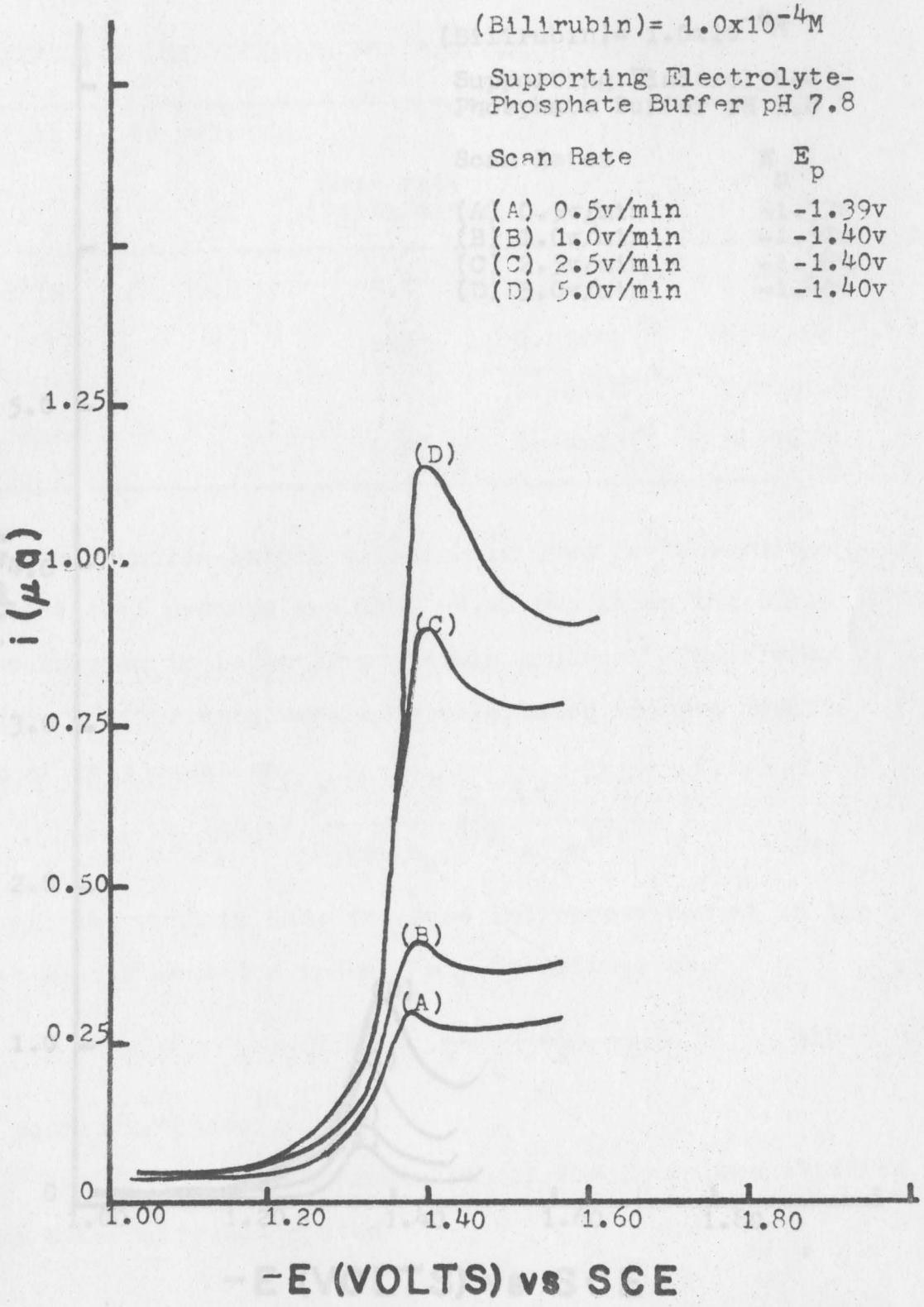


Fig.11-Bilirubin Reduction

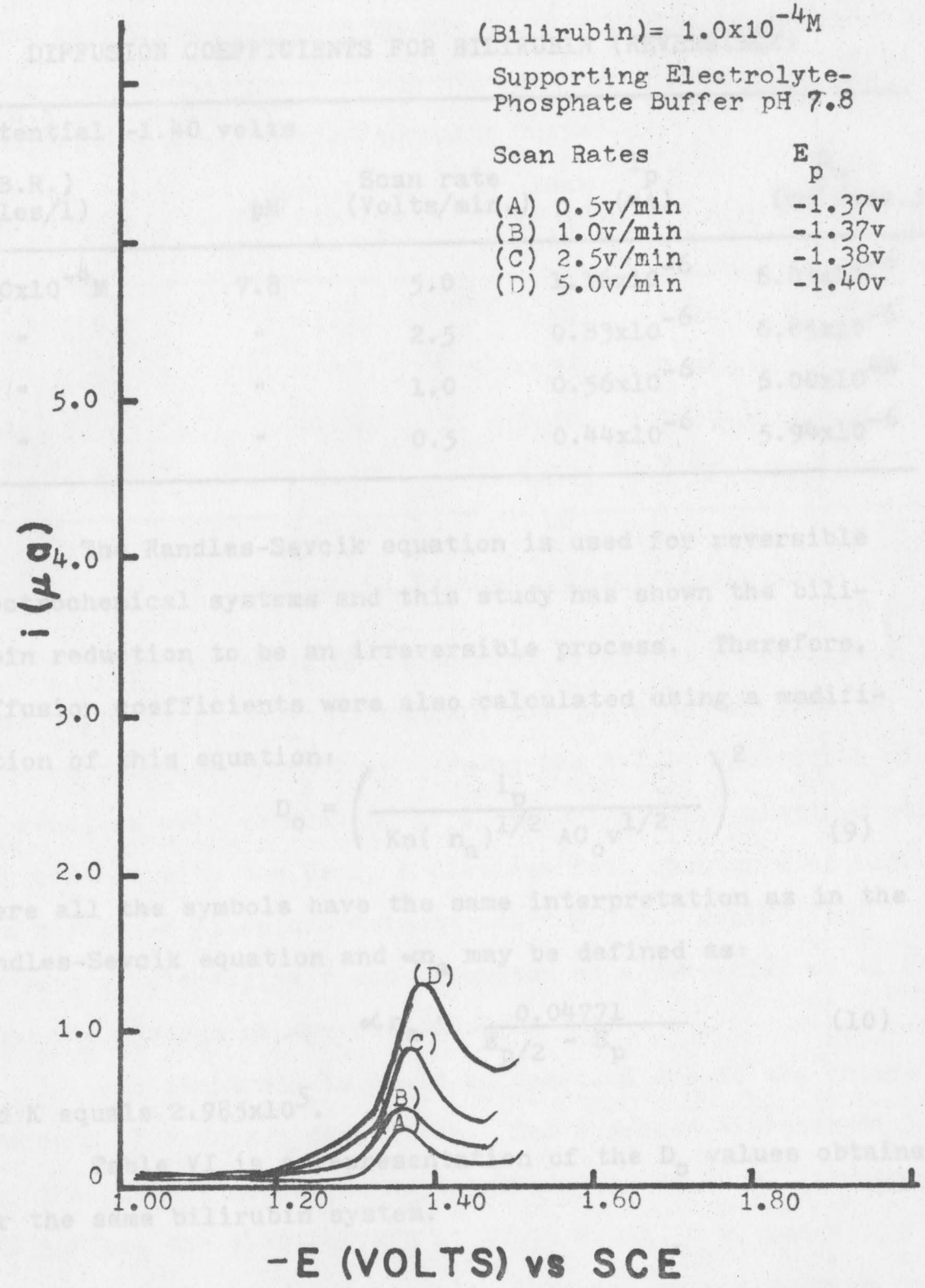


Fig.12-Bilirubin Reduction used for Diffusion Coefficient Calculations

TABLE V

DIFFUSION COEFFICIENTS FOR BILIRUBIN (REVERSIBLE)

Potential -1.40 volts (B.R.) (moles/l)	pH	Scan rate (Volts/min.)	i_p (μA)	D_o (cm^2/sec.)
1.0×10^{-4} M	7.8	5.0	1.16×10^{-6}	6.03×10^{-6}
"	"	2.5	0.83×10^{-6}	6.85×10^{-6}
"	"	1.0	0.56×10^{-6}	6.00×10^{-6}
"	"	0.5	0.44×10^{-6}	5.94×10^{-6}

The Randles-Sevcik equation is used for reversible electrochemical systems and this study has shown the bilirubin reduction to be an irreversible process. Therefore, diffusion coefficients were also calculated using a modification of this equation:

$$D_o = \left(\frac{i_p}{Kn(\alpha n_a)^{1/2} A C_o v^{1/2}} \right)^2 \qquad (9)$$

where all the symbols have the same interpretation as in the Randles-Sevcik equation and αn_a may be defined as:

$$\alpha n_a = \frac{0.04771}{E_{p/2} - E_p} \qquad (10)$$

and K equals 2.985×10^5.

Table VI is a representation of the D_o values obtained for the same bilirubin system.

TABLE VI

DIFFUSION COEFFICIENTS FOR BILIRUBIN (IRREVERSIBLE)

(B.R.) = 1.0×10^{-4} M
Supporting Electrolyte-Phosphate Buffer pH=7.8

Scan Rate (Volts/min.)	Reduction Potential (Volts)	Peak Current (μA)	αn_a	D_o (cm^2/sec.)
0.5	-1.35	0.40	1.26	8.49×10^{-6}
1.0	"	0.53	1.26	6.91×10^{-6}
2.5	-1.36	0.80	1.19	7.92×10^{-6}
5.0	-1.37	1.13	0.95	10.1×10^{-6}

E. Controlled Potential Electrolysis

The technique of controlled potential electrolysis was employed in order to confirm the two electron transfer of the bilirubin reduction. Using the N.I.L. Electrolab with a reaction cell consisting of a mercury pool, a platinum wire in contact with the pool, a platinum foil counter electrode in a fritted glass compartment and a S.C.E., controlled potential electrolysis was attempted at a potential of -1.40 v and the passage of one coulomb.

No electrolysis could be measured due to the interference of hydrogen deposition. The hydrogen overpotential of the aqueous medium is at approximately -1.40 v. Attempts to perform the electrolysis at -1.25 v, -1.30 v, and -1.35 v also proved to be unsuccessful. Attempts were also made to make the solution more alkaline. Solutions of pH 8.0 to 9.0

were unsuccessfully electrolyzed due to the hydrogen overpotential. The bilirubin solutions for this work were one ml of 1.0×10^{-2}M dissolved in 100 ml of deoxygenated phosphate buffer.

F. Electrochemistry of Bilirubin-Metal-Ion Complexes

1. Zinc (II)

A zinc solution (1.0×10^{-3}M) was prepared from zinc metal dissolved in hydrochloric acid. A 10-ml aliquot of this solution was dissolved in 100 ml of a phosphate buffer of pH 8.0. The pH was checked and found to be 8.0. This solution was deoxygenated for one-half hour with argon gas in the electrochemical cell consisting of the H.M.D.E., a platinum auxiliary electrode and a S.C.E. as shown in Figure 9. The zinc solution was electrochemically reduced and a reductive wave was found at -1.12 v vs. S.C.E. using a current span of 10μA and a potential range of 0.0 to -2.0 v. The reductive curve is illustrated in Figure 13.

A series of bilirubin concentrations was prepared. One ml of the bilirubin solution was added to the cell already containing 10 ml of zinc ion and 100 ml of phosphate buffer (pH 8.0). This solution was electrochemically reduced. The specific bilirubin concentrations and the reductive potentials of the zinc wave shift are found in Table VII and the reductive scans are illustrated in Figure 14.

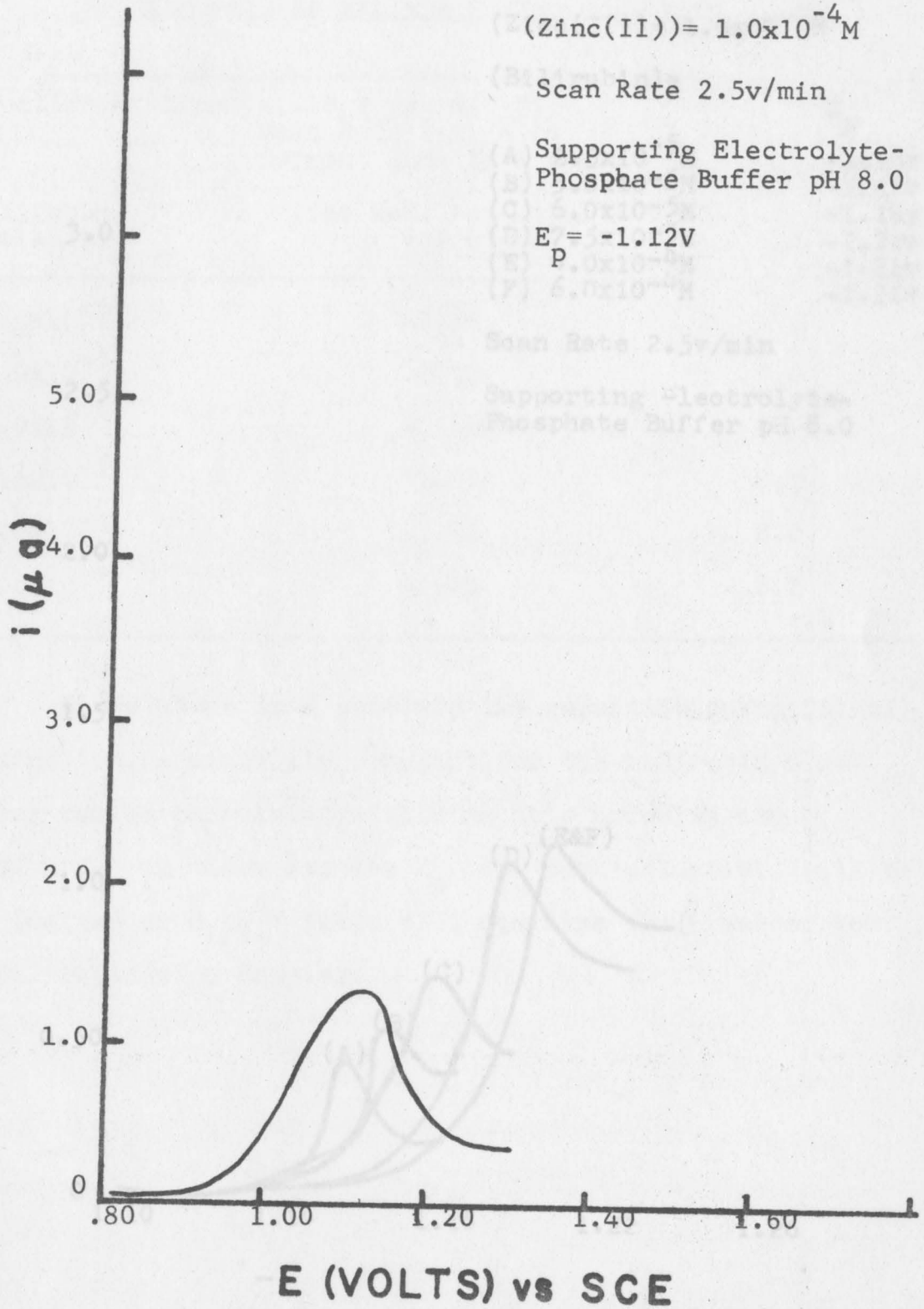


Fig.13-Zinc(II) Reduction

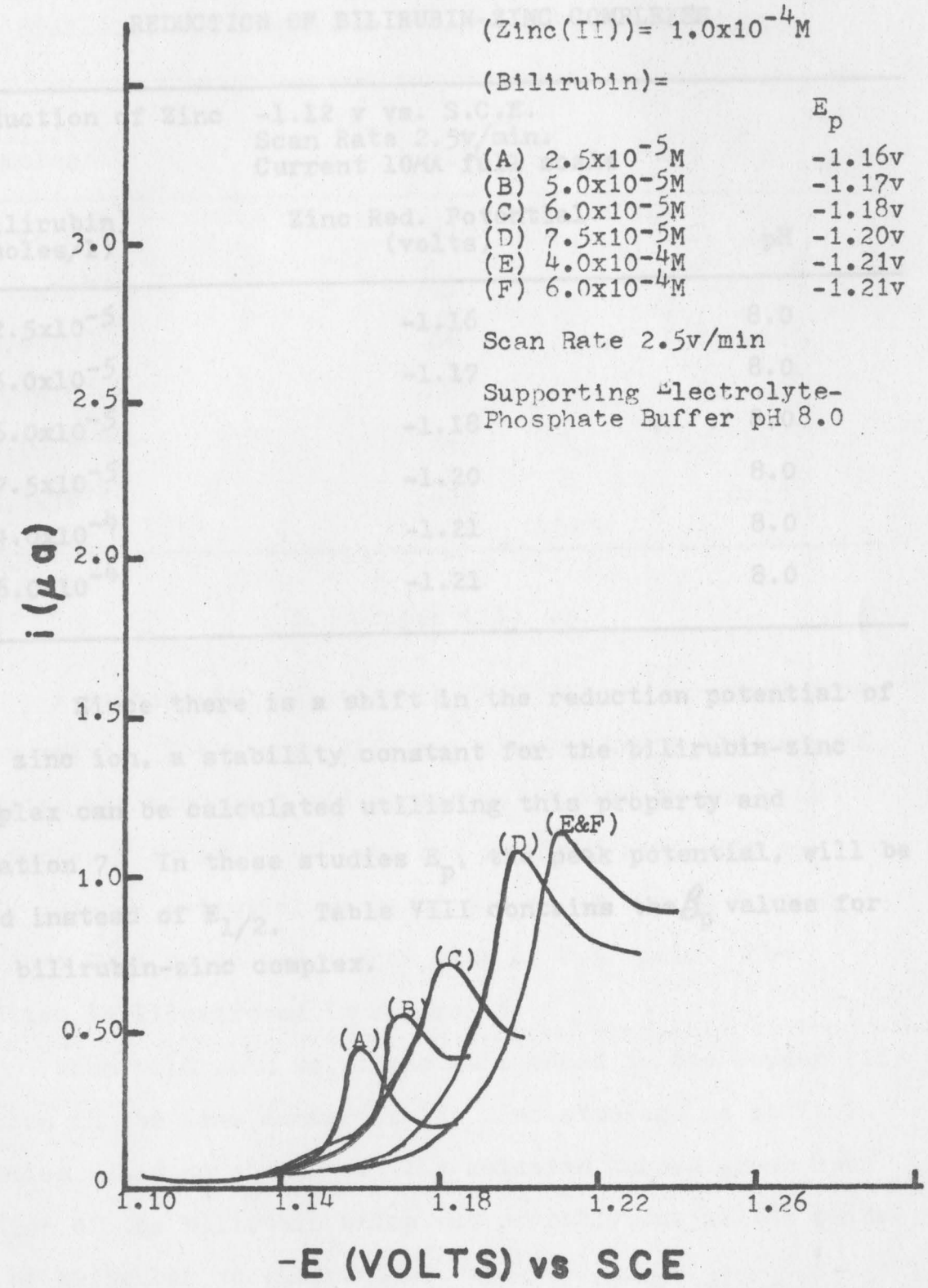


Fig.14-Bilirubin-Zinc Complex Reduction

TABLE VII

REDUCTION OF BILIRUBIN-ZINC COMPLEXES

Reduction of Zinc	-1.12 v vs. S.C.E. Scan Rate 2.5v/min. Current 10μA full scale	
(Bilirubin) (moles/l)	Zinc Red. Potential (volts)	pH
2.5×10^{-5}	-1.16	8.0
5.0×10^{-5}	-1.17	8.0
6.0×10^{-5}	-1.18	8.0
7.5×10^{-5}	-1.20	8.0
4.0×10^{-4}	-1.21	8.0
6.0×10^{-4}	-1.21	8.0

Since there is a shift in the reduction potential of the zinc ion, a stability constant for the bilirubin-zinc complex can be calculated utilizing this property and Equation 7. In these studies E_p, the peak potential, will be used instead of $E_{1/2}$. Table VIII contains the β_p values for the bilirubin-zinc complex.

TABLE VIII

STABILITY CONSTANTS FOR BILIRUBIN-ZINC COMPLEXES

(Bilirubin) (moles/l)	$(E_p)_s - (E_p)_c$ (volts)	β_p
2.5×10^{-5}	.040	1.0×10^{6}
5.0×10^{-5}	.050	1.0×10^{6}
6.0×10^{-5}	.060	2.0×10^{6}
7.5×10^{-5}	.080	7.0×10^{6}
4.0×10^{-4}	.090	3.0×10^{6}
6.0×10^{-4}	.095	3.0×10^{6}

2. Copper (II)

A copper (II) solution (1.0×10^{-3}M) was prepared from copper metal. The copper (II) was electrochemically reduced utilizing the same cell and procedure as used in the zinc ion studies. A reductive wave was obtained at -.46 v vs. S.C.E. using a potential range of 0.0 to -2.0 v, a current span 10μA full scale and a scan rate of 2.5 v/min. The reduction is illustrated in Figure 15.

When bilirubin solutions were added to the copper (II) solution in the same manner as the zinc studies, no shift in reduction could be obtained. The solution turned green upon addition of the bilirubin which was probably due to the oxidation of bilirubin to biliverdin.

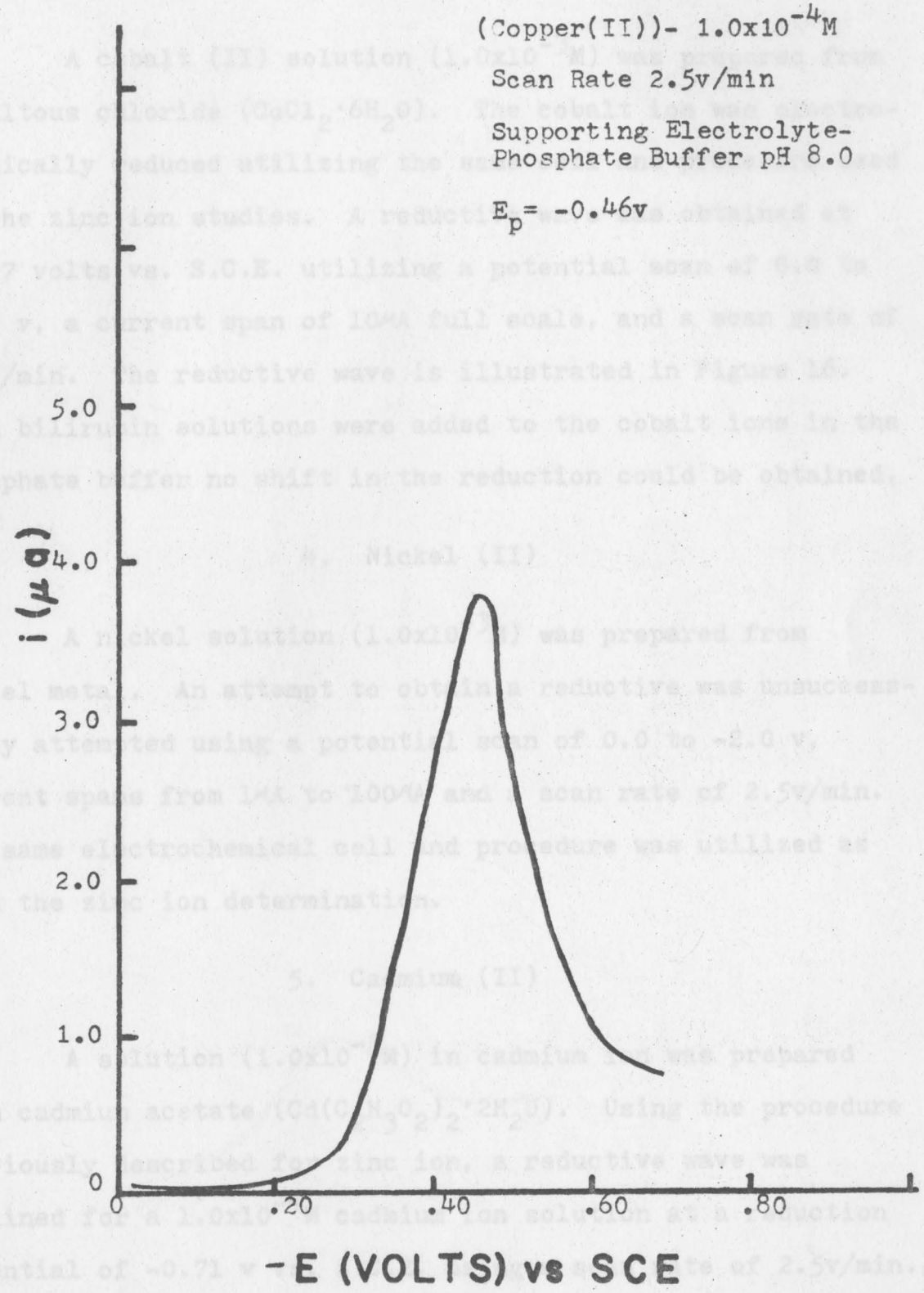


Fig.15-Copper(II) Reduction

3. Cobalt (II)

A cobalt (II) solution (1.0×10^{-3}M) was prepared from cobaltous chloride ($CoCl_2 \cdot 6H_2O$). The cobalt ion was electrochemically reduced utilizing the same cell and procedure used in the zinc ion studies. A reductive wave was obtained at -1.27 volts vs. S.C.E. utilizing a potential scan of 0.0 to -2.0 v, a current span of 10μA full scale, and a scan rate of 2.5v/min. The reductive wave is illustrated in Figure 16. When bilirubin solutions were added to the cobalt ions in the phosphate buffer no shift in the reduction could be obtained.

4. Nickel (II)

A nickel solution (1.0×10^{-3}M) was prepared from nickel metal. An attempt to obtain a reductive was unsuccessfully attempted using a potential scan of 0.0 to -2.0 v, current spans from 1μA to 100μA and a scan rate of 2.5v/min. The same electrochemical cell and procedure was utilized as with the zinc ion determination.

5. Cadmium (II)

A solution (1.0×10^{-3}M) in cadmium ion was prepared from cadmium acetate ($Cd(C_2H_3O_2)_2 \cdot 2H_2O$). Using the procedure previously described for zinc ion, a reductive wave was obtained for a 1.0×10^{-4}M cadmium ion solution at a reduction potential of -0.71 v vs. S.C.E. using a scan rate of 2.5v/min., a potential range of 0.0 to -2.0 v, and a full scale current span of 10μA. The reductive scan is illustrated in Figure 17.

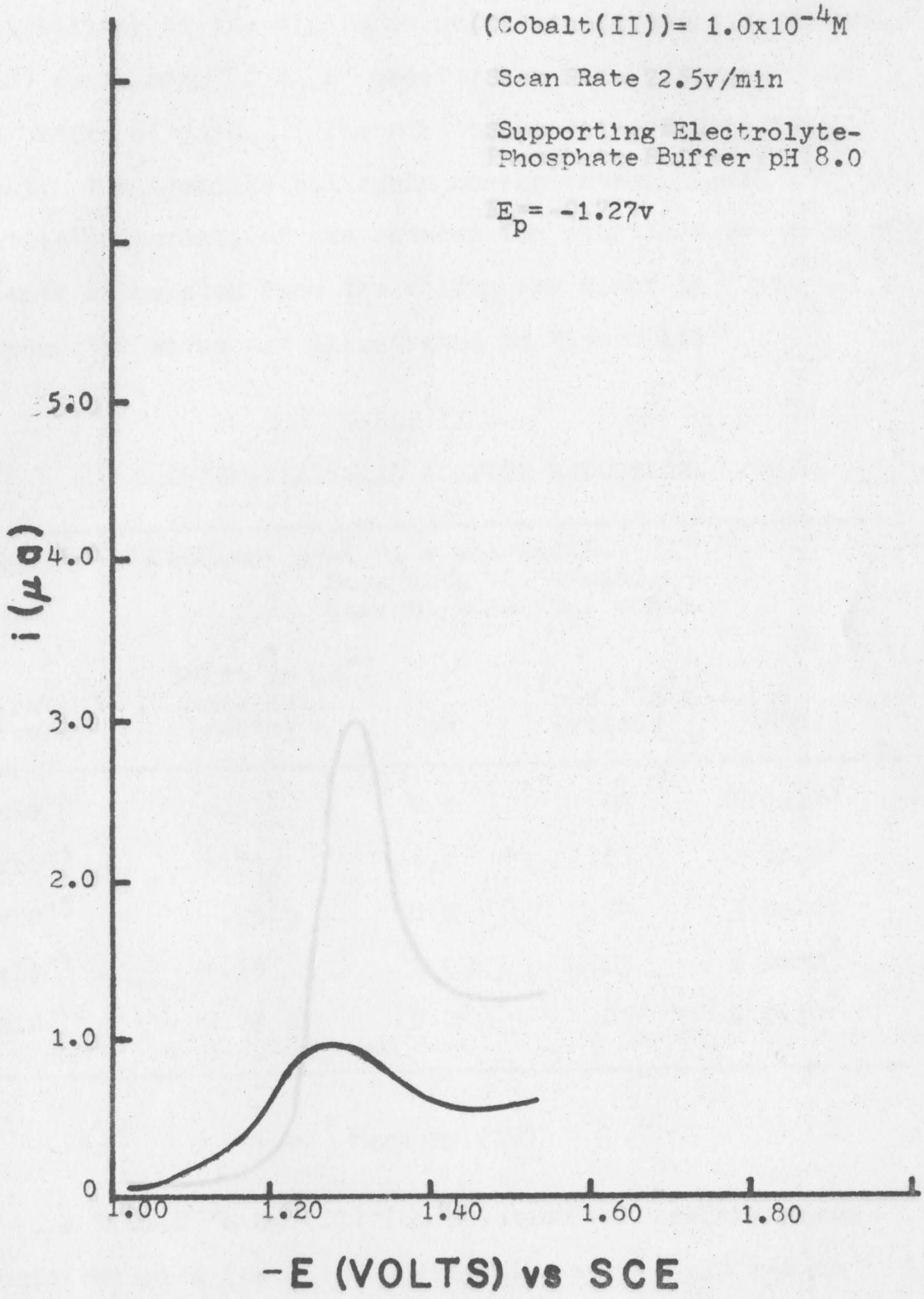


Fig.16-Cobalt(II) Reduction

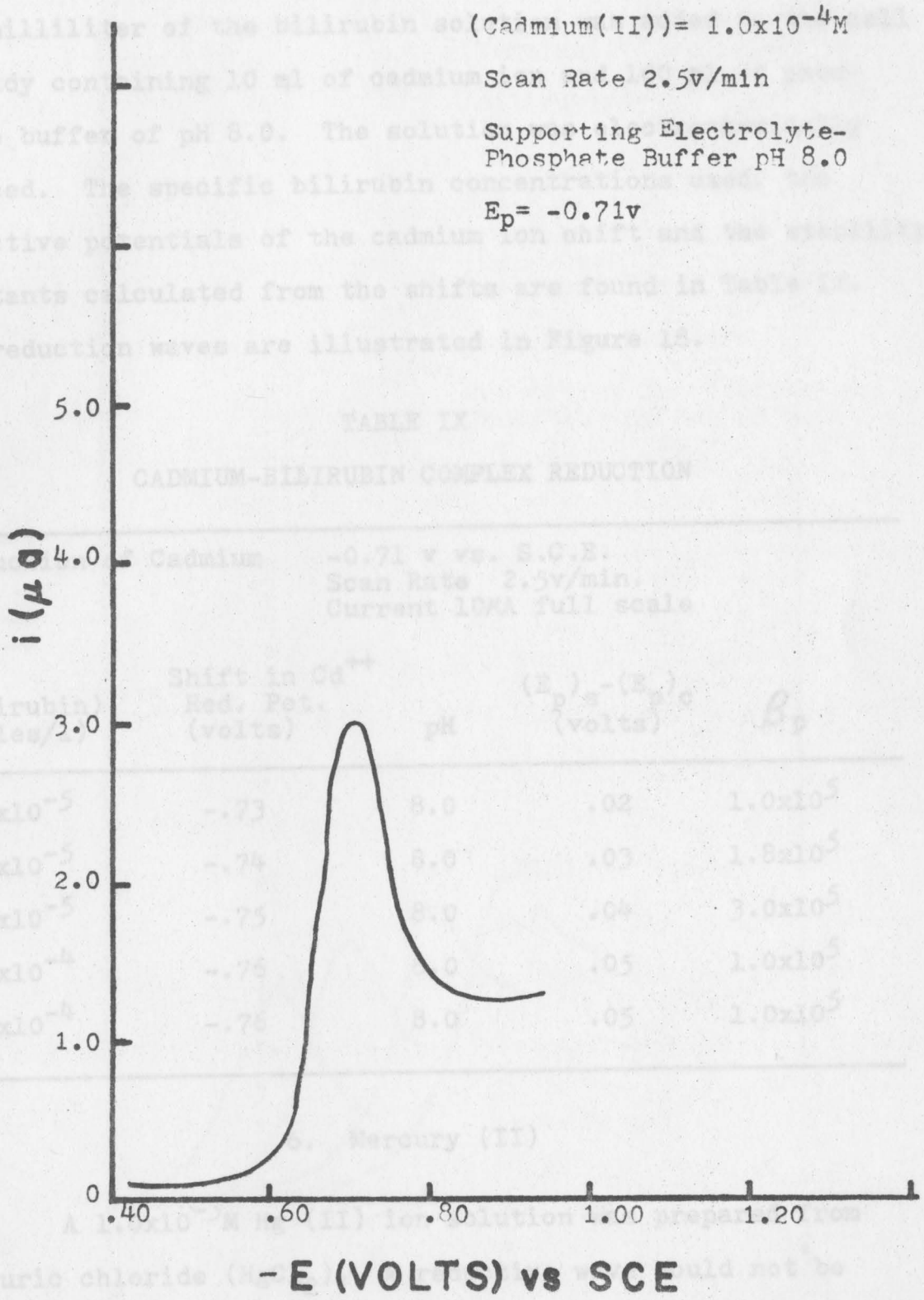


Fig.17-Cadmium(II) Reduction

A series of bilirubin concentrations was prepared. One milliliter of the bilirubin solution was added to the cell already containing 10 ml of cadmium ion and 100 ml of phosphate buffer of pH 8.0. The solution was electrochemically reduced. The specific bilirubin concentrations used, the reductive potentials of the cadmium ion shift and the stability constants calculated from the shifts are found in Table IX. The reduction waves are illustrated in Figure 18.

TABLE IX

CADMIUM-BILIRUBIN COMPLEX REDUCTION

Reduction of Cadmium -0.71 v vs. S.C.E.
Scan Rate 2.5v/min.
Current 10μA full scale

(Bilirubin) (moles/l)	Shift in Cd^{++} Red. Pot. (volts)	pH	$(E_p)_s-(E_p)_c$ (volts)	β_p
5.0×10^{-5}	-.73	8.0	.02	1.0×10^{5}
6.0×10^{-5}	-.74	8.0	.03	1.8×10^{5}
7.0×10^{-5}	-.75	8.0	.04	3.0×10^{5}
4.0×10^{-4}	-.76	8.0	.05	1.0×10^{5}
5.0×10^{-4}	-.76	8.0	.05	1.0×10^{5}

6. Mercury (II)

A 1.0×10^{-3}M Hg (II) ion solution was prepared from mercuric chloride ($HgCl_2$). A reductive wave could not be obtained using the H.M.D.E. The mercury (II) ion could be

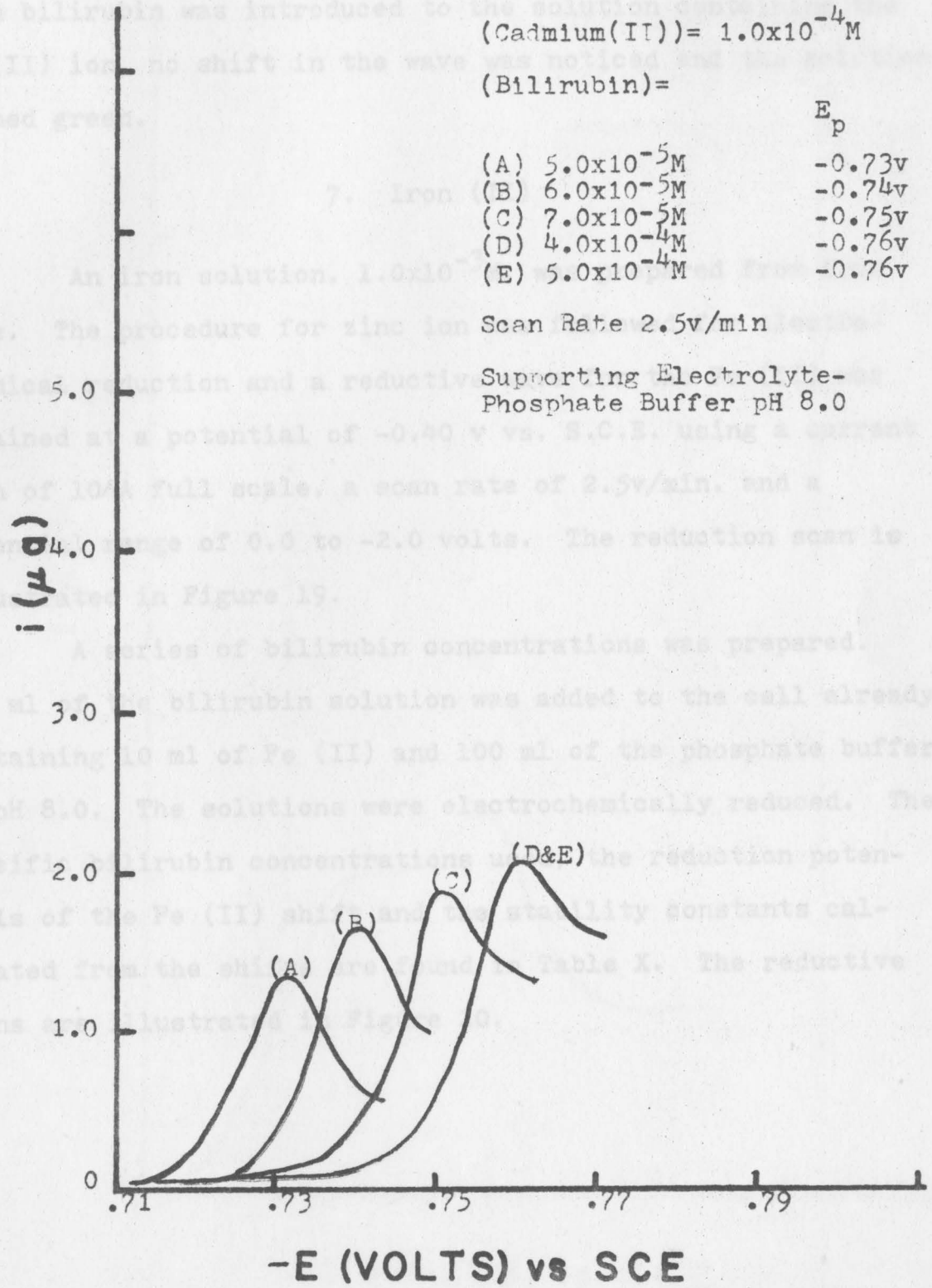


Fig.18-Cadmium(II)-Bilirubin Complex Reduction

reduced using a platinum electrode at -0.18 volts however when bilirubin was introduced to the solution containing the Hg (II) ion, no shift in the wave was noticed and the solution turned green.

7. Iron (II)

An iron solution, 1.0×10^{-3}M, was prepared from iron wire. The procedure for zinc ion was followed for electrochemical reduction and a reductive wave for the Fe (II) was obtained at a potential of -0.40 v vs. S.C.E. using a current span of 10μA full scale, a scan rate of 2.5v/min. and a potential range of 0.0 to -2.0 volts. The reduction scan is illustrated in Figure 19.

A series of bilirubin concentrations was prepared. One ml of the bilirubin solution was added to the cell already containing 10 ml of Fe (II) and 100 ml of the phosphate buffer of pH 8.0. The solutions were electrochemically reduced. The specific bilirubin concentrations used, the reduction potentials of the Fe (II) shift and the stability constants calculated from the shifts are found in Table X. The reductive scans are illustrated in Figure 20.

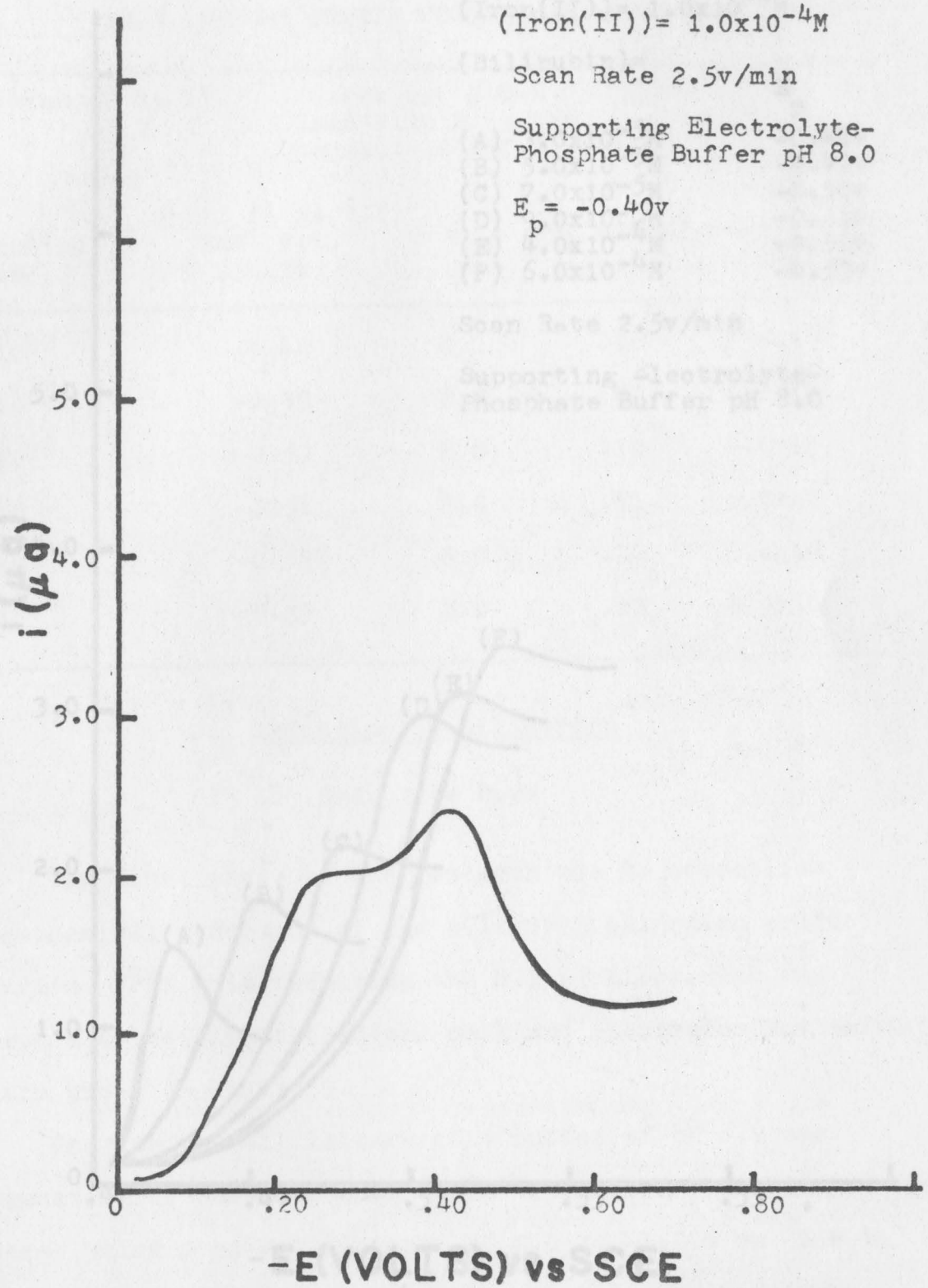


Fig.19-Iron(II) Reduction

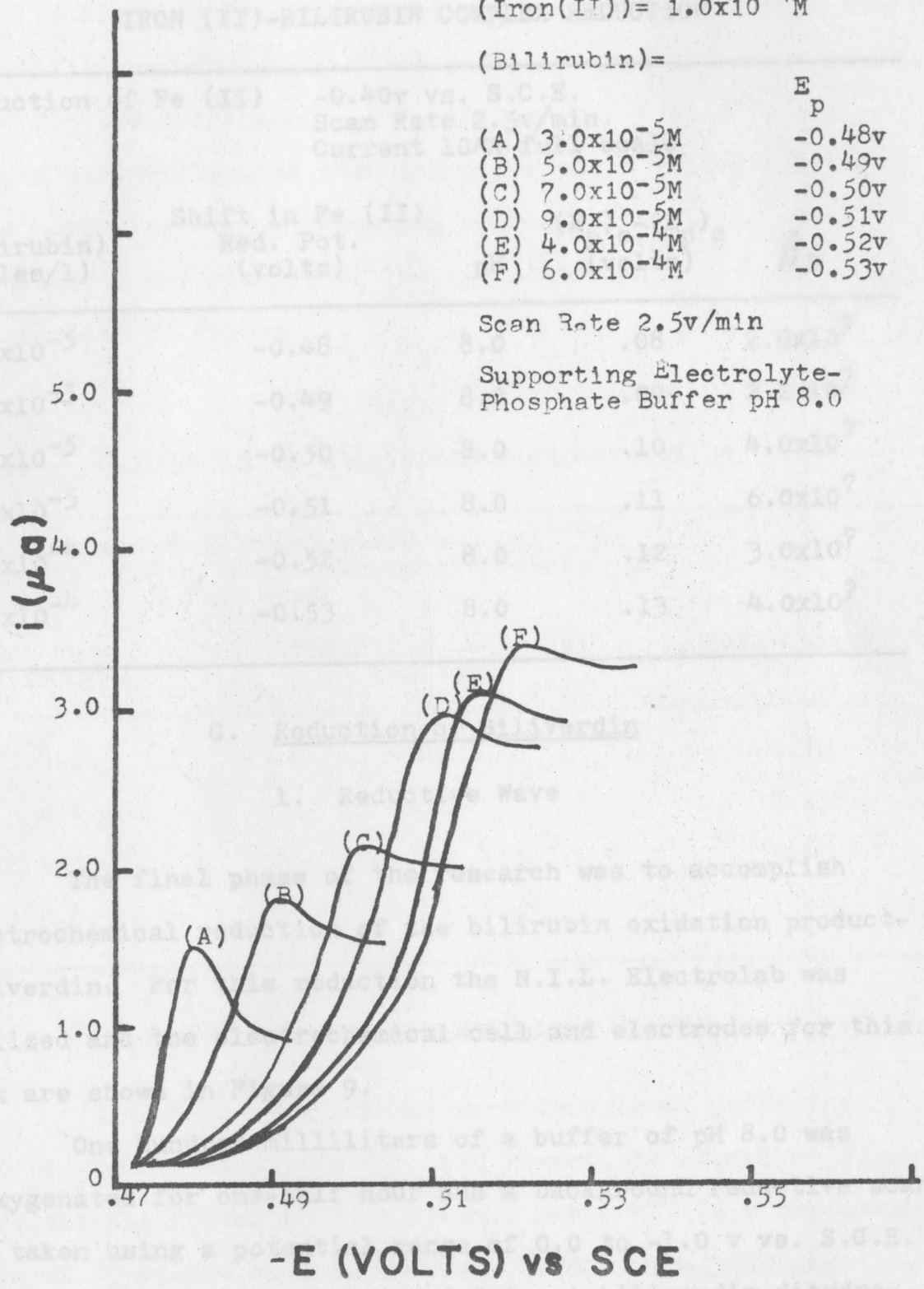


Fig.20-Iron(II)-Bilirubin Complex Reduction

TABLE X

IRON (II)-BILIRUBIN COMPLEX REDUCTION

Reduction of Fe (II) -0.40v vs. S.C.E.
Scan Rate 2.5v/min.
Current 10μA full scale

(Bilirubin) (moles/l)	Shift in Fe (II) Red. Pot. (volts)	pH	$(E_p)_s-(E_p)_c$ (volts)	β_p
3.0×10^{-5}	-0.48	8.0	.08	2.0×10^{7}
5.0×10^{-5}	-0.49	8.0	.09	2.2×10^{7}
7.0×10^{-5}	-0.50	8.0	.10	4.0×10^{7}
9.0×10^{-5}	-0.51	8.0	.11	6.0×10^{7}
4.0×10^{-4}	-0.52	8.0	.12	3.0×10^{7}
6.0×10^{-4}	-0.53	8.0	.13	4.0×10^{7}

G. Reduction of Biliverdin

1. Reductive Wave

The final phase of the research was to accomplish electrochemical reduction of the bilirubin oxidation product-biliverdin. For this reduction the N.I.L. Electrolab was utilized and the electrochemical cell and electrodes for this work are shown in Figure 9.

One hundred milliliters of a buffer of pH 8.0 was deoxygenated for one-half hour and a background reductive scan was taken using a potential range of 0.0 to -1.0 v vs. S.C.E. and a full scale current span of 5μA. A biliverdin dihydrochloride sample weighing .00334 g was added to the electro-

chemical cell. The contents of the cell were stirred for one hour to dissolve the biliverdin. The solution was then electrochemically reduced using the potential range and current scan used for the background. Reduction of the 5.0×10^{-4}M biliverdin dihydrochloride solution occurred at values ranging from -.42 v to -.46 v depending on the scan rate. Table XI illustrates the results of the reduction. The reductive scans may be seen in Figure 21.

TABLE XI

REDUCTION OF BILIVERDIN DIHYDROCHLORIDE

(Biliverdin Dihydrochloride) = 5.0×10^{-5}M
Supporting Electrolyte-Phosphate Buffer (pH 8.0)
Current 5μA full scale

Scan Rate (volts/min.)	Reduction Potential (volts)	Peak Current (μA)
0.5	-.42	0.80
1.0	-.42	0.95
2.5	-.45	1.40
5.0	-.47	1.95

2. Calculation of the Diffusion Coefficient

Diffusion coefficients were calculated using the Randles-Sevcik equations for both reversible (Equation 8) and irreversible (Equation 9) systems. Table XII is a representative of D_o values calculated for a 5.0×10^{-5}M biliverdin dihydrochloride solution.

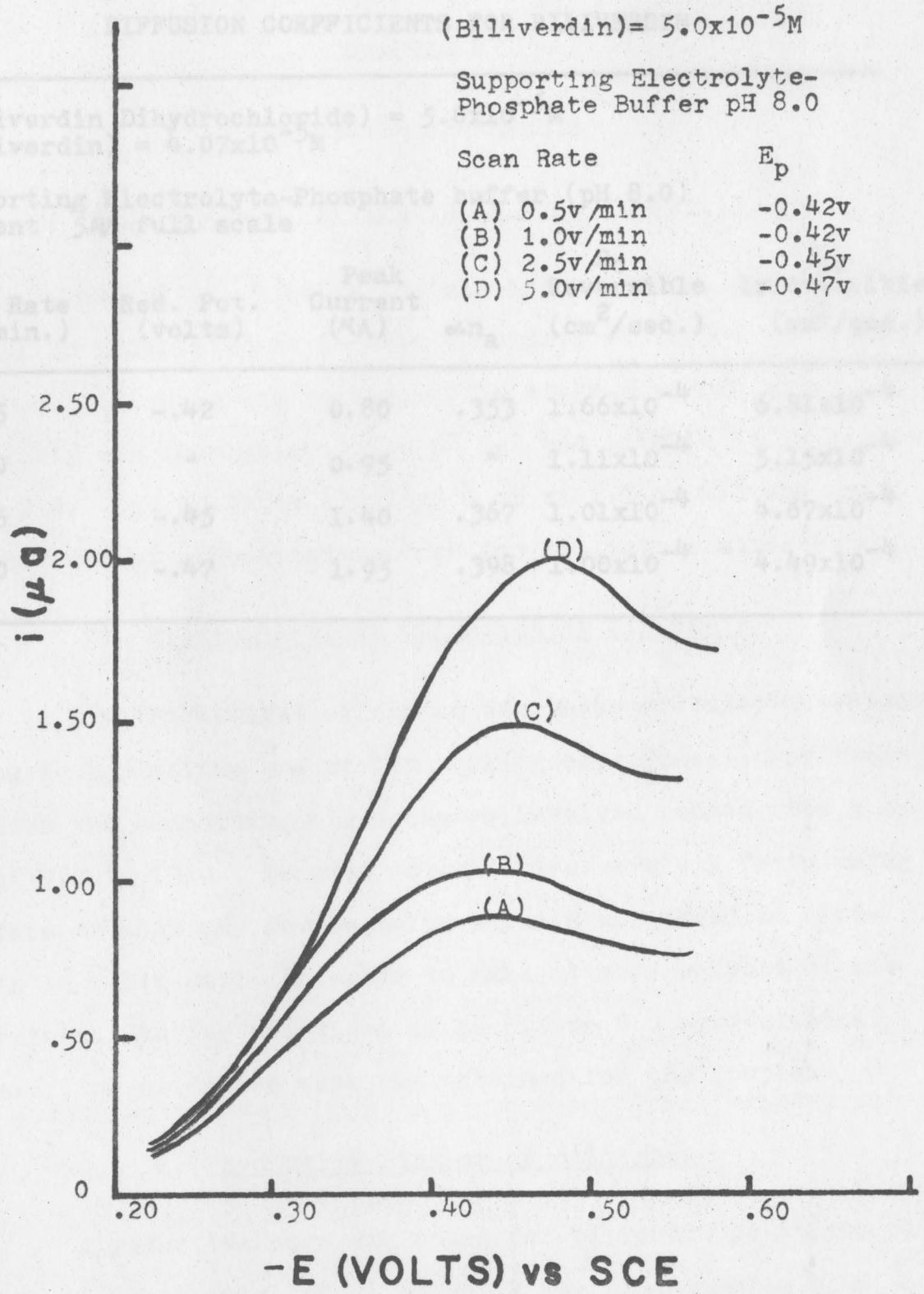


Fig.21-Biliverdin Reduction

TABLE XII

DIFFUSION COEFFICIENTS FOR BILIVERDIN

(Biliverdin Dihydrochloride) = 5.0×10^{-5}M
(Biliverdin) = 4.07×10^{-5}M

Supporting Electrolyte-Phosphate buffer (pH 8.0)
Current 5μA full scale

Scan Rate (v./min.)	Red. Pot. (volts)	Peak Current (μA)	αn_a	D_o Reversible (cm^2/sec.)	D_o Irreversible (cm^2/sec.)
0.5	-.42	0.80	.353	1.66×10^{-4}	6.81×10^{-4}
1.0	"	0.95	"	1.11×10^{-4}	5.15×10^{-4}
2.5	-.45	1.40	.367	1.01×10^{-4}	4.67×10^{-4}
5.0	-.47	1.95	.398	1.00×10^{-4}	4.49×10^{-4}

CHAPTER V

DISCUSSION OF RESULTS

A. Solubility of Bilirubin in Aqueous Solutions

The initial studies in this investigation were very useful to later studies in that they determined the pH range in which bilirubin was soluble. The studies showed that bilirubin was insoluble at low pH but was soluble at pH values over 7.0. Due to these findings a pH of 7.8 or 8.0 was used in the form of a phosphate buffer for all future studies.

B. Electrochemical Oxidation of Bilirubin

Electrochemical oxidation was unsuccessfully attempted using both platinum and carbon working electrodes. For these studies the supporting electrolytes involved ranged from a pH of 7.8 to 12.0. Because no oxidative wave was found using buffers of high pH, bovine serum albumin was added to bilirubin in a 1:1 ratio in order to make it more soluble at low pH values. Buffer solutions of pH 3.5 to 8.0 were utilized, however, no oxidative wave was obtained for the complex.

C. Reductive Studies of Bilirubin

A reductive wave was found for bilirubin solutions at a reduction potential of -1.40 volts vs. S.C.E. using the H.M.D.E. Results for various bilirubin concentrations can

be found in Table IV. The reductive wave was found to be irreversible. For this reason the diffusion coefficients for bilirubin were calculated using the Randles-Sevcik equations (Equations 8 and 9). Representative values for the D_o values are shown in Tables V and VI. The average value for the diffusion coefficient using Equation 8 is $6.21 \pm .43 \times 10^{-6}$. The average value for the diffusion coefficient using Equation 9 is $8.36 \pm 1.33 \times 10^{-6}$. The values calculated both ways were very similar and of the same order of magnitude.

D. Controlled Potential Electrolysis

The technique of controlled potential electrolysis which can be used to determine the number of electrons transferred in a reaction was employed to confirm the two-electron transfer of the bilirubin reduction. No electrolysis could be measured due to the interference of hydrogen deposition.

E. Electrochemistry of Bilirubin-Metal Complexes

1. Zinc (II)

A zinc ion solution was electrochemically reduced using a H.M.D.E. The reductive plot is shown in Figure 13. The reductive curves for the bilirubin-metal ion complexes can be seen in Figure 14. Shifts in the reductive wave due to increased bilirubin concentration and calculated stability constants are tabulated in Table VII and Table VIII. The average value for the stability constant (β_p) is $2.0 \pm 1.3 \times 10^6$.

2. Copper (II)

A copper metal ion solution was electrochemically reduced using the H.M.D.E. and the reductive scan is illustrated in Figure 15. When bilirubin solutions were added to the Cu (II) solution, no shift in reduction could be obtained. The solution changed from reddish-brown to dark green upon addition of the bilirubin which was probably caused by the oxidation of bilirubin to biliverdin enhanced by the presence of Cu (II) ions.

3. Cobalt (II)

A cobalt metal-ion solution was electrochemically reduced using the H.M.D.E. and the reductive scan is illustrated in Figure 16. When bilirubin solutions were added to the cobalt ions in the phosphate buffer, no shift in the reductive wave was obtained.

4. Nickel (II)

No reductive wave for nickel ion in a phosphate buffer could be obtained using the H.M.D.E.

5. Cadmium (II)

A reductive wave was found for the cadmium ion solution using a H.M.D.E. and the reductive scan is shown in Figure 17. The reductive scans for the bilirubin-cadmium-ion complexes are shown in Figure 18. Shifts in the reductive

wave due to increased bilirubin concentration and calculated stability constants are tabulated in Table IX. The average value for the stability constant (β_p) is $1.6 \pm .8 \times 10^5$.

6. Mercury (II)

A reductive wave for mercury ion could not be obtained using the H.M.D.E. A reductive wave was obtained using a platinum electrode, however, upon addition of bilirubin, the solution was apparently oxidized since the dark green color associated with biliverdin appeared.

7. Iron (II)

An iron (II) ion solution was electrochemically reduced using a H.M.D.E. The reductive plot is shown in Figure 19. The reductive curves for the bilirubin-iron (II) ion complexes can be seen in Figure 20. Shifts in the reductive wave due to increased bilirubin concentration and calculated stability constants are tabulated in Table X. The average value for the stability constant (β_p) is $3.5 \pm 1.5 \times 10^7$.

F. Reduction of Biliverdin

A reductive wave was found for biliverdin solutions at a reduction potential of approximately -.42 volts vs. S.C.E. using a H.M.D.E. The reductive scan is shown for various scan rates in Figure 21. The reductive wave was found to be highly irreversible and for this reason the diffusion coefficients were calculated using both Equation 8 and Equation 9.

Representative values for the D_o values are shown in Table XII. The average value for the diffusion coefficient using Equation 8 is $1.19 \pm .31 \times 10^{-4}$. The average value for the diffusion coefficient using Equation 9 is $5.28 \pm 1.06 \times 10^{-4}$. The values obtained using the irreversible equation are much higher than those obtained using the reversible equation but are of the same order of magnitude. The irreversible values are closer to those for other organic compounds in aqueous solutions than the reversible ones.[14]

CHAPTER VI

CONCLUSIONS

In this investigation, the electrochemical method of reduction was utilized in order to obtain information about bilirubin, its related metal ion complexes and biliverdin. Except for the work of Tvaroha, this is the first electrochemical study on bilirubin and related compounds. The results of this study can be divided into five main aspects:

1. Both the electrochemical processes - oxidation and reduction were attempted for dilute bilirubin solutions in various buffer systems. The oxidation process proved to be unsuccessful; however, the reduction process was successful and a reduction wave was found at a potential of -1.40 volts vs. S.C.E. Since electrochemical reduction at a H.M.D.E. is diffusion controlled, the current is proportional to concentration. The wave was found to be irreversible. The irreversibility was noted by reversing the sign of the electrochemical potential and attempting re-oxidation. The re-oxidation did not occur. Irreversibility could also be noted from the spreading out and rounding of the peak voltammograms. The reduction process was highly reproducible.

2. Through the electrochemical reduction of bilirubin, diffusion coefficients could be calulated for a theoretically reversible system and for the actual irreversible system using

the Randles-Sevcik equation. Values for D_o are very similar to those for organic species in aqueous solutions.[14]

3. Metal-bilirubin complexes were successfully formed with Zinc (II), Iron (II), and Cadmium (II). From the shift in metal-ion reduction wave upon addition of increased bilirubin concentration, stability constants were calculated. This is the first evaluation of stability constants for the metal-bilirubin complexes. Metal-ion complexes were unsuccessfully attempted for Copper (II), Cobalt (II), Nickel (II), and Mercury (II). Since there are no reported values for the stability constants of bilirubin-metal-ion complexes, no comparisons can be made. The use of this technique in direct analysis of biological fluids like serum would probably not be possible because the serum contains many other species that are reducible.

4. Electrochemical reduction was successfully attempted for biliverdin dihydrochloride. A reduction wave for the biliverdin dihydrochloride system was found at -0.42 volts vs. S.C.E. Reduction being a diffusion controlled process, the reduction current was proportional to concentration. The system proved to be even more irreversible than the bilirubin reduction using the same type of criteria and values for the reduction potential are reproducible.

5. Diffusion coefficients were calculated for a theoretically reversible system and the actual irreversible system using the Randles-Sevcik equation. Values for D_o were somewhat larger than those found for organic molecules in aqueous solutions.[14]

CHAPTER VII

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