THE CHEMISTRY OF BILIRUBIN IN N,N-DIMETHYLFORMAMIDE

by

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ABSTRACT

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The present work is concerned with the reactions of the bile pigments-bilirubin and biliverdin in N,N-Dimethylformamide (DMF) as a solvent system. Qualitative experiments in DMF were performed as a preliminary study to determine the reactivity of bilirubin with a number of oxidizing and complexing agents. It was found to readily react with a few of these reagents. Consequently, some of the reactions were followed using visible spectroscopy. The solubility and stability of bilirubin in DMF was also qualitatively compared in a number of other solvents.

Particular attention was focused on the properties of bilirubin under acidic and basic conditions. The bile pigment appeared unreactive to acids. This was confirmed by potentiometric titrations with HCl and picric acid using a glass indicator electrode. The oxidative voltammetry of bilirubin at a platinum indicator electrode was examined and showed no significant changes in the presence of picric acid. On the contrary, the acidic nature of bilirubin was indicated by its reactivity with basic impurities in the

DMF solvent. It was shown to behave as a diprotic acid as evidenced by quantitative changes occurring in its visible spectra and by potentiometric titration with tetramethylguanidine (TMG). A quantitative shift of oxidation potential from approximately +.7 volts to +.4 volts vs. Ag/AgCl(sat), NaCl(sat) reference electrode was also observed with the addition of two molar equivalents of TMG. Controlled potential coulometry of bilirubin under these basic conditions indicated that the oxidation at +.4 volts leads to the same two electron oxidation to biliverdin as occurs under neutral conditions. The oxidative potential of biliverdin was also observed to shift to lower potentials with the addition of excess TMG.

The reactivity of bilirubin with the Zinc(II) ion was also investigated under neutral conditions and in the presence of TMG as a base. Visible spectroscopy indicated that with the addition of TMG a reversible metal ion-bilirubin complex forms with a corresponding shift in maximum absorbance from 450nm to 530nm. An examination of the dependence of complex formation on the concentrations of TMG and Zinc(II) was also undertaken utilizing visible spectroscopy and potentiometry. The results tend to indicate that a possible complex equilibrium is established between Zinc(II) as a Lewis acid, bilirubin as a diprotic acid, and TMG as a common base.

The formation of the Zinc(II)-bilirubin complex was also correlated with a shift in the oxidation potential of bilirubin from +.4 volts to approximately 0.0 volts using both platinum and vitreous carbon indicator electrodes. An attempt was made to elucidate the oxidative process at 0.0 volts using constant potential coulometry in combination with visible spectroscopy. The experimental data are discussed and interpreted as resulting from the formation of a mixture of products. The oxidation of the Zinc(II) complex with I_2 is also followed spectrophotometrically and compared to the results of the electrolysis.

A separate study of the reactivity of biliverdin with TMG and Zinc(II) was necessitated to clarify the voltammetric and spectral changes observed in the bilirubin reactions. Biliverdin was also found to readily complex with Zinc(II) under basic conditions and to undergo significant spectral shifts. Changes in the anodic voltammetry of biliverdin with the addition of TMG and Zinc(II) are reported and are similar to the changes observed with bilirubin. An electrolysis of the Zinc(II)-biliverdin complex was also performed and the results used to help interpret the spectral data obtained from the Zinc(II)-bilirubin complex oxidation.

A short study of the reaction of the Copper(II) ion with bilirubin and biliverdin was also undertaken. As in the case with Zinc(II), no reactions occurred until TMG was added. The visible spectra following the course of the

reaction of Copper(II) with both bile pigments is presented. Both reactions appeared to progress in a quantitative fashion leading to the formation of the same apparent product The reactions were irreversible and tend to indicate that the bile pigments undergo oxidation.

ACKNOWLEDGEMENTS

I wish to thank Dr. John D. Van Norman, my thesis advisor, for his indispensable guidance, suggestions, and help in the completion of this research. I would also like to extend my appreciation to the faculty of the Department of Chemistry for their continuous encouragement and aid and give particular thanks to Dr. James Reeder, Dr. Richard Phillips, Dr. Thomas Dobbelstein, and Mr. Dale Manos for helpful discussions and suggestions pertaining to various aspects of this work.

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LIST OF SYMBOLS

SYMBOL	DEFINITION	UNITS
DMF	N,N-Dimethylformamide	
DMSO	Dimethylsulfoxide	
BR	Bilirubin	
BV	Biliverdin	
E_{cell}	Cell potential	volts
$E^{o}_{ox,RED}$	Standard electrode potential	volts
Ox	Oxidized form of an arbitrary substance	
Red	Reduced form of an arbitrary substance	
E_{Ref}	The electrode potential of any arbitrary reference electrode	volts
E_g	Potential measured at a glass membrane electrode	volts
$(H^+)_{UNK}$	Proton concentration of unknown solution	moles/liter
$(H^+)_{STD}$	Proton concentration of a known standard solution	moles/liter
R	Universal gas constant	
T	Temperature	Kelvin units
n	Number of moles of electrons exchanged per mole of reactant	equivalents/ mole
F	Faraday constant	96,500 coulombs/ equivalent
i_L	Limiting diffusion current	amperes
D	Diffusion coefficient	cm^2/sec
C	Concentration	moles/liter
δ	Nernst diffusion layer thickness	cm

LIST OF SYMBOLS (CONT'D)

SYMBOL	DEFINITION	UNITS
$E_{\frac{1}{2}}$	Half-wave potential	volts
i	Measured current	amperes
$(i_L)_c$	Limiting cathodic current	amperes
$(i_L)_a$	Limiting anodic current	amperes
i_p	Peak current	amperes
A	Area of the electrode	cm^2
ν	The rate of voltage scan	volts/sec
α	The transfer coefficient	
N_a	The number of electrons in the rate-controlling step of the electron-transfer process	
Q	Quantity of electricity	coulombs
M	Amount of material electrolyzed	moles
i_o	Value of initial current	amperes
V	Volume of the electrolyzed solution	liters
TMG	Tetramethylguanidine	
Zn_2BR	The Zinc(II)-bilirubin complex	
$ZnBV^-$	The Zinc(II)-biliverdin complex	
S.C.E.	Saturated calomel electrode	
v	Volts	volts
mv	Millivolts	millivolts
ε	Molar absorptivity	liter/mole-cm

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CHAPTER I

INTRODUCTION

A. Nature of Bilirubin

Bilirubin is a yellow tetrapyrrole belonging to a class of compounds found in the human body called bile pigments. Approximately 300 milligrams of bilirubin are formed daily from the enzymatic catabolism of hemoglobin taking place primarily in the reticuloendothelial cells in the liver, bone marrow, and spleen. Being insoluble at the pH of the blood, bilirubin is transported to the liver bound to the serum protein, albumin (Figure 1). In the endoplasmic reticulum of the liver cells bilirubin is separated from the albumin protein and is converted into water soluble mono- and di-glucuronides by reacting with uridine diphosphate glucuronate under the influence of UDP glucuronyl transferase. This esterification is essential, since a defect in any stage of the process results in an impairment of the liver's capability for removing bilirubin from the blood stream. Impairment of this process is manifested by the accumulation of bile pigment in the plasma in sufficient amounts to impart a yellowish tint to the skin. This is known as jaundice.

Conjugated bilirubin can be excreted via the bile to the intestine where it is hydrolyzed and undergoes a series of reductions by bacterial enzymes to form a series of

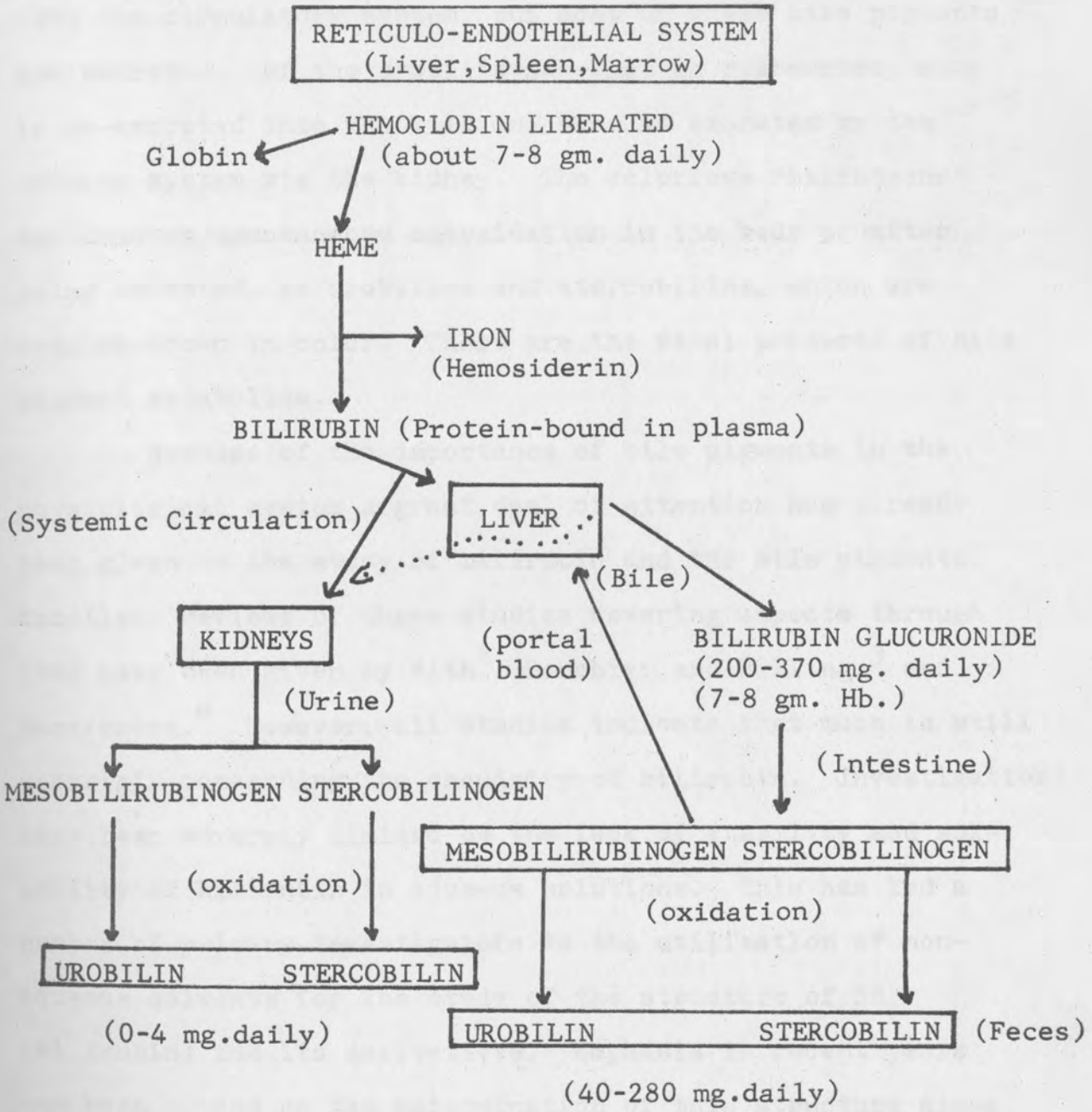


Figure 1. Hemoglobin Degradation and the Formation of Bile Pigments.

colorless tetrapyrroles known collectively as bilinogens or fecal "urobilinogen". Some of the urobilinogen is reabsorbed into the circulatory system, but most of these bile pigments are excreted. Of the urobilinogen that is reabsorbed, some is re-excreted into the bile and some is excreted by the urinary system via the kidney. The colorless "bilinogens" may undergo spontaneous autoxidation in the body or after being excreted, to urobilins and stercobilins, which are reddish-brown in color. These are the final products of bile pigment metabolism.

Because of the importance of bile pigments in the physiological system a great deal of attention has already been given to the study of bilirubin and the bile pigments. Excellent reviews of these studies covering aspects through 1967 have been given by With[2], Bouchier and Billing,[3] and Hargreaves.[4] However, all studies indicate that much is still uncertain concerning the chemistry of bilirubin. Investigations have been severely limited by the lack of stability and solubility of bilirubin in aqueous solutions. This has led a number of primary investigators to the utilization of non-aqueous solvents for the study of the structure of BR (bilirubin) and its derivatives. Emphasis in recent years has been placed on the determination of this structure since it is believed to play an extremely important role in the chemistry of BR. These studies have been made primarily on the basis of visible spectroscopy, IR, and NMR data. The characteristic differences between BR and its esters in terms

of solubility and reactivity have been explained on the basis of tautomerism and intramolecular hydrogen bonding. However, controversy has only recently been again aroused in this issue. The results to date thus still appear inconclusive.

One of the characteristic properties of most bile pigments was found to be their ability to form complex salts with Zinc ions. For a long time it was generally accepted, however, that rubins did not form complex salts due to their relative acidity as compared with the other bile pigments. However, only recently BR has been found to complex with a large number of transition and rare-earth elements.[5] Complexation has also been shown to catalyze the oxidation of BR by either an isomerization or chemical oxidation.[5,6] However, the only studies reported to this date have been with respect to complex stability and spectra. No real efforts have been made to quantitatively characterize the BR complexes or to elucidate their characteristics and structures.

B. Statement of the Problem

The literature available on the studies of bile pigments present only four investigations which deal with direct electroanalytical studies of bilirubinoid systems. These have been limited in scope. Thus, not much is known concerning the electrochemical behavior of BR and its analogues. Nevertheless, these investigations have shown the feasibility and promise of applying polarography, voltammetry, and controlled potential coulometry to the bilirubinoid system for the purposes

of identification, accurate quantitative determination, and synthesis. These methods are also advantageous in that measurements can be made without the addition of other chemical redox reagents or without changing the bulk solution composition.

Van Norman has shown the feasibility of combining electroanalytical and spectral studies of BR in N,N-dimethylformamide.[7] The utilization of dimethylformamide as a widely used electrolytic solvent system for a wide range of polar and nonpolar organic compounds and inorganic salts has also been well documented. Acid and base relationships have also been recently characterized for a wide variety of compounds in dimethylformamide (DMF).[34]

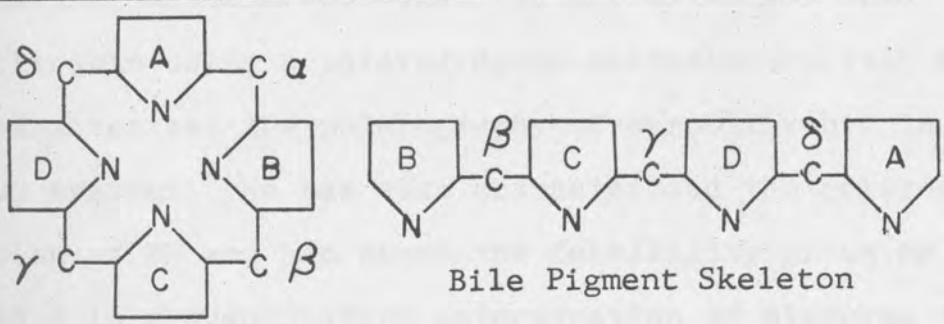
This investigation is, therefore, an attempt to utilize spectrophotometric and electrochemical methods for the study of BR in DMF as a solvent system. A preliminary qualitative study is made regarding the reactivity of BR in DMF with acid-base and redox reagents. This is followed by a potentiometric and voltammetric study of BR under acid and base conditions and under the conditions which lead to complexation with Zinc. The electrochemical methods are concurrently followed by visible spectroscopy in order to elucidate the chemical changes taking place.

CHAPTER II

THEORY

A. The Chemistry of Bilirubin

Bile pigments may be defined as compounds that consist of a chain of four pyrrole rings linked together by methene ($-CH_2-$) or methyne ($-CH=$) groups. They are classified according to the number and position of the double bonds joining the bridge carbon atoms to the pyrrole nuclei.[2] All naturally occurring bile pigments from higher animals are derived from protoporphyrin-IX by oxidative removal of the α -carbon linkage.



Porphyrin Skeleton

Bile Pigment Skeleton

Figure 2

Although the bile pigments are often represented as linear tetrapyrroles for convenience, the cyclic nearly planar arrangement as in porphyrin is a more precise representation in that it accounts for many of the properties of bile pigments. These include tautomeric and isomeric forms,[8,9] prototropic isomerisation or autoxidation reactions,[9,10,11]

the ability to form metal complexes,[8,9,10] and the optical activity of urobilins and stercobilins.[11,12]

Recently an enzyme-catalyzed mechanism has been proposed for the 'in vitro' formation of BR from the oxidative degradation of haemoglobin.[13] This is presented in Figure 3 primarily to indicate the structural relationships between biliverdin and bilirubin and the intermediate role of biliverdin. The exact mechanism of BR conversion into the other reduced bile pigments has not been elucidated. However, a synthetic reductive pathway is well known for the formation of urobilin from bilirubin.[12,14] This series of reactions is presented in Figure 4.

B. Tvaroha[15] has also indicated the feasibility of synthesizing mesobilirubinogen (or urobilinogen) from mesobilirubin using a polarographic microelectrolysis and has characterized the polarography of mesobilirubin in basic aqueous systems. He has also characterized the polarographic reduction of BR and has shown the feasibility of using this technique in a quantitative determination of mixtures of these two species.[16] In his work, Tvaroha also reported a polarographic anodic wave for mesobilirubin with a pH-dependent half-wave potential at about -.1 Volts vs. the S.C.E. The changes in the electrocapillary maxima with concentration indicate that this is probably a 4 electron oxidation of a Hg-mesobilirubin complex formed on the surface of the mercury drop.

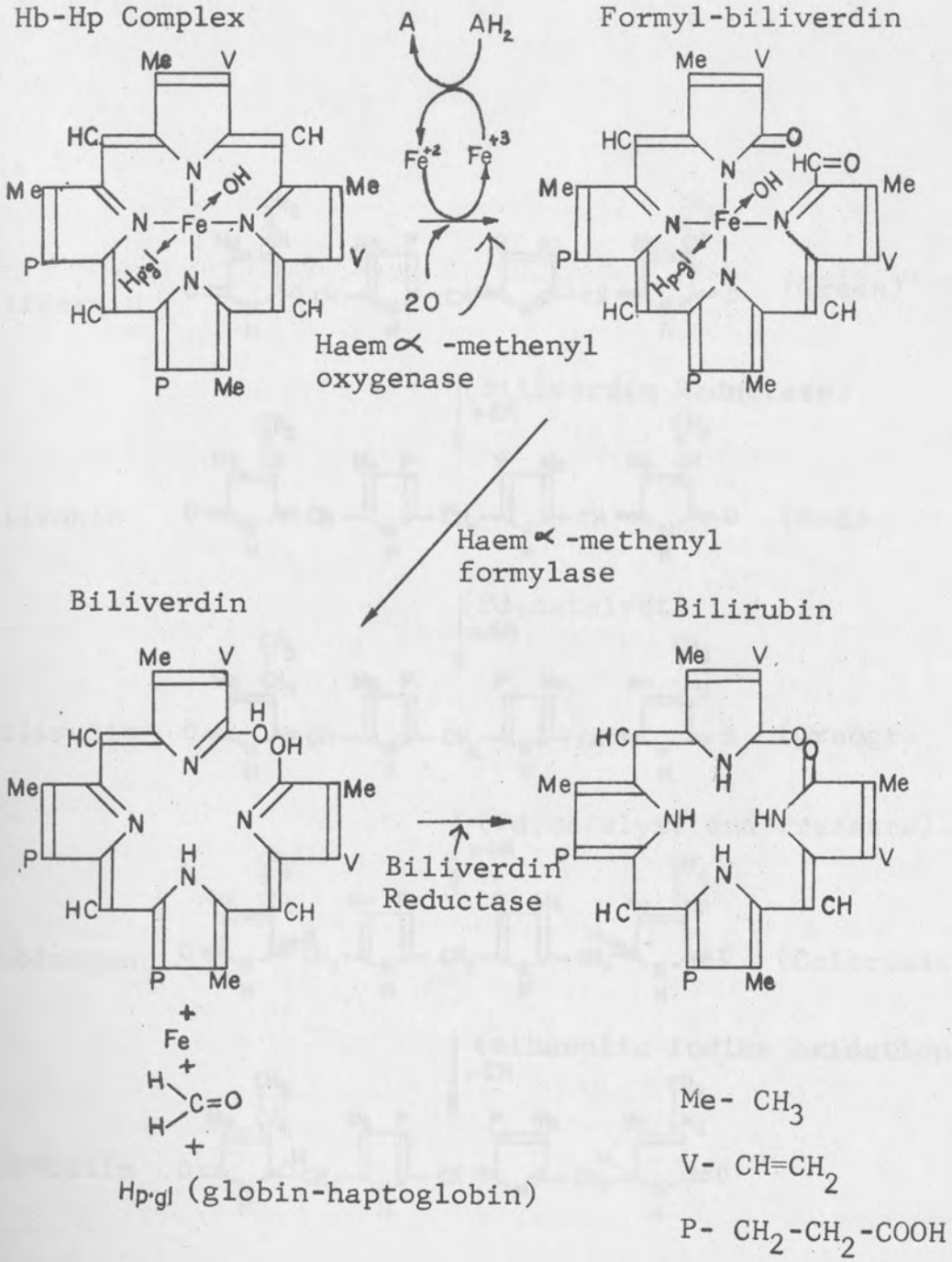


Figure 3- Possible Mechanism of Haemoglobin Degradation.

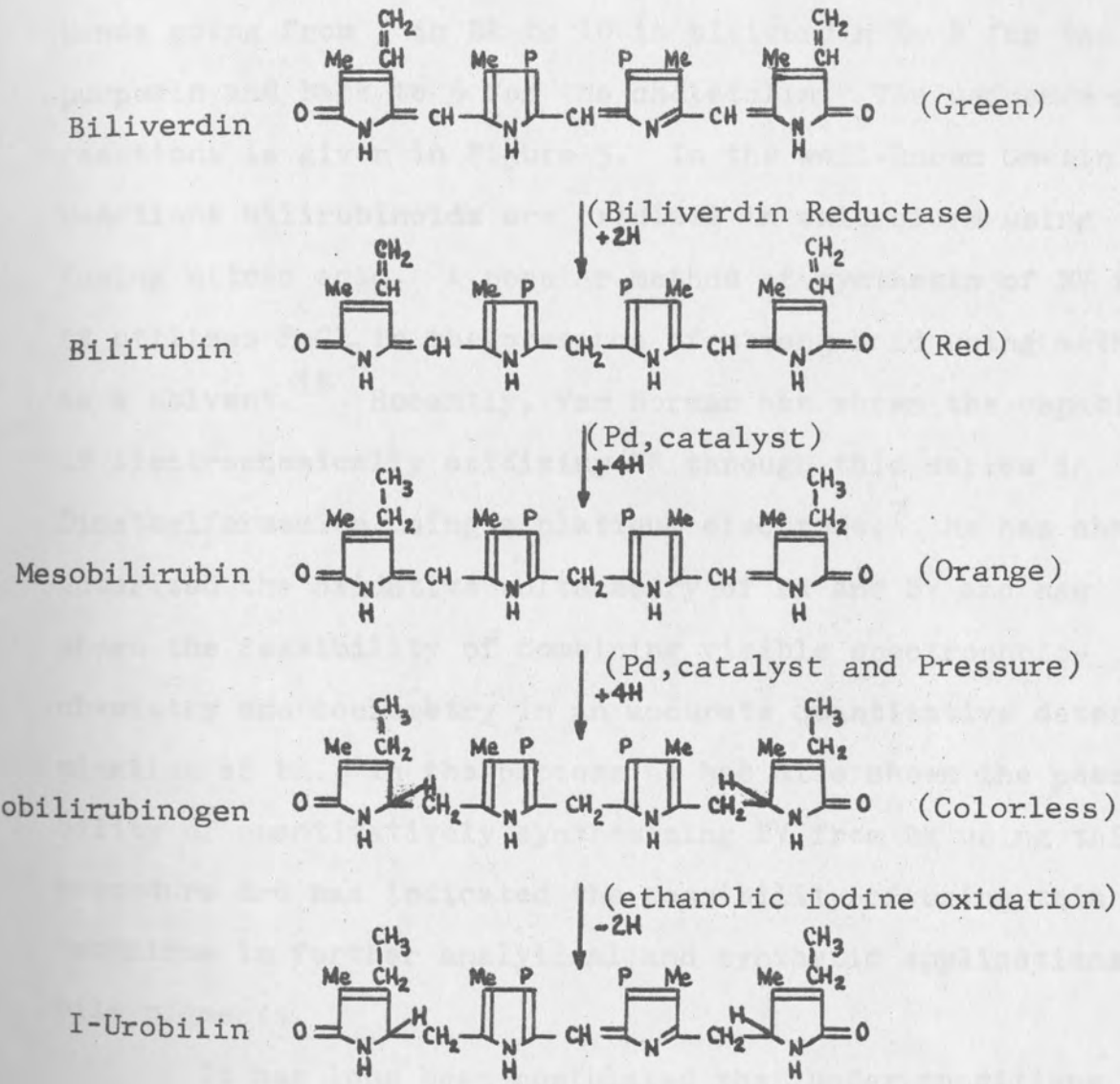


Figure 4. Reductive Pathway of Bile Pigments

The oxidative behavior of BR has also been well known and characterized. The spectral series associated with the oxidative changes has been reported by Zak et al.[17] using oxidizing agents of varying strengths. The color changes can be attributed to the change in the number of conjugated double bonds going from 5 in BR to 10 in biliverdin to 8 for the purpurin and back to 5 for the choletolin. The sequence of reactions is given in Figure 5. In the well-known Gmelin reactions bilirubinoids are oxidized in chloroform using fuming nitric acid. A popular method of synthesis of BV from BR utilizes $FeCl_3$ in the presence of strong acid using methanol as a solvent.[18] Recently, Van Norman has shown the capability of electrochemically oxidizing BR through this series in Dimethylformamide using a platinum electrode.[7] He has characterized the oxidative voltammetry of BR and BV and has shown the feasibility of combining visible spectrophotochemistry and coulometry in an accurate quantitative determination of BR. In the process he has also shown the possibility of quantitatively synthesizing BV from BR using this procedure and has indicated the possibility of using this technique in further analytical and synthetic applications to bile pigments.

It has long been postulated that under conditions in which the normal physiological reductive pathway is impaired, alternate routes might exist for the oxidative catabolism of BR in animals[19] with glucuronyl transferase deficiencies. It is also known that BR readily undergoes photooxidation to the

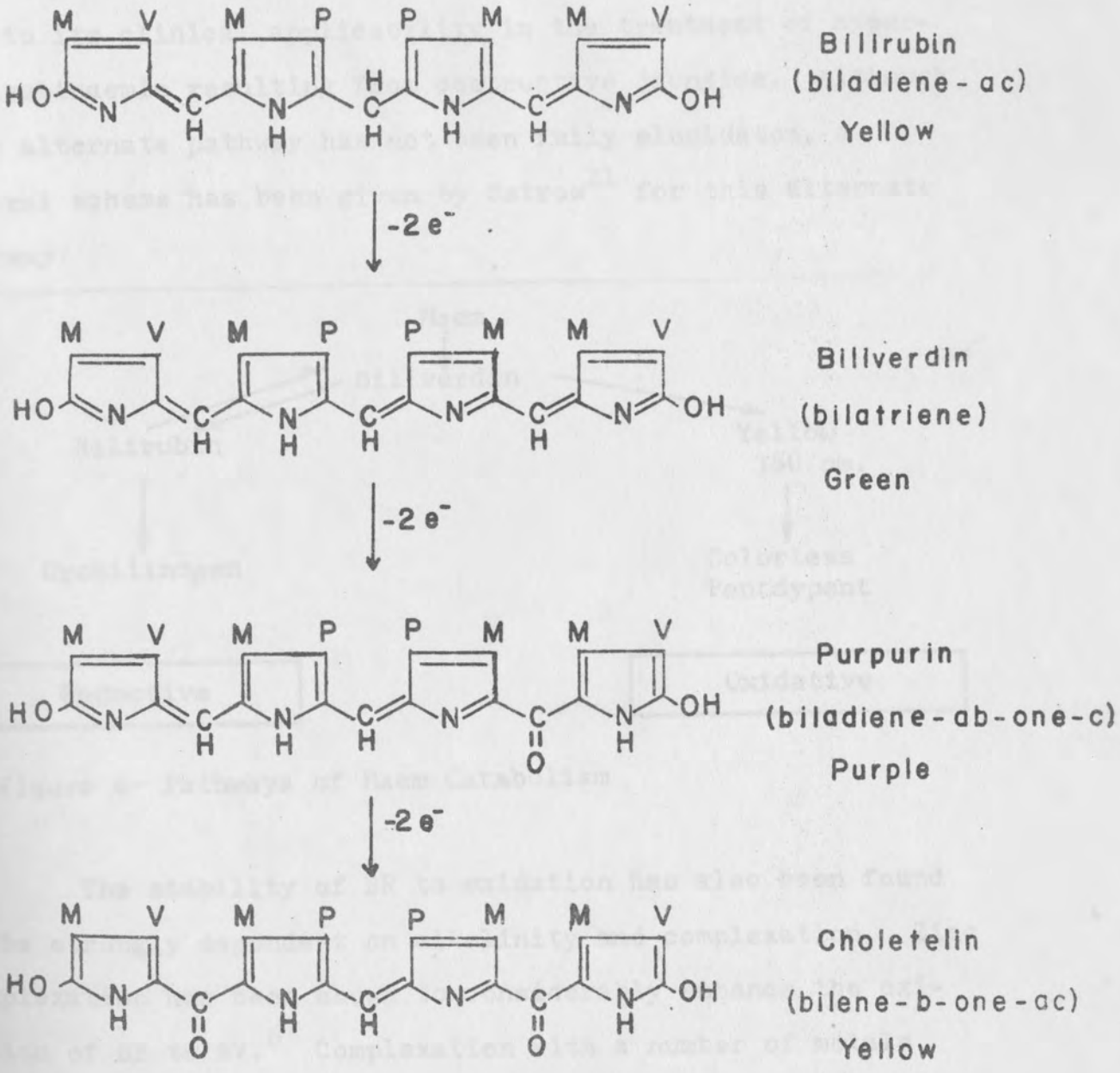


Figure 5- Oxidation Sequence of Bilirubin

verdinoid under both aerobic and aenerobic conditions.[5,20] The coupling of BR to the protein was shown to considerably enhance this aerobic and aenerobic oxidative process.[20] Importance has been focused on this alternate oxidative process due to its clinical applicability in the treatment of hyperbilirubinaemia resulting from obstructive jaundice. Although this alternate pathway has not been fully elucidated, a general scheme has been given by Ostrow[21] for this alternate pathway:

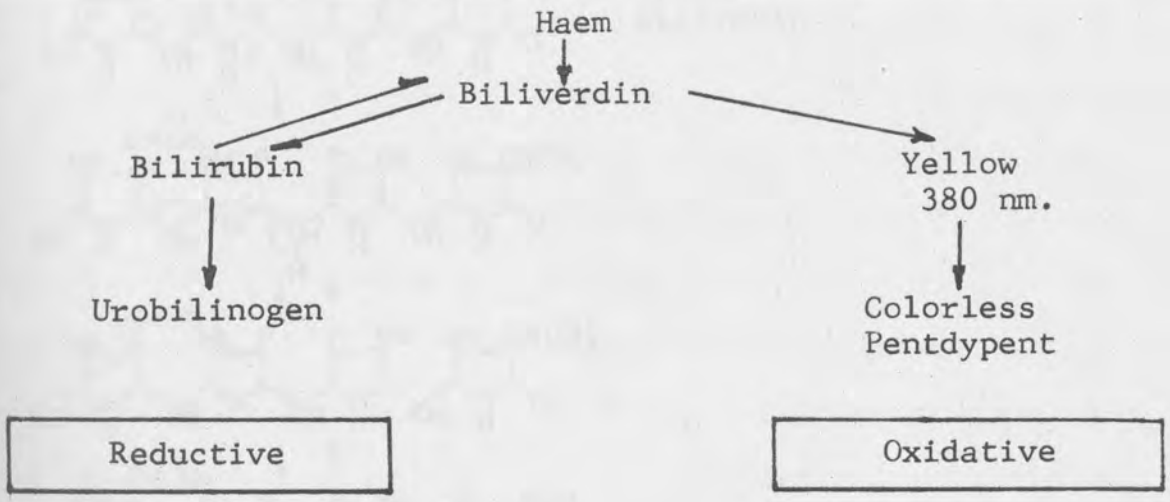
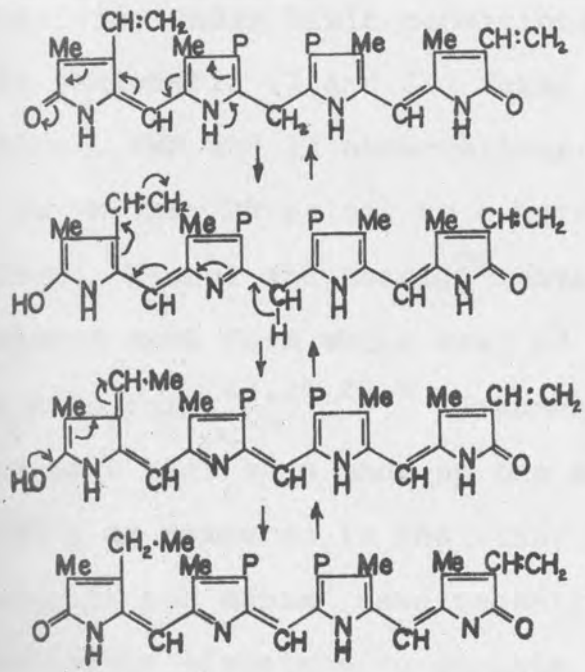


Figure 6- Pathways of Haem Catabolism

The stability of BR to oxidation has also been found to be strongly dependent on alkalinity and complexation. Zinc complexation has been shown to considerably enhance the oxidation of BR to BV.[6] Complexation with a number of metals has been shown to catalyze this process even under aenerobic conditions.[5] This ease of BR oxidation under the variety of conditions mentioned above is explained in terms of the lability of the central methylene bridge, the oxidation of

which results in the formation of the more stable verdinoid product. The autoxidation of BR to verdin even in the absence of an oxidizing agent is explained in terms of an intramolecular oxidation or prototropy. In this process, an isomerization occurs in which an unsaturated ethylene side chain of BR is reduced in a proton exchange which results in the oxidation of the methylene bridge. A scheme for this process is given as follows:



Bilirubin

Dihydrobiliverdin

Figure 7- Isomerization of Bilirubin to Dihydrobiliverdin

The product of this process is not BV but a reduced analogue, dihydrobiliverdin. This type of prototropic isomerization has also been shown to occur in other bile pigments.[9,22] It has been suggested that this process is catalyzed by irradiation with light of wavelengths between 400nm and 600nm and by metal complexation.[5,20]

The exact tautomeric structure for BR in solution has been a subject of debate ever since its discovery. It is commonly accepted that the relative insolubility of BR in neutral aqueous solutions is a result of strong intramolecular bonding of the type presented in Figure 9. This type of hydrogen bonding has also been used to explain the relative stability of BR to reaction as compared with its esters or conjugates,[24,25] its metal complexes, and as compared to its reactivity under basic conditions.[20,6,26] Arguments favoring both tautomeric (I and II) forms have been presented based on optical, NMR, and IR observations.[23,27,28,29] An uncertainty as to whether BR exists as a keto or enol isomer has also arisen. Nichol and Morell[29] have argued in favor of the predominant enol form while most of the other investigators favor the keto form.[23,24,27,8] Arguments favoring the keto structure have also been made on the basis of bilirubin's relative acidity as compared to the other bile pigments.[8,30] However, Velapoldi and Menis[5] have recently proposed a mono-lactam-mono-lactim structure to explain the ability of BR to form metal complexes. This is similiar to the structures proposed for stercobilin, i-urobilin, and d-urobilin.[8,12]

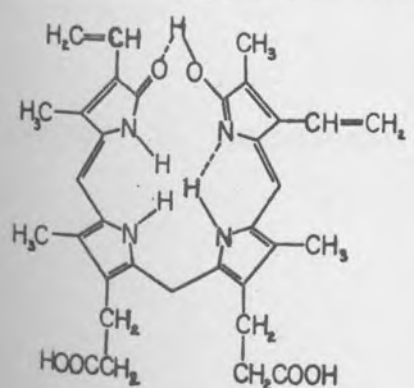


Figure 8- Mono-Lactam-Mono-Lactim Form of Bilirubin

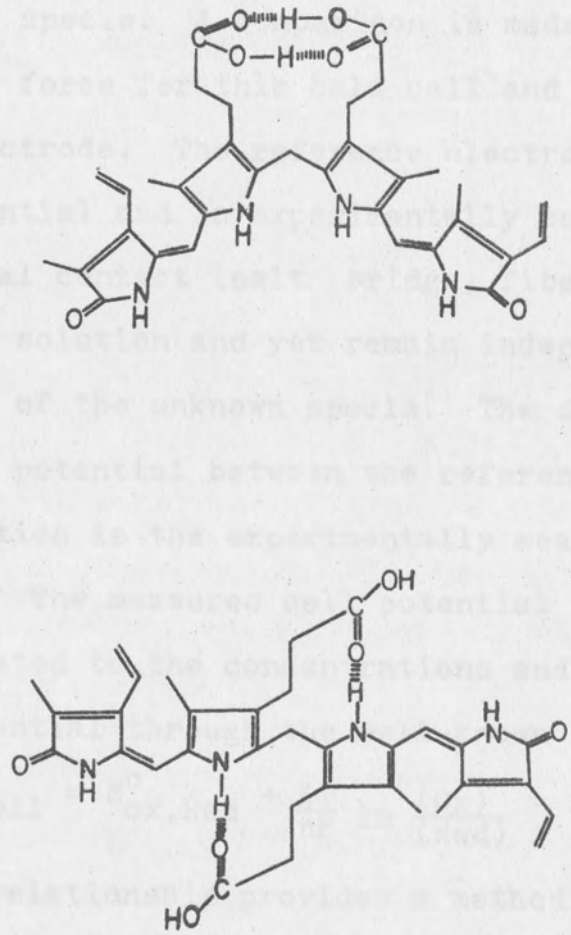


Figure 9. Tautomeric Forms of Bilirubin, Hydrogen Bonding

B. Direct Potentiometry

In this method analytical use is made of the thermodynamic relations existing between cell potentials and concentrations existing in a dynamic equilibria. Measurement is made of the electromotive force at zero faradaic current resulting at an indicator electrode due to the activity of the electroactive specie. A comparison is made between the electromotive force for this half cell and that of a stable reference electrode. The reference electrode has a constant relative potential and is experimentally constructed to make electrochemical contact (salt bridge, fiber, capillary, etc.) with the test solution and yet remain independent of the concentration of the unknown specie. The difference in electromotive potential between the reference half cell and the test solution is the experimentally measured cell potential (E_{cell}). The measured cell potential for a given redox system is related to the concentrations and to the reference electrode potential through the well-known Nernst equation:

$$E_{cell} = E^{o}_{ox,Red} + \frac{RT}{nF} \ln \frac{(Ox)}{(Red)} - E_{Ref}$$

This relationship provides a method for following the course of a reaction or titration where the ratio (Ox)/(Red) is changed and results in a corresponding change in the observed E_{cell}. One can plot the E_{cell} vs milliliters of reactant added and follow the equilibrium relationships of the redox system as the familiar potentiometric titration curve.

For most of the potentiometric experiments in this study a pH-sensitive glass electrode is used as the indicator electrode. This device is based upon the fact that thin membranes of certain varieties of glass are responsive to hydrogen ions. If two solutions are separated by this membrane, a difference of potential will arise between its two surfaces which 'ideally' can be represented by the Nernst equation:

$$E_g = \frac{RT}{F} \ln \frac{(H^+)_{UNK}}{(H^+)_{STD}}$$

or

$$E_g = .059 \ln \frac{(H^+)_{UNK}}{(H^+)_{STD}} \text{ at } 25^\circ \text{ C}$$

The same principles which apply to aqueous solutions can be applied to establish pH scales in nonaqueous solvents. However, no thermodynamically acceptable procedure has as yet been devised to correlate pH scales in different solvents.

C. Single-Sweep Peak Voltammetry

The study of current potential relationships of electrode reactions in stirred or quiet solutions is known as voltammetry. In the method utilized in this study, the potential at the indicator electrode (platinum) is changed linearly as a function of time (ramp function) and the current resulting from reactions at the electrode surface is analyzed. The magnitude of the current observed is dependent on the electrode potential, the rate of mass transport, and the kinetics of the electron-transfer reaction at the electrode

surface. The maximum current of oxidation or reduction is governed by the rate of mass transport of the electroactive specie from the solution to the electrode surface and is proportional to its bulk concentration.

A quantitative treatment for this type of reaction may be initiated by using Fick's first law to approximate conditions of semi-infinite linear diffusion.[32] Using the Nernst diffusion layer concept and Fick's law an expression for the limiting current may be derived:

$$i_L = \frac{nFADC}{\delta}$$

If the reaction is considered reversible the extent of reaction or current at the electrode is dictated by the thermodynamic equilibria of the redox system. Fick's first law may be applied to the Nernst equation to derive an expression relating current and potential:

$$E = E_{\frac{1}{2}} + \frac{.059}{n} \log \frac{i_L - i}{i}$$

where

$$E_{\frac{1}{2}} = E^{o}_{Ox,Red} + \frac{.059}{n} \log \frac{D_{red}}{D_{ox}}$$

In the case where both oxidizable and reducible electroactive species are present, a more general expression for a composite anodic-cathodic process is:

$$E = E_{\frac{1}{2}} + \frac{.059}{n} \log \frac{(i_L)_c - i}{i - (i_L)_a}$$

This equation can be used to describe the current-potential relationship as long as the potential sweep is slow enough

so that the diffusion layer is not changed. If however, the potential is swept rapidly so that a depletion effect occurs about the electrode (increases), a peak current is observed followed by a decay in current. For a reversible electrode reaction involving soluble reactants and products, the value of the peak current is given by the Randles-Sevcik equation:

$$i_p = 2.72 \times 10^5 \, N^{3/2} A D^{1/2} C_{ox} \nu^{\frac{1}{2}} \text{ at } 25^{o}C$$

The potential at which the peak occurs is related to the polarographic half-wave potential by

$$E_{peak} = E_{\frac{1}{2}} - 1.1 \frac{RT}{nF}$$

The value of the peak current varies, however, with the reversibility of the electrode reaction, as does the potential at which the peak occurs. The peak current for an irreversible electrode reaction is given by

$$i_p = 3.01 \times 10^5 \, n(\alpha N_a)^{\frac{1}{2}} A D^{\frac{1}{2}} C \nu^{\frac{1}{2}}$$

The potential-sweep technique may thus be utilized to relate the concentration of an electroactive specie to the observed peak current since constant experimental conditions imply a linear relationship between current and concentration. The potential at peak current (E_{peak}) may also be used to identify the electroactive specie since it is characteristic of the compound electrolyzed and may be related to the polarographic half-wave potential by the expression given above.

D. Controlled Potential Electrolysis and Coulometry

In controlled potential electrolysis the potential of the working electrode is held at a fixed potential on the plateau of the polarographic or voltammetric wave, and the electroactive specie is oxidized or reduced completely. An electrode of large surface area is used and the solution is stirred vigorously in order to complete the reaction in a reasonable period of time. When the total quantity of electricity flowing during the electrolysis is determined, the technique is called controlled potential coulometry. The relationship between the amount of current passed and the quantity of material electrolyzed is given by the following:

$$Q = nFM$$

The value of the initial current at the start of electrolysis is $i_o = \frac{nFDAC}{\delta}$

where δ is the thickness of the diffusion layer (cm) and depends on the rate of stirring. During the course of the electrolysis the current falls off exponentially according to the equation $i = i_o \exp(-Bt)$

where i is the current after time t and $B = \frac{DA}{V\delta}$

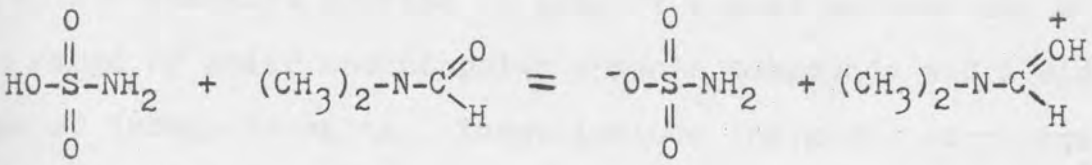
The total quantity of electricity which has passed during the electrolysis is given by

$$Q = \int_0^t i \, dt$$

This technique can thus be used to determine the n value for a reaction provided the concentration is known or vice versa. This can be determined by either taking the area under the graph of current vs. time or it can be determined by inserting a current integrator in series with the cell.

E. N,N-Dimethylformamide as a Solvent System

Dimethylformamide (DMF) is a protophylic aprotic solvent and a slightly weaker base than dimethylsulfoxide or pyridine. In this respect it can almost be regarded as a neutral solvent. It is protophylic in the sense that it is a weak base and can accept protons:[33]



Sulphamic Acid DMF

However, unlike water, neutral DMF does not undergo autoprotolysis nor does it serve as a proton donor to form a deprotonated solvent (lyate) anion. The basic character of DMF serves to level the strength of weak bases, but on the other hand this property and its relatively low dielectric constant (37) serve to accentuate the differences between acids. Thus, it serves as an excellent media for titrations of acids and acid analogues with strong bases. The pH range of DMF extends over 18 pH units and thus makes it suitable for such differentiating titrations.[33] Recently Kolthoff et al.[34] have shown the feasibility of calibrating the glass

electrode in DMF and have determined the dissociation constants for a large number of organic acids and bases.

In non-aqueous media with relatively low dielectric constant a factor must be taken into consideration which in aqueous media is of secondary importance. This is the possibility or tendency of solvated ions to associate to form ion pairs and larger aggregates. The theory for these types of interactions is not yet well developed. For analytical purposes, ion pair formation can be neglected when the dielectric constant is greater than 40.[35] Thus, DMF appears to be a border line solvent for this consideration.

The relatively polar character of DMF and its dielectric constant combine to make it a good solvent for a wide range of polar and nonpolar organic compounds and a wide range of inorganic salts. These include inorganic perchlorates, alkali and alkaline earth iodides, and LiCl. Chloride salts tend to be insoluble while nitrates are soluble but tend to decompose. Dimethylformamide has become of special interest to electrochemists as a medium for the reduction of organic and organometallic compounds and as a media for polarography due to its wide accessible potential range, especially in the extreme negative region. Using $NaClO_4$ as the supporting electrolyte and platinum as the working electrode it has a potential range of +1.6 to -1.6 volts vs. the saturated calomel electrode (aqueous). A number of supporting electrolytes, working, and reference electrodes have been successfully applied in DMF.[36,37]

DMF has a liquid range of (-61° to $+153^{\circ}$), a viscosity comparable to water, and a relatively low vapor pressure at room temperature. It can be utilized as a solvent for absorption spectroscopy in the visible and near u.v. down to 270nm. However, acids and bases catalyze a decomposition or hydrolysis of DMF to yield dimethylamine, carbon monoxide, other amines, and formic acid. A number of procedures are available for the purification of DMF to conductivity levels of less than $1X10^{-7}$ $ohm^{-1}cm^{-1}$.[36]

CHAPTER III

Materials and Apparatus

A. Materials

All chemicals used were of reagent grade and were used without recrystallization. Table 1 gives the chemicals used, the manufacturer, and the grade of the reagent. The solvent dimethylformamide was prepared by storage over molecular sieves and then was distilled (25°-40°C) under reduced pressure (2mm-10mm Hg) in the presence of anhydrous $CuSO_4$ as a drying agent and as an agent for the removal of amine impurities.[36] Distillation was performed under an argon or nitrogen atmosphere retaining the middle fraction (3/5) for use. Fresh solvent was prepared as a matter of necessity on approximately a bi-weekly basis.

The bilirubin was obtained from the Sigma Chemical Company with a "purity of 99%" and had a molar absorptivity in chloroform of 60,000 ± 600. The biliverdin dihydrochloride (approximately 80% biliverdin) was also obtained from Sigma Chemical. Both Argon and Nitrogen were used for the de-aeration of solutions (~30 min.) and as inert atmospheres in this study. The argon was purified by passing over clean copper chips at 600°C.

TABLE I

REAGENTS USED

Material	Formula	Grade Reagent	Manufacturer of Distributor
N,N-Dimethylformamide	$HCON(CH_3)_2$	Certified A.C.S.	Fisher Scientific
Ferrous ammonium sulfate	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	"	"
Zinc chloride	$ZnCl_2 \cdot X\ H_2O$	"	"
Potassium permanganate	$KMnO_4$	"	"
Potassium persulfate	$K_2S_2O_8$	"	"
Potassium periodate	KIO_4	"	"
Ceric ammonium nitrate	$(NH_4)_2\ Ce\ (NO_3)_6$	"	"
Pyridine	N:CHCH:CHCH:CH	"	"
Cobaltous nitrate	$Co(NH_3)_3 \cdot 6H_2O$	Certified Reagent	"
Ferric chloride	$FeCl_3$	"	"
Nickel (ous) chloride	$NiCl_2 \cdot 6H_2O$	"	"
Acetonitrile	CH_3CN	Certified	"
Glacial acetic acid	CH_3COOH	Reagent	"

Material	Formula	Grade Reagent	Manufacturer or Distributor
Zinc metal	Zn		Fisher Scientific
Zinc iodide	ZnI_2	N.F. VIII	"
Ethylenediamine	$H_2NCH_2CH_2NH_2$	Purified	"
Molecular sieves (Davison)	base: Alumina-silicate cation: calcium	Type 5A Grade 522	"
Cupric sulfate, anhydrous	$CuSO_4$	Analyzed	Baker Chemical Co.
Sodium chloride	NaCl	"	"
Iodine	I_2	"	"
Bromine	Br_2	"	"
Potassium ferrocyanide trihydrate	$K_4Fe(CN)_6 \cdot 3H_2O$	"	"
Potassium dichromate	$K_2Cr_2O_7$	"	"
Dimethylsulfoxide	$(CH_3)_2SO$	"	"
Chloroform	$CHCl_3$	"	"
EDTA disodium, dihydrate crystal		Analyzed A.C.S.	"

Material	Formula	Grade Reagent	Manufacturer or Distributor
Tetrabutylammonium hydroxide (25% in methanol)	$(CH_3CH_2CH_2CH_2)_4NOH$	Baker Grade	Baker Chemical Co.
Potassium biphthalate	$1\text{-}KOCOC_6H_4\text{-}2COOH$	Primary Standard	"
Sodium perchlorate	$NaClO_4$		Ventron (Alfa Inorganics)
Silver nitrate	$AgNO_3$	Reagent, A.C.S.	Sargent-Welch Scientific
1,1,3,3 - Tetramethylguanidine	$(CH_3)_2NC(:NH)N(CH_3)_2$	White Label	Eastman Kodak Co.
Picric acid	$C_6H_3N_3O_7$	"	"
Perchloric acid (70%)	$HClO_4$ (aq)	Reagent, A.C.S.	Allied Chemical
Ferrous sulfate	$FeSO_4 \cdot X\ H_2O$	Reagent	" "
Sodium bisulfite, meta (anhydrous)	$Na_2S_2O_5$	"	" "
Triethylenetetramine	$(H_2N\ (CH_2)_2NHCH_2)_2$	Technical	Matheson Coleman and Bell
2,4-Pentanedione	$CH_3COCH_2COCH_3$	Practical	" "

Material	Formula	Grade Reagent	Manufacturer or Distributor
Diethylenetriamine	$(H_2N(CH_2)_2)_2NH$	Practical	Matheson Coleman and Bell
Argon (gas)	Ar	Pre-Purified	Matheson
Hydrogen chloride (gas)	HCl	Technical	"
Nitrogen (gas)	N_2	Pre-Purified	AERCO
Zinc acetate	$Zn(C_2H_3O_2)_2 \cdot 2H_2O$	Analytical	Mallinckrodt Chemical Works
Ferrocene	$(C_5H_5)_2Fe$	CP Grade	Research Organic/Inorganic Chemical Co.

B. Apparatus

All solutions from crystalline or liquid reagents were prepared by weight using a Mettler H20 semimicro balance with a readability of .00001 gram. Zn^{++} solutions in DMF were also prepared electrochemically using a zinc metal strip as the anode and a Sargent Model IV Coulometric Current Source as the current measuring and controlling instrument. Electrochemical generation was performed in the presence of 0.1M $NaClO_4$ as the electrolyte and using a platinum foil counter electrode separated from the prime solution by a fine porosity glass fritted compartment. The zinc metal strip was cleaned with nitric acid, rinsed with DMF, and a preelectrolysis was performed in a separate solution of DMF prior to preparation of the stock Zn^{++} solution.

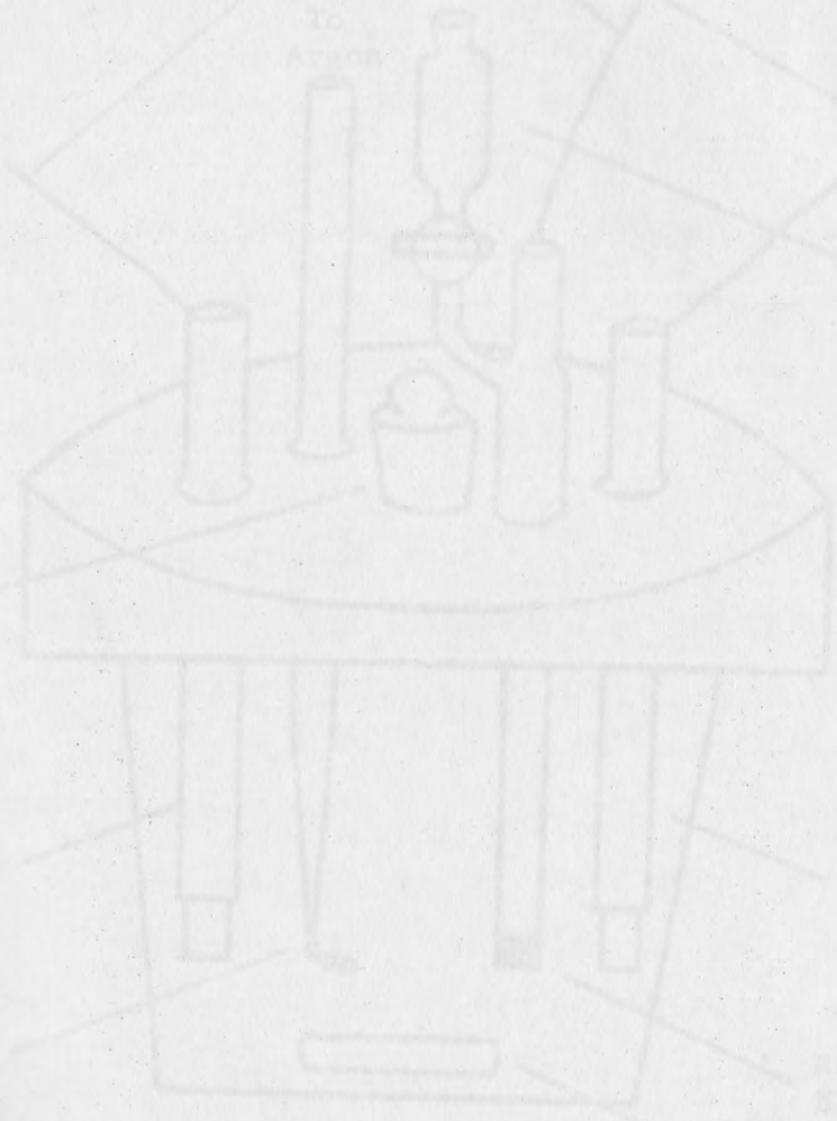
Near ultraviolet and visible spectra were obtained using a Cary Model 14M recording spectrophotometer or a Beckman Model DB spectrophotometer both calibrated using a didymium glass filter. Infrared spectra were obtained with a Beckman Model IR5 spectrophotometer using polystyrene for frequency calibration.

All electrochemical measurements were obtained with a National Instrument Laboratory "Electrolab" in conjunction with a Val Tech Model 1024 X-Y Recorder or a PAR Model 170 Electrochemistry System.[49] Potentiometric measurements were made using an Orion Model 801/digital pH meter.

Several different types of electrodes were used in this study. Ag/.01M $AgNO_3$ (DMF) and Ag/AgCl(sat), NaCl(sat) (DMF) reference electrodes were prepared by dipping a silver wire into solution contained in a 5mm o.d. glass tube that was sealed at the lower end containing an asbestos fiber as an electrolytic contact. Ag/AgCl(sat), NaCl(sat) and Ag/AgCl(sat), NaCl(sat), $NaClO_4$(.1M) reference electrodes were also prepared using a Metrohm Ag/AgCl Nonaqueous Reference Electrode (EA425) assembly making solution contact with a 5mm o.d. glass tube that was closed at the lower end by a fine-porosity frit. The electrodes for voltammetry were two Sargent-Welch Pt indicator electrodes with an area of approximately $2cm^2$ or one Pt indicator electrode in conjunction with a vitreous carbon working electrode (Beckwith Carbon Corp.). The vitreous carbon electrode was made by pressure fitting a vitreous carbon crucible (5/8"o.d. X 1/2" hgt.) onto a hollow teflon tube and using an inner mercury pool in contact with a copper wire for electrical contact. Controlled potential coulometric measurements were taken using a large platinum foil and a platinum counter electrode in an isolated fritted compartment. Potentiometric measurements were made using a Coleman glass electrode in conjunction with one of the above reference systems.

The electrochemical cells for potentiometry, voltammetry, and coulometry were Metrohm Model EA 874 titration vessels (EA 875-50, EA 875-20, EA 876-20) which had five openings in the upper portion to permit insertion of the

appropriate electrodes, gas bubblers, and pipets. The cell as used for voltammetry is illustrated in Figure 10.



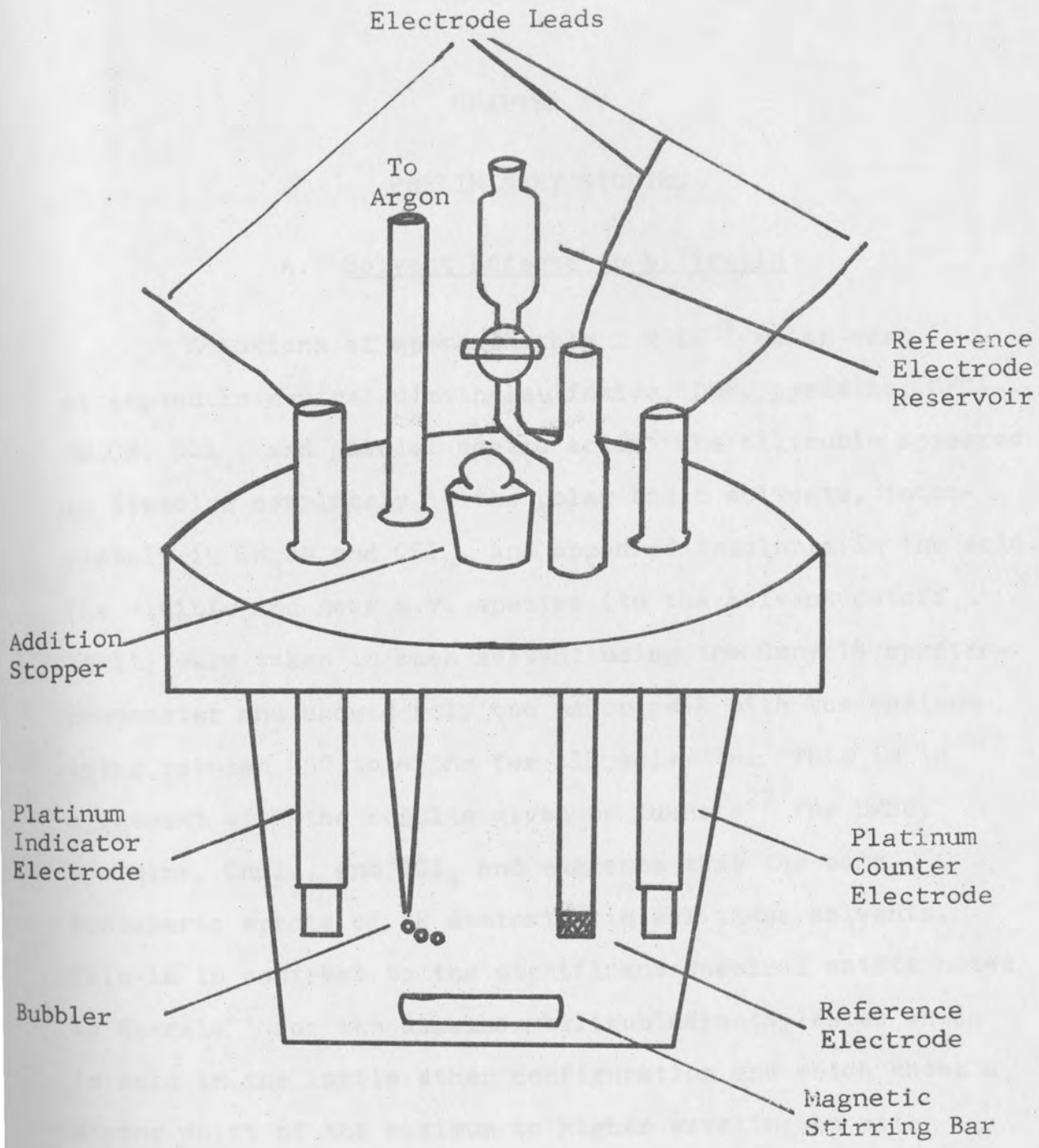


Figure 10. Electrochemical Cell for Voltammetry

CHAPTER IV

PRELIMINARY STUDIES

A. Solvent Effects on Bilirubin

Solutions of approximately 1 x 10^{-4} molar were attempted in neutral dimethylsulfoxide, DMF, pyridine, $CHCl_3$, CH_3CN, CCl_4, and glacial acetic acid. The bilirubin appeared to dissolve completely in the polar basic solvents, incompletely in CH_3CN and CCl_4, and appeared insoluble in the acid. The visible and near u.v. spectra (to the solvent cutoff limit) were taken in each solvent using the Cary 14 spectrophotometer and showed only one major peak with the maximum lying between 450 to 455nm for all solvents. This is in agreement with the results given by Kuenzle[27] for DMSO, pyridine, $CHCl_3$, and CCl_4 and suggests that the same tautomeric specie of BR dominates in all these solvents. This is in contrast to the significant spectral shifts noted by Kuenzle[27] for the dimethoxybilirubindimethylester which is held in the lactim ether configuration and which shows a strong shift of the maximum to higher wavelingths with increasing polarity of the solvent. With BR there did appear a slight broadening in the peaks resulting in DMSO and pyridine, but these were not significant enough to warrant investigation. All solutions were purged with nitrogen for thirty minutes and appeared stable to light and if stored in

the freezer (-30°C) for at least a few days. No study was made of the degradation of these solutions. However, solutions of BR in $CHCl_3$ and CCl_4 appeared to undergo only slight oxidation even after two months of storage (~10% as determined spectrophotometrically), whereas the solution in pyridine was highly oxidized (~50%). The DMSO solution could not be stored (freezing pt - 18.5°C) and oxidized in a few days at room temperature in the dark.

Assuming a molar absorptivity of 6×10^4 for BR (450nm) in CCl_4 and CH_3CN, the solubility of BR is approximated at 1×10^{-4} in CCl_4 and 3×10^{-6} in acetonitrile at room temperature. Kuenzles data indicates that the molar absorptivity (ε) of BR does not change significantly with solvent (ε = 58,000-62,000) except in the case of pyridine (ε = 54,300). In DMF using a molar absorptivity of 60,000 the solubility of BR was determined as 6.7×10^{-4} by diluting a saturated solution and taking the visible spectra.

An attempt was made to take the infrared spectrum of BR in saturated solutions of DMF, DMSO, and $CHCl_3$ using a light path of 0.5 mm with pure solvent in the reference cell. However, the concentrations were too low and in each case the spectra of solvent only was observed. Broderson et al.[28] have obtained a partial IR spectra of 3mM BR in chloroform, but all other IR work has been limited to dispersions in mineral mulls or KBr discs.[29,38,39,24] KBr discs were prepared by mixing and grinding 1-2% mixtures of BR in KBr and subjecting the mixtures to 8 tons of hydraulic pressure for

one minute under vacuum. The IR spectra of the resulting 1/2 inch diameter discs clearly reproduced the results already published. The same procedure was applied to biliverdindi-hydrochloride. Although numerous trials appeared reproducible, the IR spectra of the hydrochloride salt did not contain the distinctive features of the BR spectra even though different concentrations were tried. In any event, it appears that infrared analysis of weakly soluble bile pigments must be limited to crystalline products which can be subjected to KBr discs or dispersed in mineral mulls. Figures 11 and 12 are examples of the IR spectra obtained using the IR-5 and KBr discs.

B. Reactivity of Bilirubin in DMF

The reactivity of bilirubin with a variety of oxidizing and complexing agents was qualitatively undertaken by simply adding dropwise concentrated solutions of these reagents to concentrated solutions of bilirubin in DMF and observing any noticeable color changes visually. These of course,represent a change in the conjugation of the bile pigment and thus an oxidation or complexation. The following table summarizes the results.

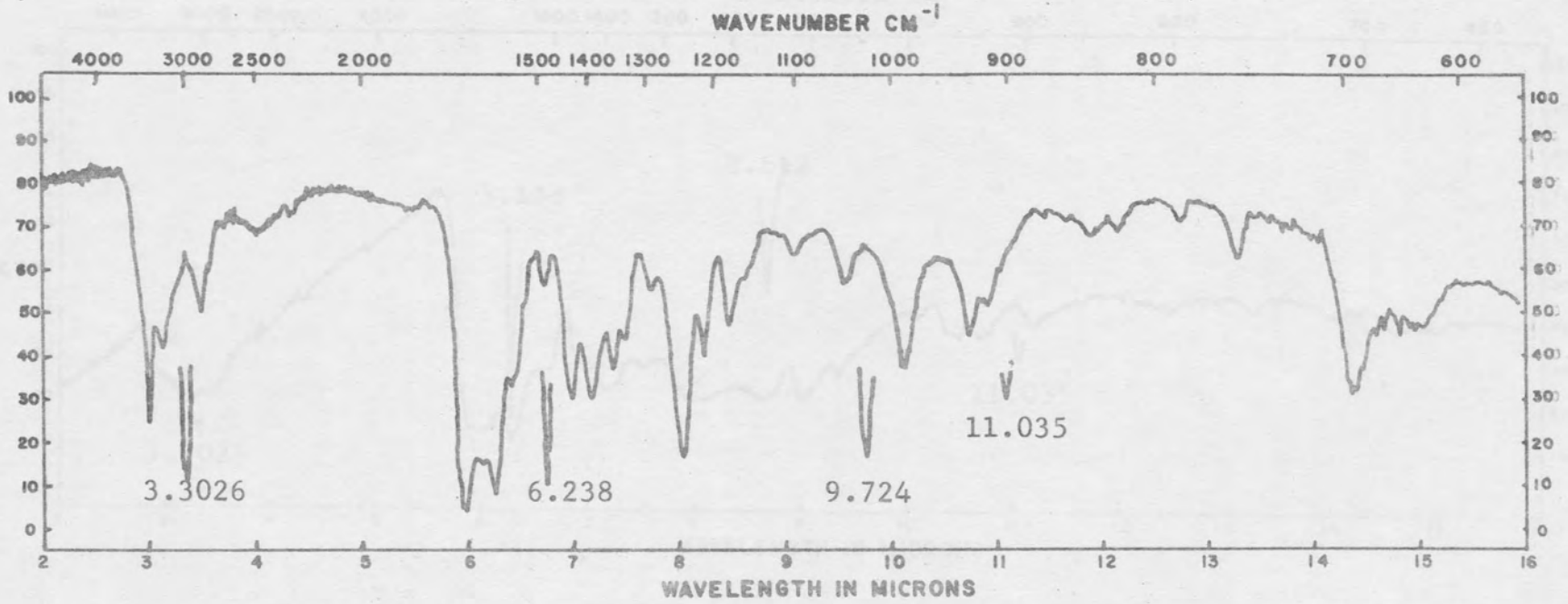


Figure 11- The Infrared Spectrum of Bilirubin- KBr Pellet.

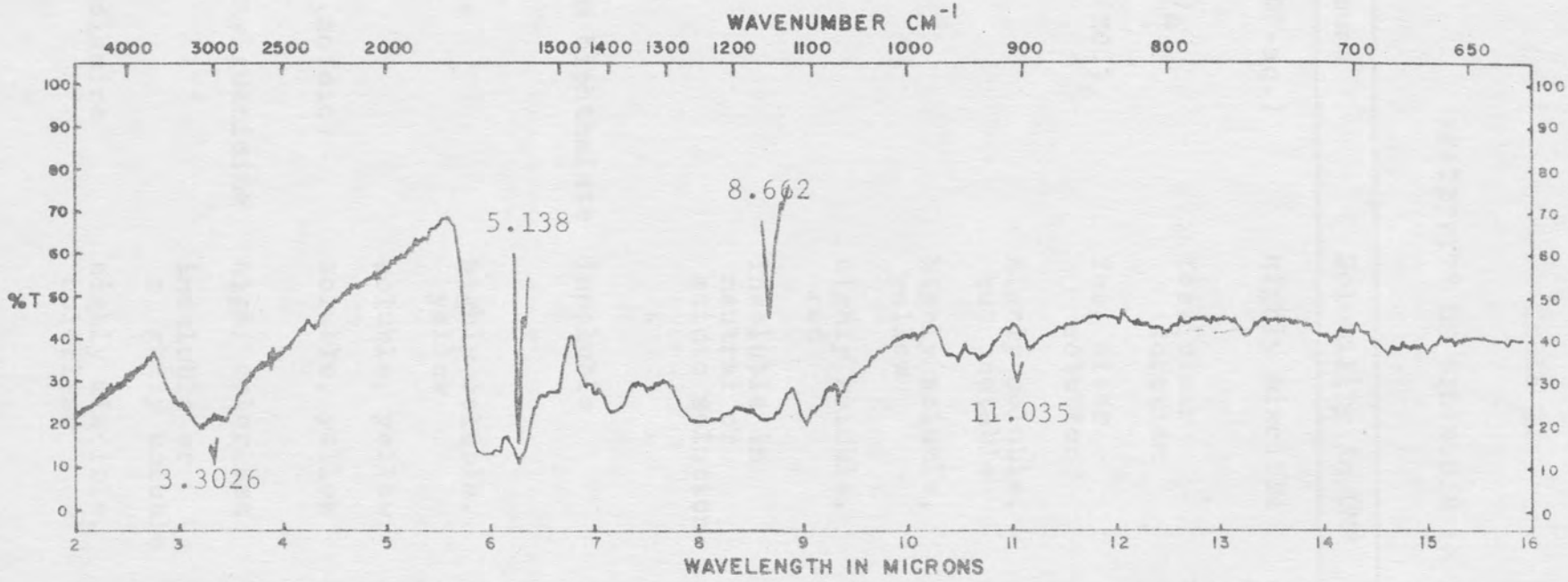


Figure 12- The Infrared Spectrum of Biliverdin Dihydrochloride-KBr Pellet

TABLE II

REACTIVITY OF BILIRUBIN IN DMF

Compound	Solubility in DMF	Reaction with BR
$HClO_4$ (10F-aq.)	highly miscible	oxidation to verdinoid green
$H_4Ce(SO_4)_4$	Yes, clear solution	oxidation through Gmelin series
$(NH_4)_2Ce(NO_3)_6$	Yes, clear solution	" "
$KMnO_4$	highly soluble, but unstable	" "
I_2	highly soluble, yellow	rapid oxidation to verdinoid green
Br_2	highly soluble, red	" "
$K_2S_2O_8$	insoluble in neutral or acidic solution	None
KIO_4	" "	None
Potassium biphthalate	insoluble	None
$Na_2S_2O_5$	"	"
Ferrocene	highly soluble, yellow	no apparent reaction
$K_2Cr_2O_7$	soluble, yellow	" " "
$K_2Cr_2O_7$ (acidic)	soluble, yellow	oxidation to verdinoid green
tetramethylguanidine	high, colorless	no immediate effect
EDTA	insoluble or slightly soluble	None
Ethylenediamine	highly miscible, colorless	no apparent reaction

TABLE II (Cont'd)

Compound	Solubility in DMF	Reaction with BR
Triethylenetetramine	highly miscible, colorless	no apparent reaction
$Cu(ClO_4)_2 \cdot 6H_2O$	highly soluble, blue solution	rapid lightening of color
$FeCl_3$	high, yellow color	rapid shift to green-yellow
$ZnCl_2 \cdot XH_2O$	high, colorless	shift to pink color
$Zn(CH_3COO)_2 \cdot 6H_2O$	high, colorless	shift to red color
$Co(No_3)_2 \cdot 6H_2O$	high, bluish red	rapid shift to amber color
$FeSO_4 \cdot XH_2O$	insoluble	None
$K_4Fe(CN)_6 \cdot 3H_2O$	insoluble or only slightly soluble (?)	reacts on shaking, weaker color
$NiCl_2 \cdot 6H_2O$	high, green blue	rapid shift to yellow green
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	insoluble	None

The term insoluble in the table refers to the fact that no salt crystals of the inorganic compound were visually observed to dissolve and the solution remained clear in color. All solutions of compounds soluble in DMF appeared qualitatively stable except the solutions of $KMnO_4$ which repeatedly turned to the brown color of MnO_2 on standing for about a half-hour. Addition dropwise of 10F(aq.) $HClO_4$ resulted in a gradual change of bilirubin to the verdinoid color. This seemed dependent on the amount of acid added but

was slow enough even in a relatively concentrated solution of acid in DMF to be easily followed spectrophotometrically with the Cary 14. The verdinoid peak at 380nm clearly appeared with the simultaneous disappearance of the peak at 450nm for bilirubin. This appears to be a unique situation since bilirubin appears to be insoluble in acidic aqueous solutions and reacts very rapidly to decomposition with the addition of concentrated $HClO_4$ (aqueous) to chloroform solutions. The chloroform product was red and readily extracted into an aqueous layer at the surface of the chloroform. This indicates that perhaps the product was a mixture of Neoxanthobilirubic acids or dipyrroles which result with vigorous oxidations that cleave the methene bridge.

$K_2Cr_2O_7$ did not appear to react with bilirubin in neutral DMF but appeared to react more vigorously in acid media than the acid by itself. The spectra of $K_2Cr_2O_7$ was taken in neutral DMF and showed seven peaks occurring between 330 and 400nm. This fine structure was subsequently lost with the addition of $HClO_4$ (aqueous) and was converted into one broad band over the same region. This indicates the $K_2Cr_2O_7$ is either undergoing a structural change or equilibrium shift to a specie which appears more reactive under acid (aqueous) conditions in DMF, perhaps analogous to that found in aqueous solutions.

No Fe(II) salts were found which readily dissolved in DMF, although shaken solutions of $K_4Fe(CN)_6 \cdot 3H_2O$ seemed to react with BR. The other transition metals in the form of their hydrated salts were highly soluble in DMF and showed reactivity with BR. In order to qualitatively ascertain the nature of the reactions, concentrated solutions ($\sim$ .1%) of the transition metal salts in DMF listed in Table 1 were added dropwise to BR samples stored in the spectral cuvettes ($\sim$ 3ml) and the resulting changes were followed in the visible region using the Beckmann D.B. Figures 13, 14, and 15 show the results obtained in the cases of Fe(III), Co(II), and Ni(II). The numbers on the spectral lines indicate the number of drops of solution added to the BR and thus show a progression of the reactions. In each case isosbestic points are implied but perhaps not precise due to dilution effects. The spectra of the pure metal ion solutions in DMF were also taken to assess whether they interfere with the reaction spectra. In the case of Fe(III) and Cu(II) (max. 430nm) no interference is expected. Co(II) shows a single peak at 520nm which should not interfere excessively with the results obtained. However, Ni(II) does absorb at 420nm, but the large change in ε (extinction) indicates that BR is undergoing a reaction. In all cases excess metal and the passage of time resulted in even further oxidation. Zn(II) also reacted readily with BR resulting in a shift from yellow to deep red (max. 530nm). The Zinc (II) product appeared to be highly stable when stored in the freezer

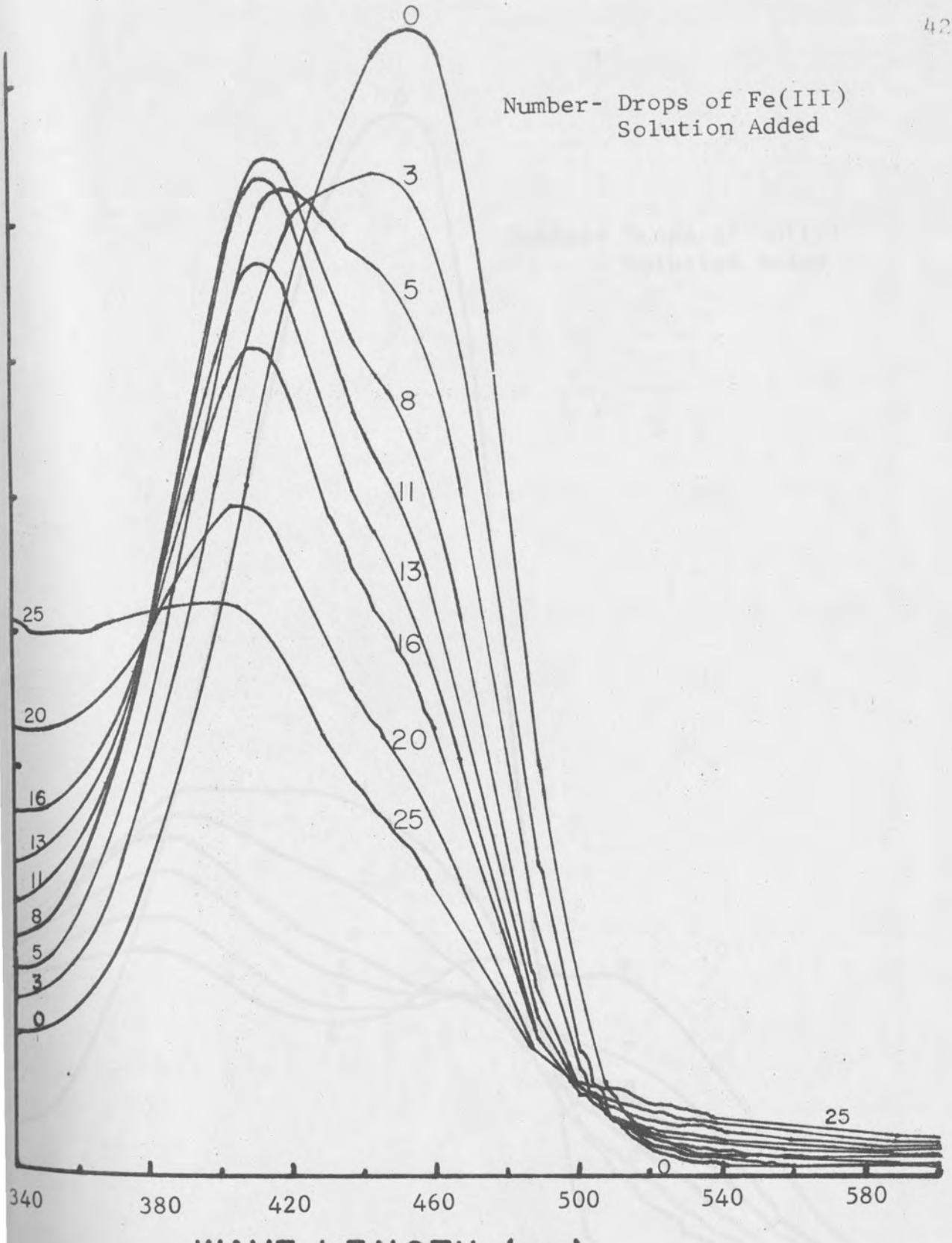


Figure 13. The visible spectra of the reaction of $FeCl_3 \cdot 6H_2O$ with Bilirubin in DMF- 10 mm. cell.

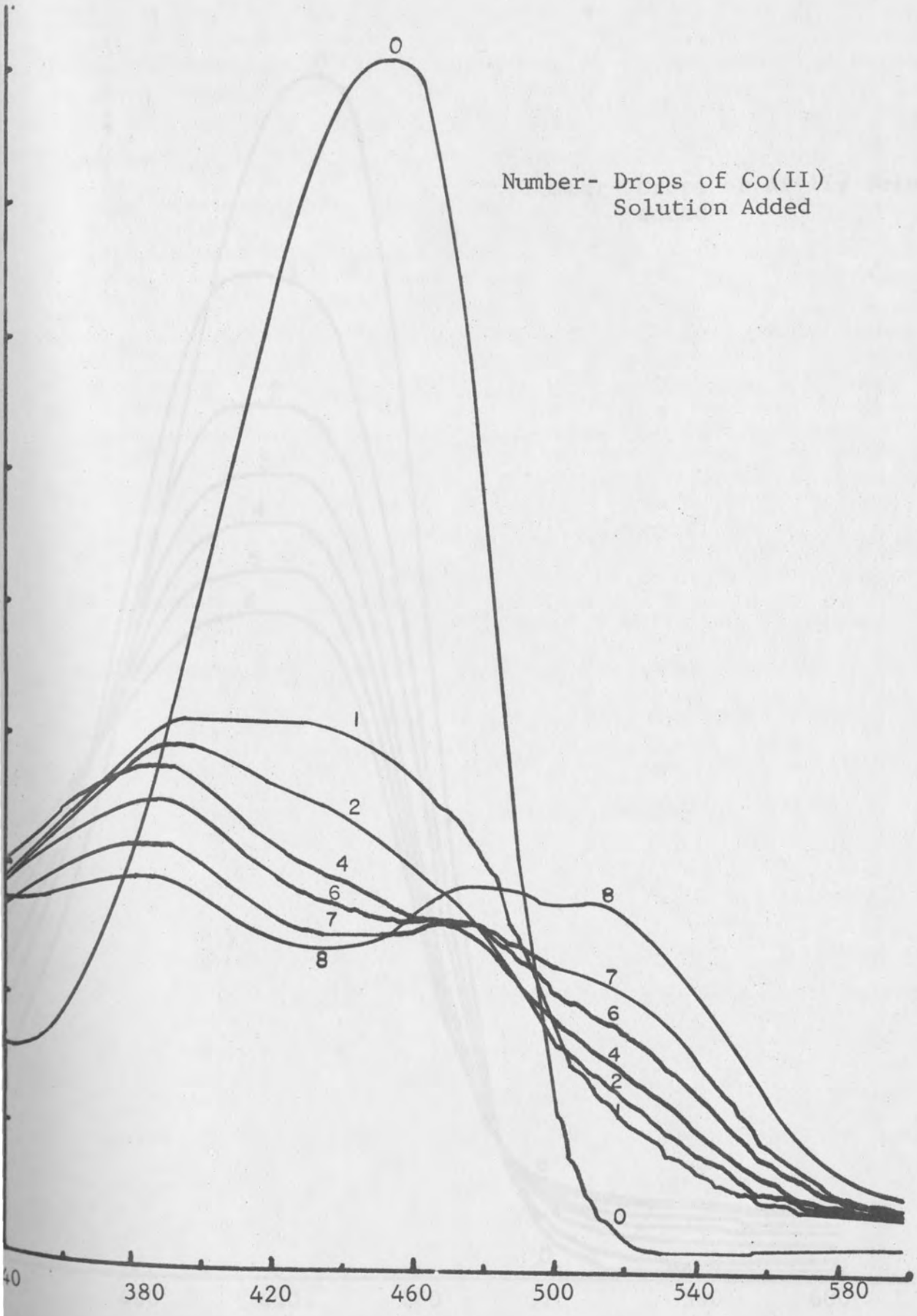


Figure 14. The Visible Spectra of the reaction of $Co(NO_3)_2 \cdot 6H_2O$ with Bilirubin

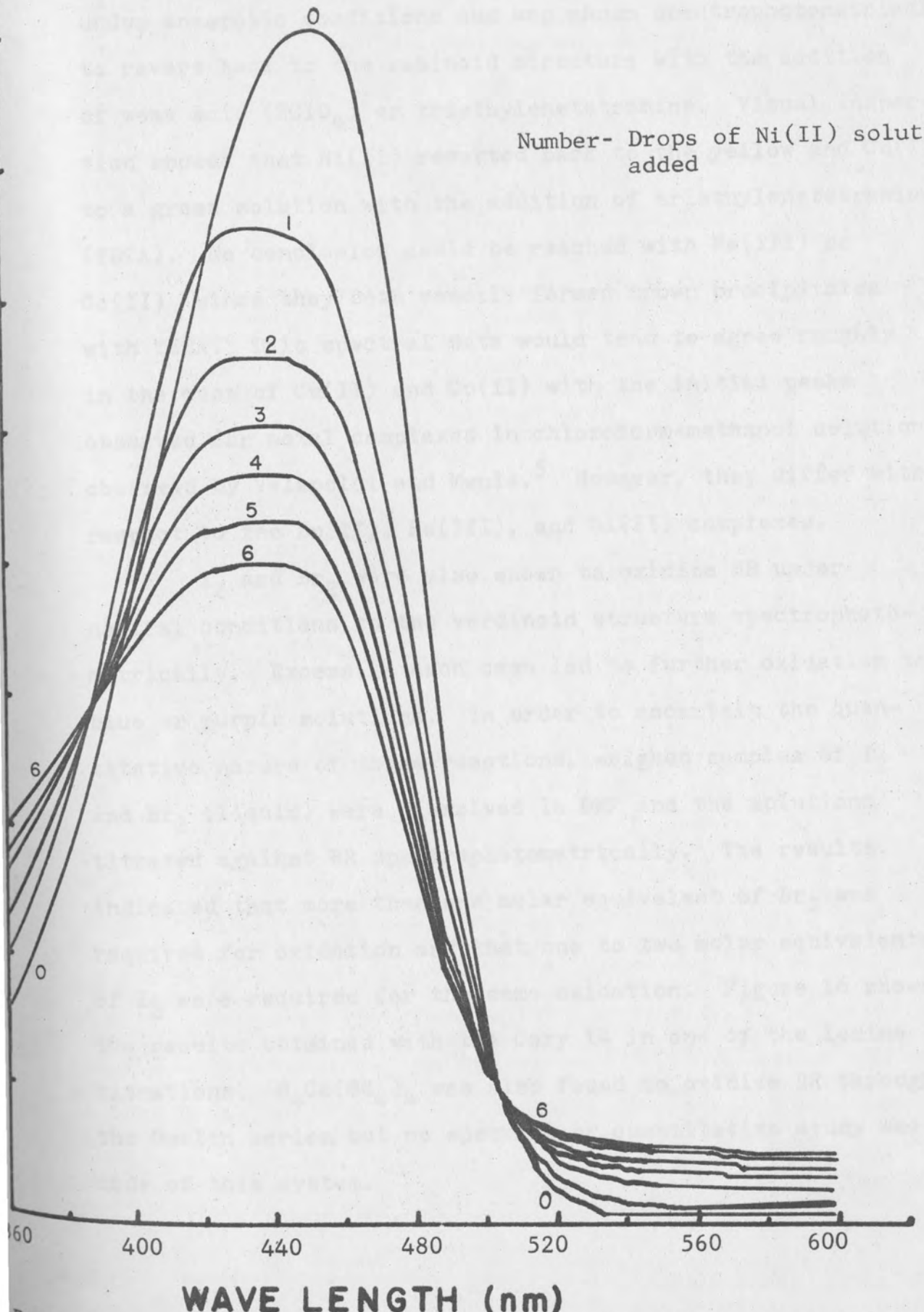


Figure 15. The Visible Spectra of the Reaction of $NiCl_2.6H_2O$ with

under anaerobic conditions and was shown spectrophotometrically to revert back to the rubinoid structure with the addition of weak acid ($HClO_4$) or triethylenetetramine. Visual inspection showed that Ni(II) reverted back to the yellow and Cu(II) to a green solution with the addition of triethylenetetramine (TETA). No conclusion could be reached with Fe(III) or Co(II) since they both readily formed brown precipitates with TETA. This spectral data would tend to agree roughly in the case of Cu(II) and Co(II) with the initial peaks observed for metal complexes in chloroform-methanol solutions observed by Velapoldi and Menis.[5] However, they differ with respect to the Zn(II), Fe(III), and Ni(II) complexes.

I_2 and Br_2 were also shown to oxidize BR under neutral conditions to the verdinoid structure spectrophotometrically. Excess in each case led to further oxidation to blue or purple solutions. In order to ascertain the quantitative nature of these reactions, weighed samples of I_2 and Br_2 (liquid) were dissolved in DMF and the solutions titrated against BR spectrophotometrically. The results indicated that more than one molar equivalent of Br_2 was required for oxidation and that one to two molar equivalents of I_2 were required for the same oxidation. Figure 16 shows the results obtained with the Cary 14 in one of the iodine titrations. $H_4Ce(SO_4)_4$ was also found to oxidize BR through the Gmelin series, but no spectral or quantitative study was made of this system.

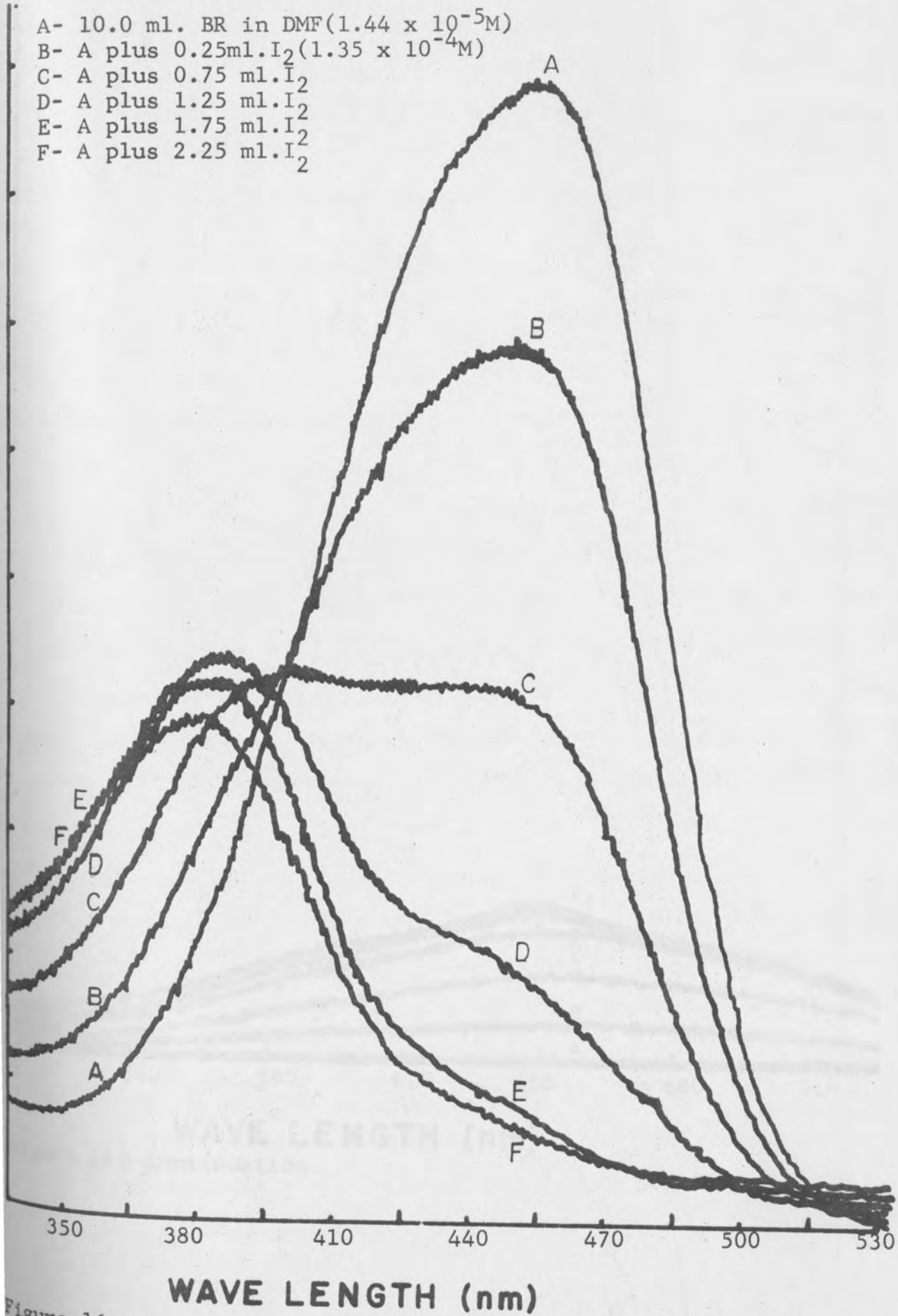


Figure 16A. Spectra of Bilirubin Titrated with Iodine in DMF.

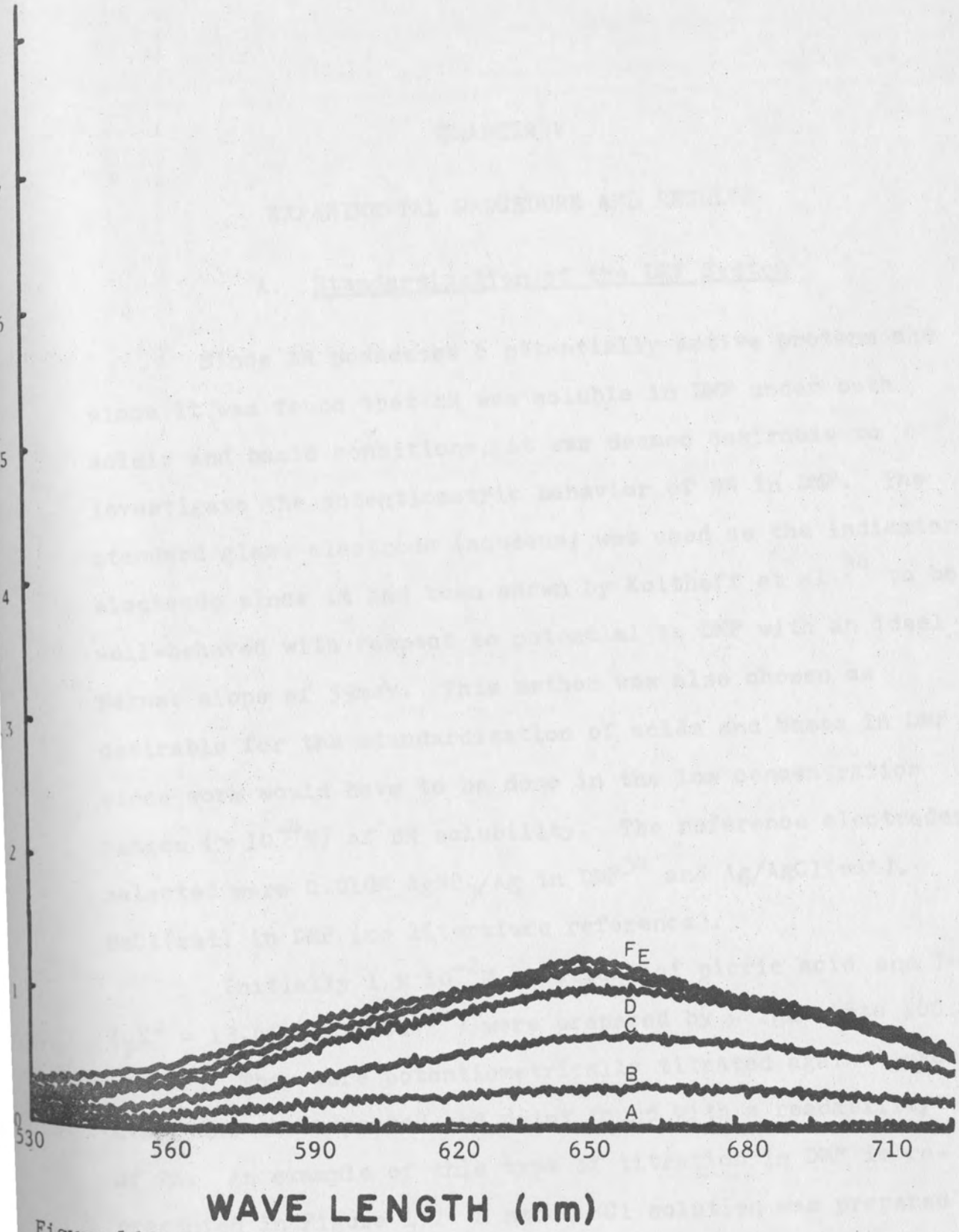


Figure 16 B-Continuation

CHAPTER V

EXPERIMENTAL PROCEDURE AND RESULTS

A. Standardization of the DMF System

Since BR possesses 6 potentially active protons and since it was found that BR was soluble in DMF under both acidic and basic conditions, it was deemed desirable to investigate the potentiometric behavior of BR in DMF. The standard glass electrode (aqueous) was used as the indicator electrode since it had been shown by Kolthoff et al.[34] to be well-behaved with respect to potential in DMF with an ideal Nernst slope of 59mev. This method was also chosen as desirable for the standardization of acids and bases in DMF since work would have to be done in the low concentration ranges ($\sim 10^{-4}$M) of BR solubility. The reference electrodes selected were 0.010M $AgNO_3$/Ag in DMF[34] and Ag/AgCl(sat), NaCl(sat) in DMF (no literature reference).

Initially 1 x 10^{-2}M solutions of picric acid and TMG ($_pK^d$ = 13.65 for $TMGH^+$[34]) were prepared by weight into 100 ml of DMF. They were potentiometrically titrated against each other and the expected end point found with a readability of 2%. An example of this type of titration in DMF is represented in Figure 17. A stock HCl solution was prepared by bubbling HCl through DMF for 30 minutes (HCl$\sim$5.4M). For standardization and use this solution was diluted to

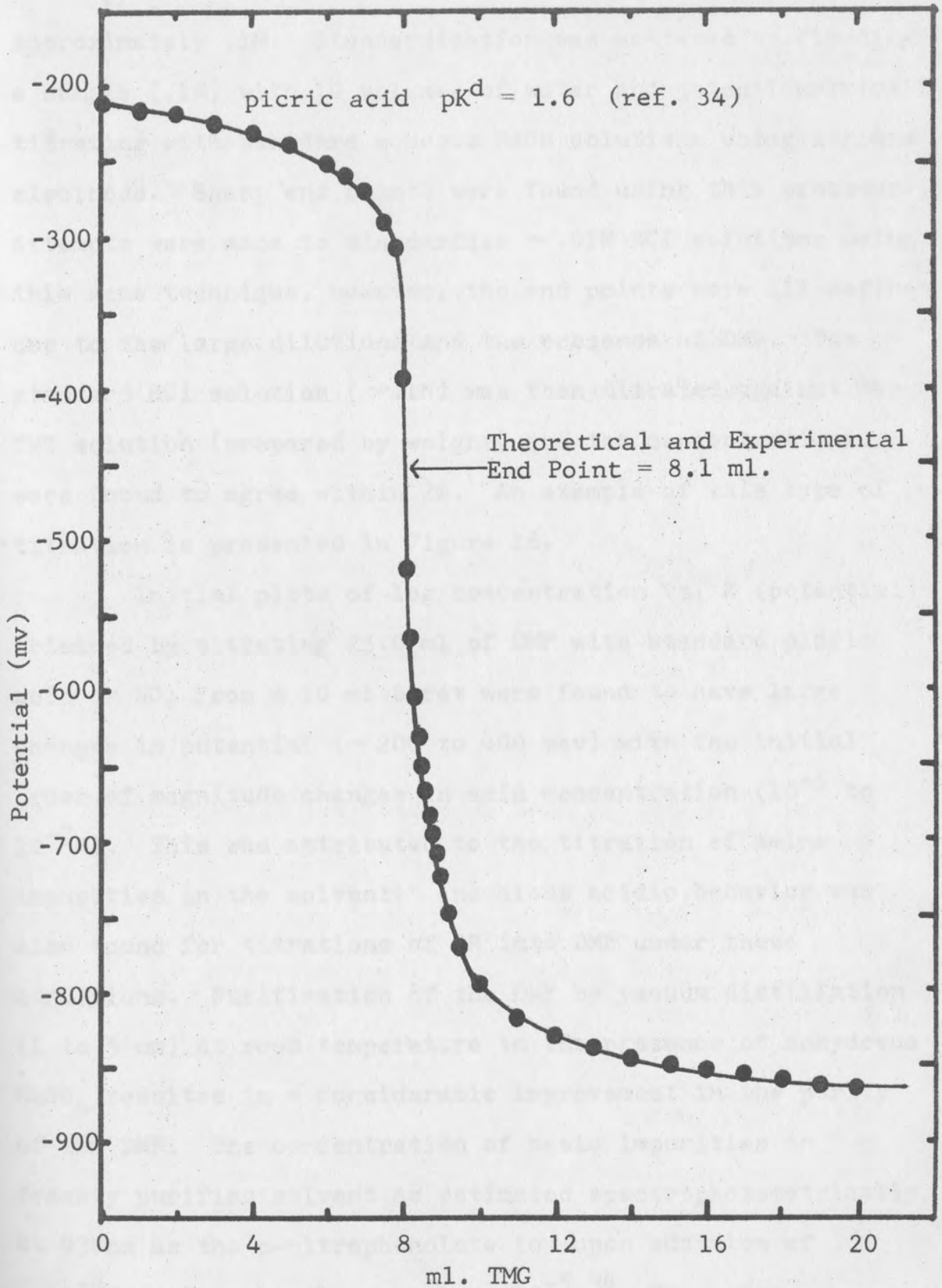


Figure 17 . Potentiometric Titration of 28.0 ml. Picric Acid (2.86×10^{-3}M) with TMG(9.9×10^{-3}M) in DMF- Indicator Electrode, Glass Electrode; Reference Electrode, $Ag/AgNO_3$,.01M.

approximately .1M. Standardization was achieved by flooding a sample (.1M) with 10 volumes of water and potentiometrically titrating with standard aqueous NaOH solutions using a glass electrode. Sharp end points were found using this procedure. Attempts were made to standardize ~ .01M HCl solutions using this same technique, however, the end points were ill-defined due to the large dilutions and the presence of DMF. The standard HCl solution (~.1N) was then titrated against the TMG solution (prepared by weight) and the concentrations were found to agree within 2%. An example of this type of titration is presented in Figure 18.

Initial plots of log concentration vs. E (potential) obtained by titrating 25.0 ml of DMF with standard picric acid or HCl from a 10 ml buret were found to have large changes in potential (~ 200 to 400 mev) with the initial order of magnitude changes in acid concentration (10^{-5} to 10^{-3}M). This was attributed to the titration of amine impurities in the solvent. Anomalous acidic behavior was also found for titrations of BR into DMF under these conditions. Purification of the DMF by vacuum distillation (1 to 5 mm) at room temperature in the presence of anhydrous $CuSO_4$ resulted in a considerable improvement in the purity of the DMF. The concentration of basic impurities in freshly purified solvent as estimated spectrophotometrically at 430nm as the p-nitrophenolate ion upon addition of 10^{-4}M p-nitrophenol was ~ 1. x 10^{-5}.[34] The molar

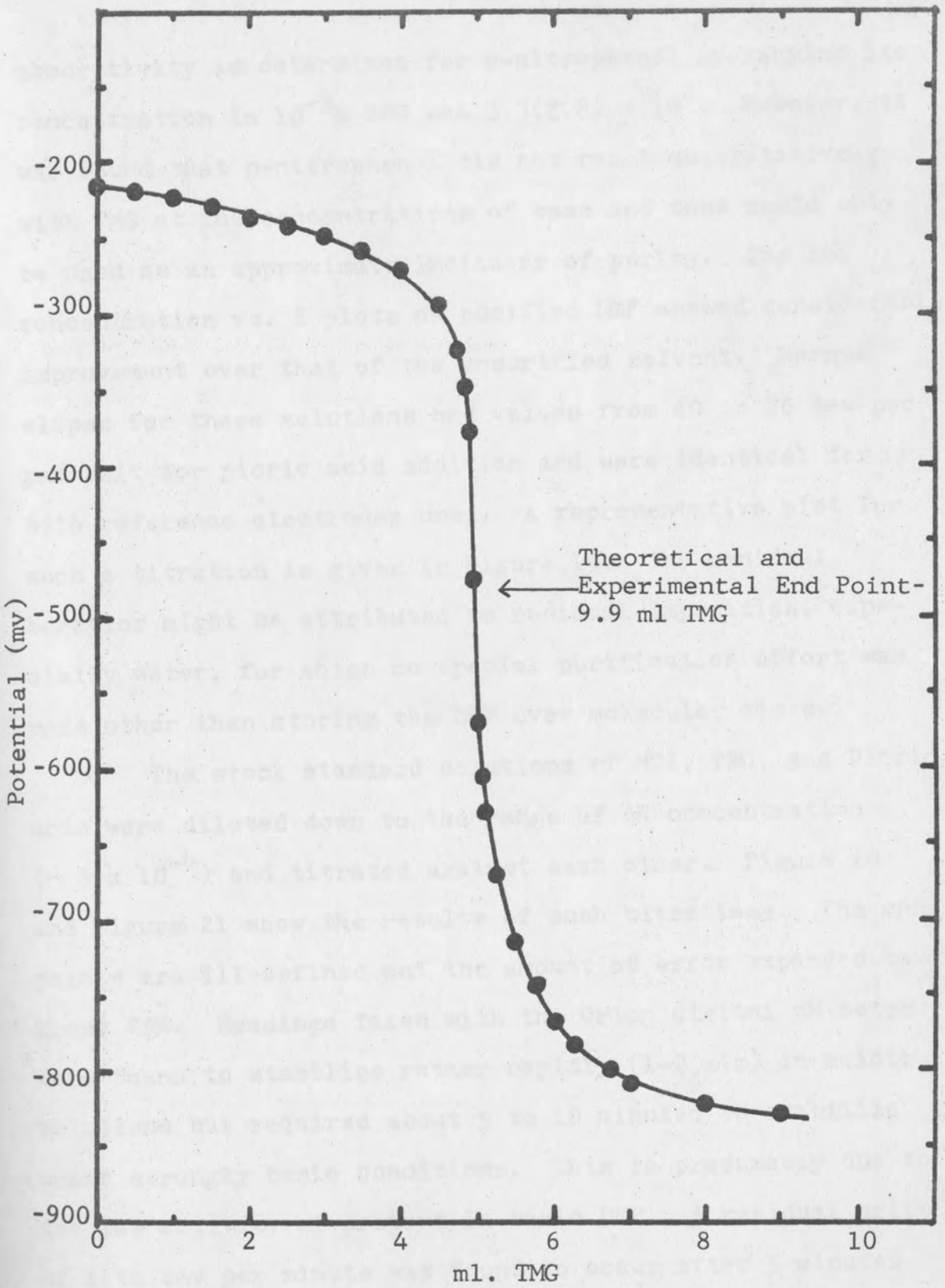


Figure 18 . Potentiometric Titration of 25.0 ml. HCl(1.89×10^{-3}M) with TMG(4.8×10^{-3}M) in DMF-Indicator Electrode,Glass Electrode; Reference Electrode, $Ag/AgNO_3$,.01M.

absorptivity as determined for p-nitrophenol by varying its concentration in 10^{-3}M TMG was 3.3(±.2) x 10^{4}. However, it was found that p-nitrophenol did not react quantitatively with TMG at low concentrations of base and thus could only be used as an approximate indicator of purity. The log concentration vs. E plots of purified DMF showed considerable improvement over that of the unpurified solvent. Nernst slopes for these solutions had values from 60 to 70 mev per paH unit for picric acid addition and were identical for both reference electrodes used. A representative plot for such a titration is given in Figure 19. The nonideal behavior might be attributed to residual impurities, especially water, for which no special purification effort was made other than storing the DMF over molecular sieves.

The stock standard solutions of HCl, TMG, and Picric acid were diluted down to the range of BR concentration ($\sim 5 \times 10^{-4}$) and titrated against each other. Figure 20 and Figure 21 show the results of such titrations. The end points are ill-defined and the amount of error expanded to about ±5%. Readings taken with the Orion digital pH meter were found to stabilize rather rapidly (1-2 min) in acidic solutions but required about 5 to 10 minutes to stabilize under strongly basic conditions. This is presumably due to the low activity of protons in basic DMF. A residual drift of 1 to 2mv per minute was found to occur after 5 minutes of waiting which occurred in the direction of the titration (acid or base). Readings were taken at constant intervals in the hope of minimizing this effect.

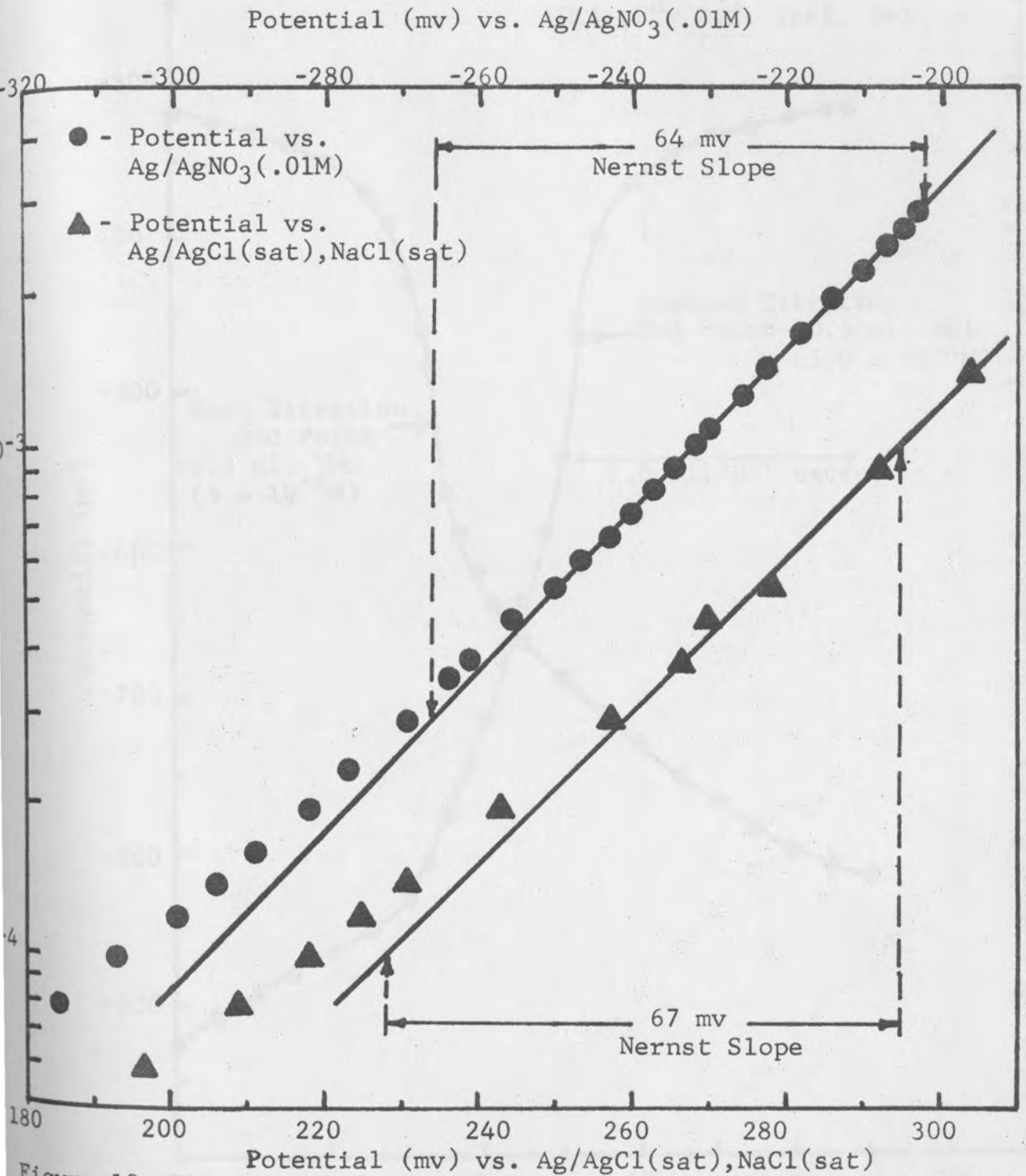


Figure 19. The Potential of various concentrations of Picric Acid in DMF versus log of Picric Acid Concentration- Indicator Electrode, Glass Electrode; Two Reference Electrodes used alternately.

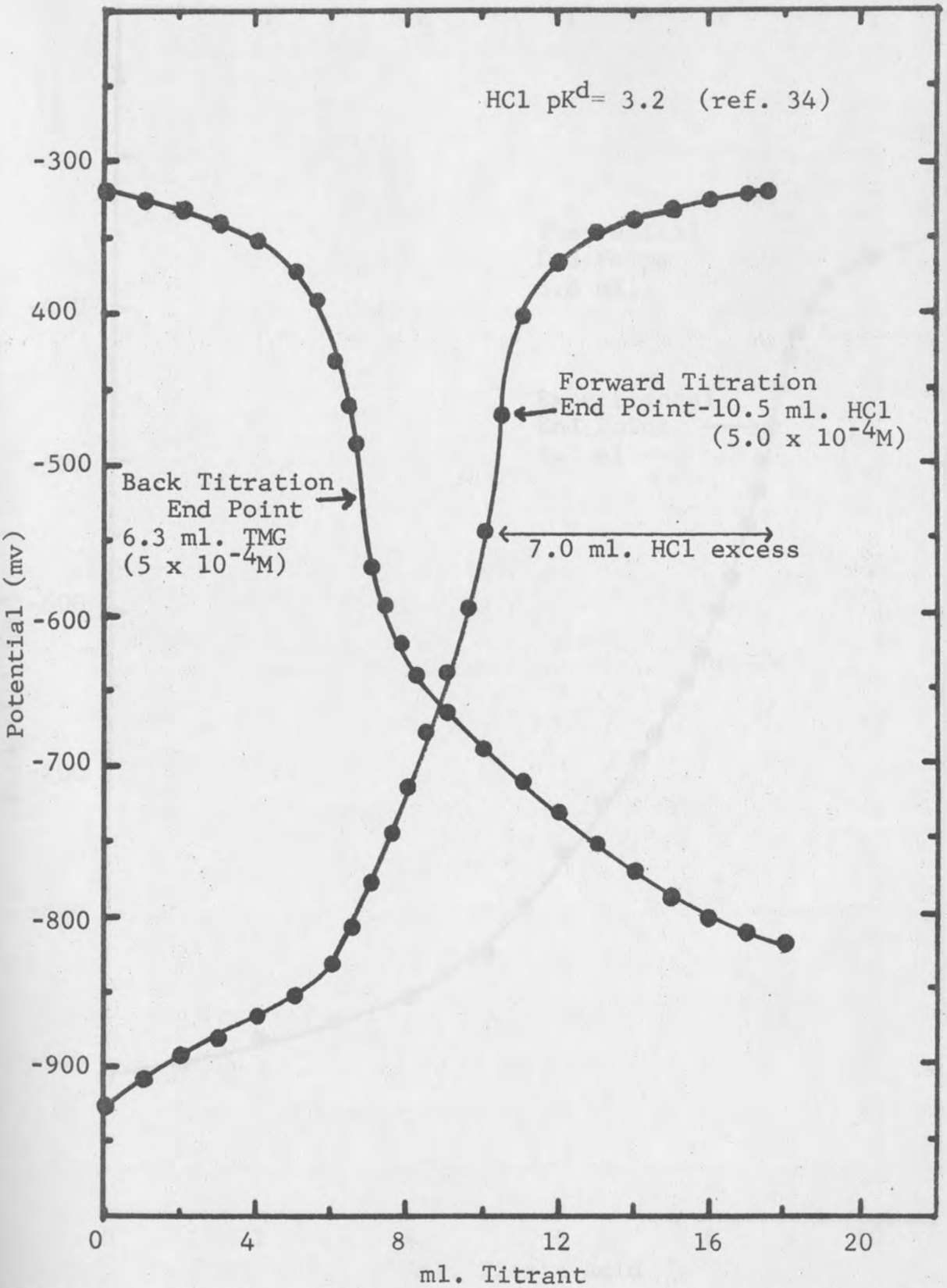


Figure 20 . Potentiometric Titration of TMG with HCl followed by a Back-titration with TMG in DMF-Indicator Electrode,Glass Electrode; Reference Electrode, Ag/$AgNO_3$,0.01M.

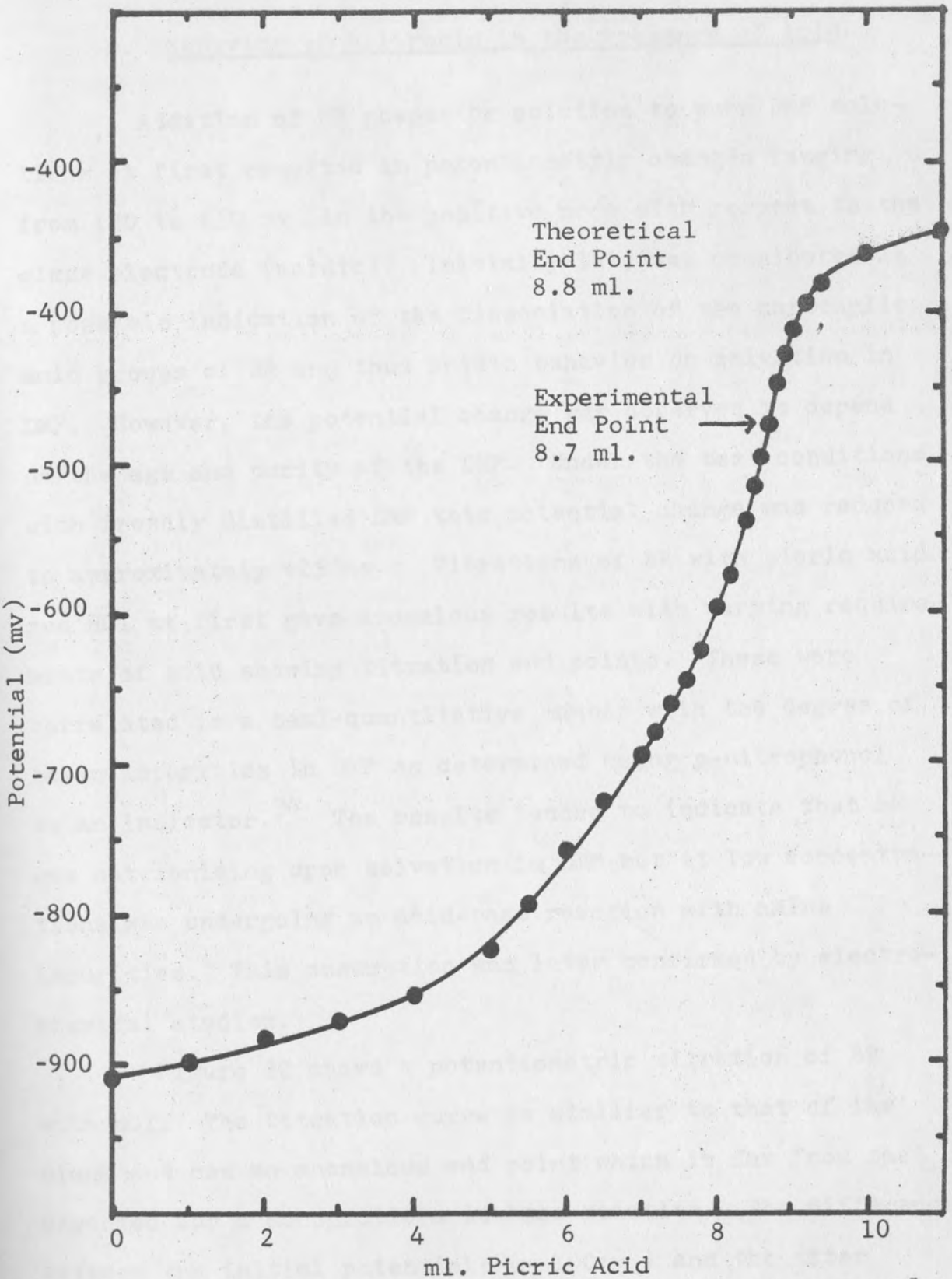


Figure 21. Potentiometric Titration of 20.0 ml. TMG(2.5×10^{-5}M) with Picric Acid(5.7×10^{-4}M) in DMF-Indicator Electrode,Glass Electrode; Reference Electrode,$Ag/AgNO_3$,.01M.

B. Behavior of Bilirubin in the Presence of Acid

Addition of BR powder or solution to pure DMF solutions at first resulted in potentiometric changes ranging from 100 to 150 mv in the positive mode with respect to the glass electrode (acidic). Initially this was considered as a possible indication of the dissociation of the carboxylic acid groups of BR and thus acidic behavior on solvation in DMF. However, the potential change was observed to depend on the age and purity of the DMF. Under the best conditions with freshly distilled DMF this potential change was reduced to approximately +25 mv . Titrations of BR with picric acid and HCl at first gave anomalous results with varying requirements of acid showing titration end points. These were correlated in a semi-quantitative manner with the degree of basic impurities in DMF as determined using p-nitrophenol as an indicator.[34] The results tended to indicate that BR was not ionizing upon solvation in DMF but at low concentrations was undergoing an acid-base reaction with amine impurities. This assumption was later confirmed by electrochemical studies.

Figure 22 shows a potentiometric titration of BR with HCl. The titration curve is similiar to that of the blank and has an anomalous end point which is far from that expected for a monoprotic acid-base reaction. The difference between the initial potentials (~ 100mv) and the titer values at -600mv and -475mv might be accounted for by

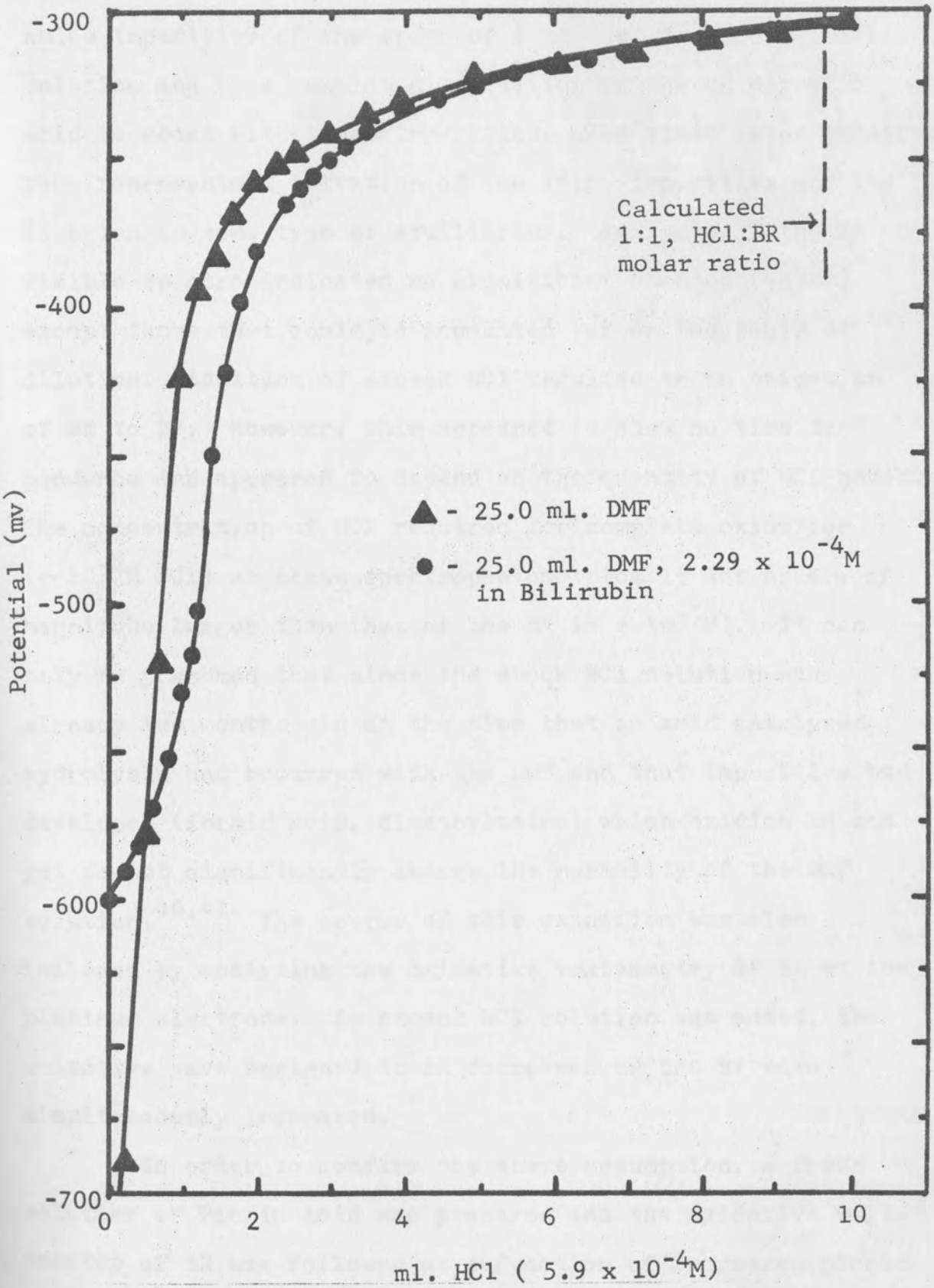


Figure 22 . Potentiometric Titration of Bilirubin with HCl in DMF- Indicator Electrode, Glass Electrode; Reference Electrode, $Ag/AgNO_3$,.01M.

amine impurities of the order of 1 to 2 x 10^{-5}M for 25ml of solution and by a partial dissociation of the BR diprotic acid to react with these impurities. The titer value obtained thus represents a titration of the amine impurities and the BR anion in some type of equilibrium. Analysis of the BR visible spectra indicated no significant changes (453nm) except those that could be accounted for on the basis of dilution. Addition of excess HCl resulted in an oxidation of BR to BV. However, this appeared to show no time dependence and appeared to depend on the quantity of HCl added. The concentration of HCl required for complete oxidation ($\sim 10^{-2}$M HCl) as shown spectrophotometrically was orders of magnitude larger than that of the BR (8 x 10^{-5}M). It can only be presumed that since the stock HCl solution was already two months old at the time that an acid catalyzed hydrolysis had occurred with the DMF and that impurities had developed (formic acid, dimethylamine) which oxidize BR and yet do not significantly change the normality of the DMF solution.[40,41] The course of this oxidation was also followed by analyzing the oxidative voltammetry of BR at the platinum electrode. As excess HCl solution was added, the oxidative wave assigned to BR decreased as the BV wave simultaneously increased.

In order to confirm the above assumption, a fresh solution of Picric Acid was prepared and the oxidative voltammetry of BR was followed as a function of increased picric

acid concentration. Figure 23 shows the results of this experiment. There is no shift or change in the profile of the oxidative wave for BR (+.7v vs. Ag/AgCl(sat), NaCl(sat) electrode). The decrease in peak height can be accounted for quantitatively on the basis of dilution effects. The change in the BV wave (+.85v) might be explained on the basis of BVH^+ formation. These results are in significant contrast to those observed above with old HCl solutions. The visible spectra of BR in the presence of picric acid could not be followed due to the interfering absorption of picric acid. However, analysis of the region from 600 to 700 nm indicated that less than 3% of the BR had been oxidized to BV during the course of the experiment. This fraction might be explained as a result of the electrolysis occurring at the Pt electrode.

Attempts were also made to follow the reduction of BR at the Pt electrode. Under neutral conditions a cathodic wave for BR was observed at -1.2v vs. Ag/AgCl(sat), NaCl(sat). Upon addition of acid, however, irregular results were obtained. These were attributed to reduction and adsorbtion of H^+ and a fouling of the Pt electrode under acid conditions. Attempts were also made to follow the reductive processes using a Hanging drop mercury electrode. However, even after repeated cleanings of the electrode apparatus, the results were very irregular and the drop size was hard to control. These difficulties were attributed to an instability of the hanging drop in DMF due to surface tension or wetting effects.

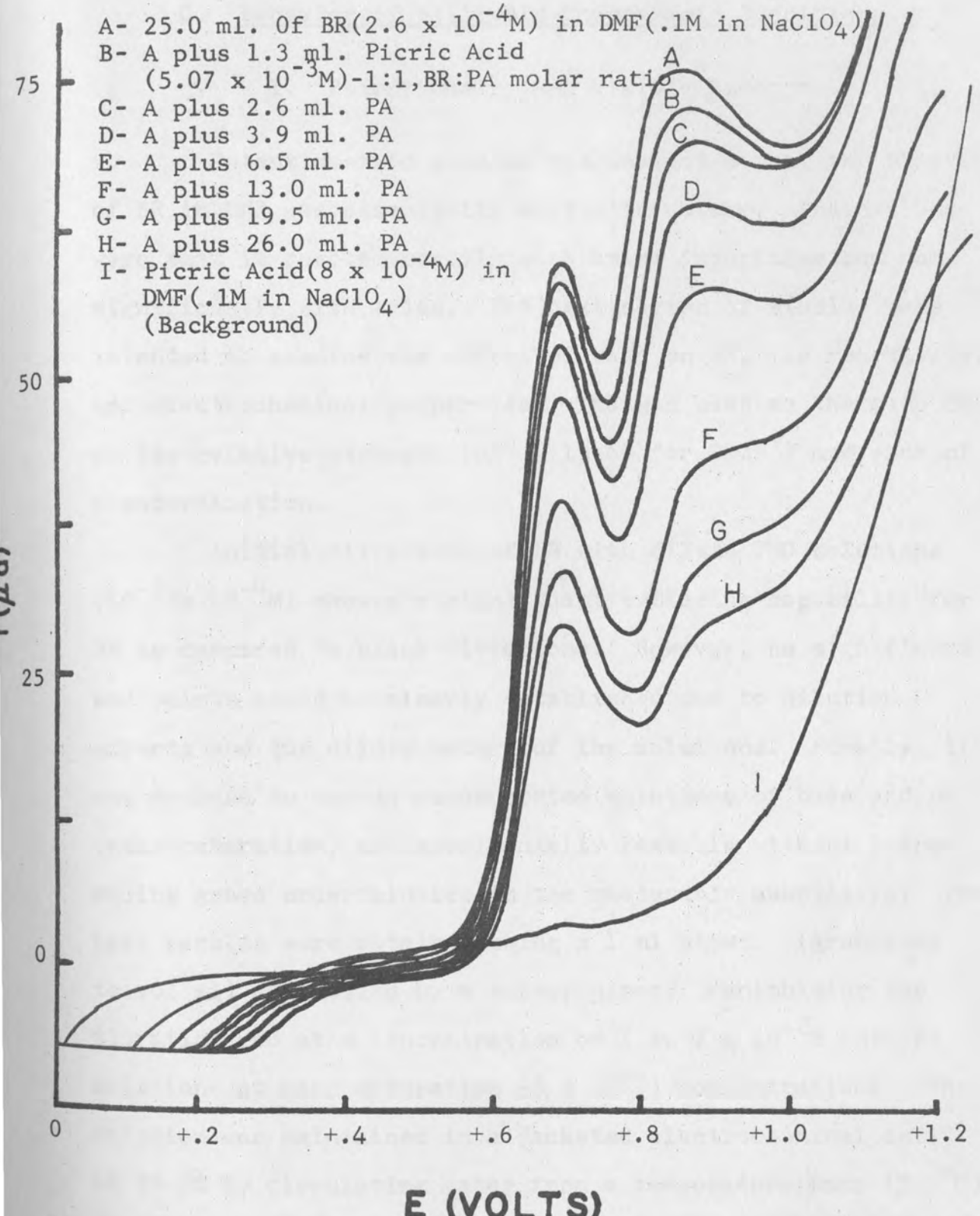


Figure 23. Oxidation of Bilirubin under Acidic Conditions in DMF-Indicator Electrode, Pt; Reference Electrode, Ag/AgCl(sat),NaCl(sat); Scan Rate,1.0 v/min.

C. Behavior of Bilirubin Under Basic Conditions

1. Potentiometry and Visible Spectra

Potentiometric studies had indicated that the behavior of BR in DMF was essentially acidic in nature. Indications were that it reacted readily with amine impurities but not significantly with acids. The next series of studies were intended to examine the effect of base on BR, its reactivity, and electrochemical properties. TMG was used as the base due to its relative strength (pK^d = 13.65 for $TMGH^+$) and ease of standardization.

Initial titrations of BR with dilute TMG solutions (10^{-3} to 10^{-4}M) showed a significant buffering capability for BR as compared to blank titrations. However, no significant end points could be clearly established due to dilution effects and the dilute nature of the solutions. Finally, it was decided to use as concentrated solutions of base and BR (near saturation) as experimentally feasible without introducing gross uncertainties in the measurable quantities. The best results were obtained using a 1 ml pipet (graduated to .01 ml) controlled by a rubber pipet manipulator for titrating TMG at a concentration of 1 to 2 x 10^{-2}M into BR solutions at near saturation (6 x 10^{-4}) concentrations. The solution was maintained in a jacketed electrochemical cell at 25.5°C by circulating water from a temperature bath (±.1°C). TMG was added in .05 ml quantities and potential readings taken at 5 minute intervals. Readings were found to stabilize

within a two minute period after titrant addition followed by a drift of 1 to 2 mv per minute in the basic direction (negative potential). It was hoped that taking readings at constant intervals would nullify this effect.

Figure 24 shows the result of one such titration and includes a blank titration with TMG under identical experimental conditions on the same day. The calculated end points and the experimentally determined end point inflections appear within reasonable proximity (±10%). Thus, it is implied that at least two protons are quantitatively released by BR under basic conditions. No more inflection points were established potentiometrically although it was noted that a buffering effect by BR continues even with the addition of excess TMG as compared to the blank. Under strongly basic conditions, however, the response of the glass electrode becomes progressively more sluggish. This is presumed a result of extremely low proton activity under such conditions in this solvent system.

That this is indeed a quantitative reaction is also reflected by spectroscopic results. Figures 25A and 25B show the change in spectra with the addition of TMG. With the addition of the first equivalent of base a decrease in molar absorptivity and a slight broadening of the absorption is observed. Although the effect is twice that which would be expected from a dilution effect, it is still within experimental uncertainty. With the addition of the second equivalent of TMG a progressive increase in absorbance

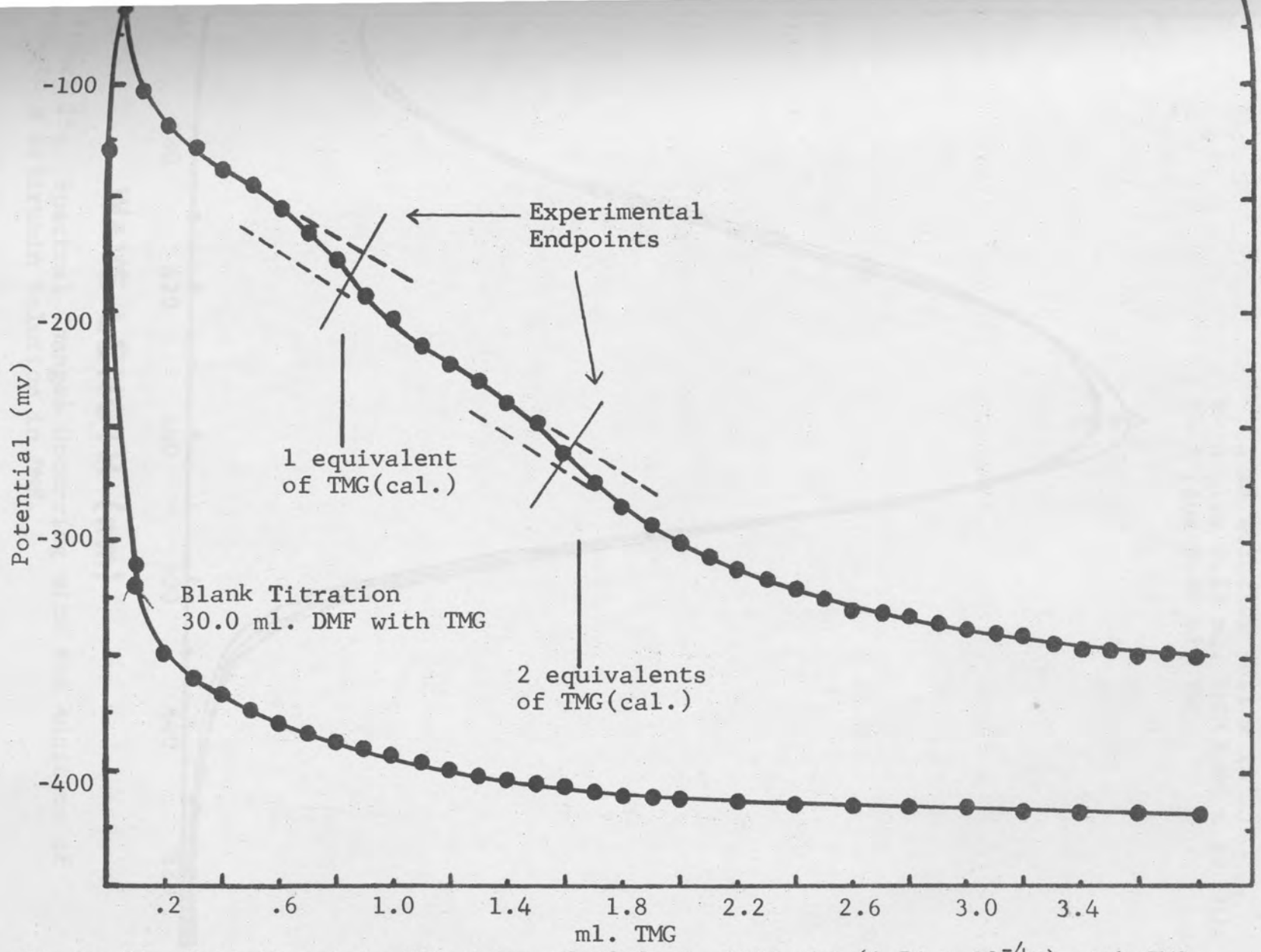


Figure 24. Potentiometric Titration of 30.0 ml. Bilirubin(4.76×10^{-4}M) with TMG (1.75×10^{-2}M) in DMF at 25.5°C-Indicator Electrode,Glass Electrode,Reference Electrode, Ag/AgCl(sat),NaCl(sat).

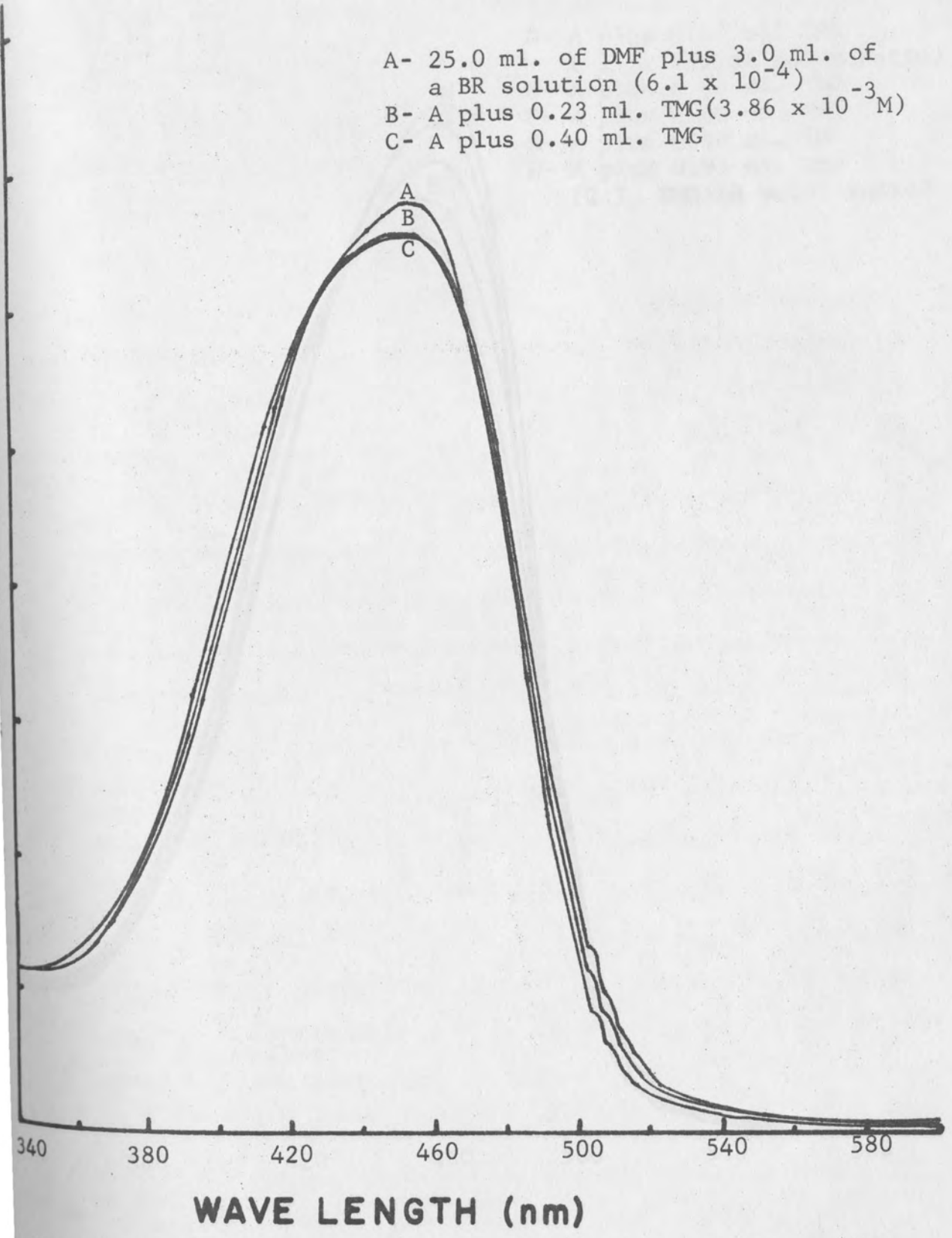


Figure 25A. Spectral Changes Occurring with the Addition of TMG to a Bilirubin Solution in DMF.

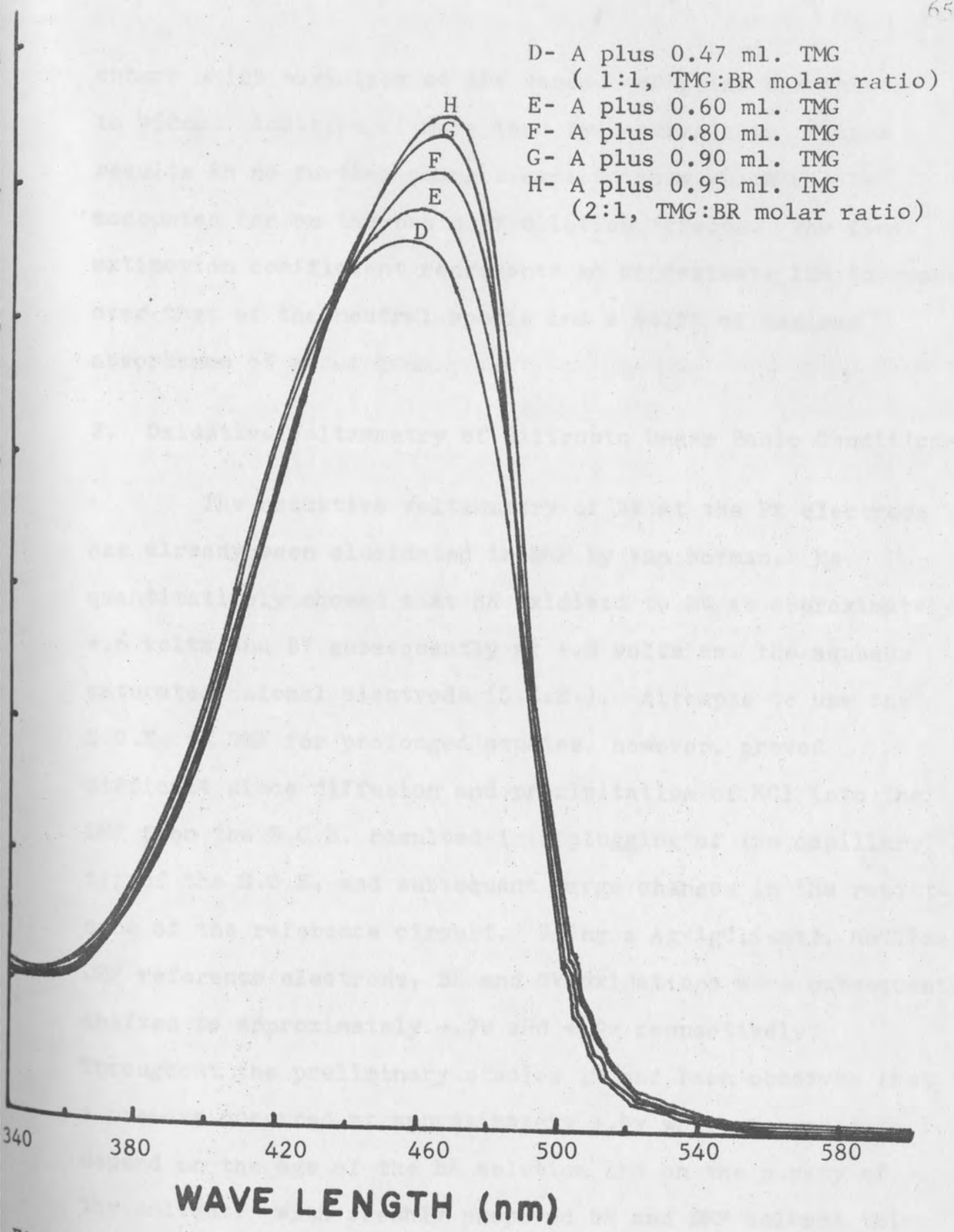


Figure 25 B- Continuation

occurs which maximizes at the second end point and shifts to 462nm. Addition of more than two equivalents of base results in no further changes except those which can be accounted for on the basis of dilution effects. The final extinction coefficient represents an approximate 10% increase over that of the neutral specie and a shift of maximum absorbance of about 10nm.

2. Oxidative Voltammetry of Bilirubin Under Basic Conditions

The oxidative voltammetry of BR at the Pt electrode has already been elucidated in DMF by Van Norman. He quantitatively showed that BR oxidized to BV at approximately +.6 volts and BV subsequently at +.8 volts vs. the aqueous saturated calomel electrode (S.C.E.). Attempts to use the S.C.E. in DMF for prolonged studies, however, proved difficult since diffusion and precipitation of KCl into the DMF from the S.C.E. resulted in a plugging of the capillary tip of the S.C.E. and subsequent large changes in the resistance of the reference circuit. Using a Ag/AgCl(sat), NaCl(sat) DMF reference electrode, BR and BV oxidations were subsequently shifted to approximately +.7v and +.9v respectively. Throughout the preliminary studies it had been observed that a prewave occurred at approximately +.4v which appeared to depend on the age of the BR solution and on the purity of the solvent. With freshly prepared BR and DMF solvent this prewave was totally eliminated. The same prewave existed in the voltammograms of Van Norman before electrolysis.[7]

The nature of the prewave at +.4v was affirmatively resolved by the analysis of BR under basic conditions. Figures 26A and 26B show the chronological changes resulting from the stepwise addition of exactly two molar equivalents of TMG to a BR solution. The oxidative wave at +.7v is quantitatively converted into the wave at +.45v while the verdinoid oxidation remains relatively unchanged in its peak current potential. Addition of excess TMG (2 to 3 equiv.) resulted in a slight increase in the wave at +.45v and a loss in the current of the second wave. However, a quantitative study was precluded by adsorption effects which seemed to occur after the addition of more than two equivalents of TMG. The peak height of both peaks was progressively diminished and distorted with repetitive scans but could be resotred with a cleaning of the Pt electrode in nitric acid and subsequent rinsing in DMF or by a 'crude' vigorous wiping of the electrode with a "Kimwipe" and then rinsing. Figure 27 shows the increase in current at +.4v using this 'crude' technique to follow the changes occurring with increasing quantities of TMG. That the BV oxidation does indeed shift to lower potential under more strongly basic conditions ($\sim$ 2 equivalents TMG) and merges with the BR oxidation was shown in an independent study of the voltammetry of BV. Figure 28 shows the progressive shift of BV oxidation with increasing base concentration from +0.9v to 0.2v. Although there is a significant increase in the background current as a result of relatively large amounts of TMG (Fig. 21 and Fig. 22), this

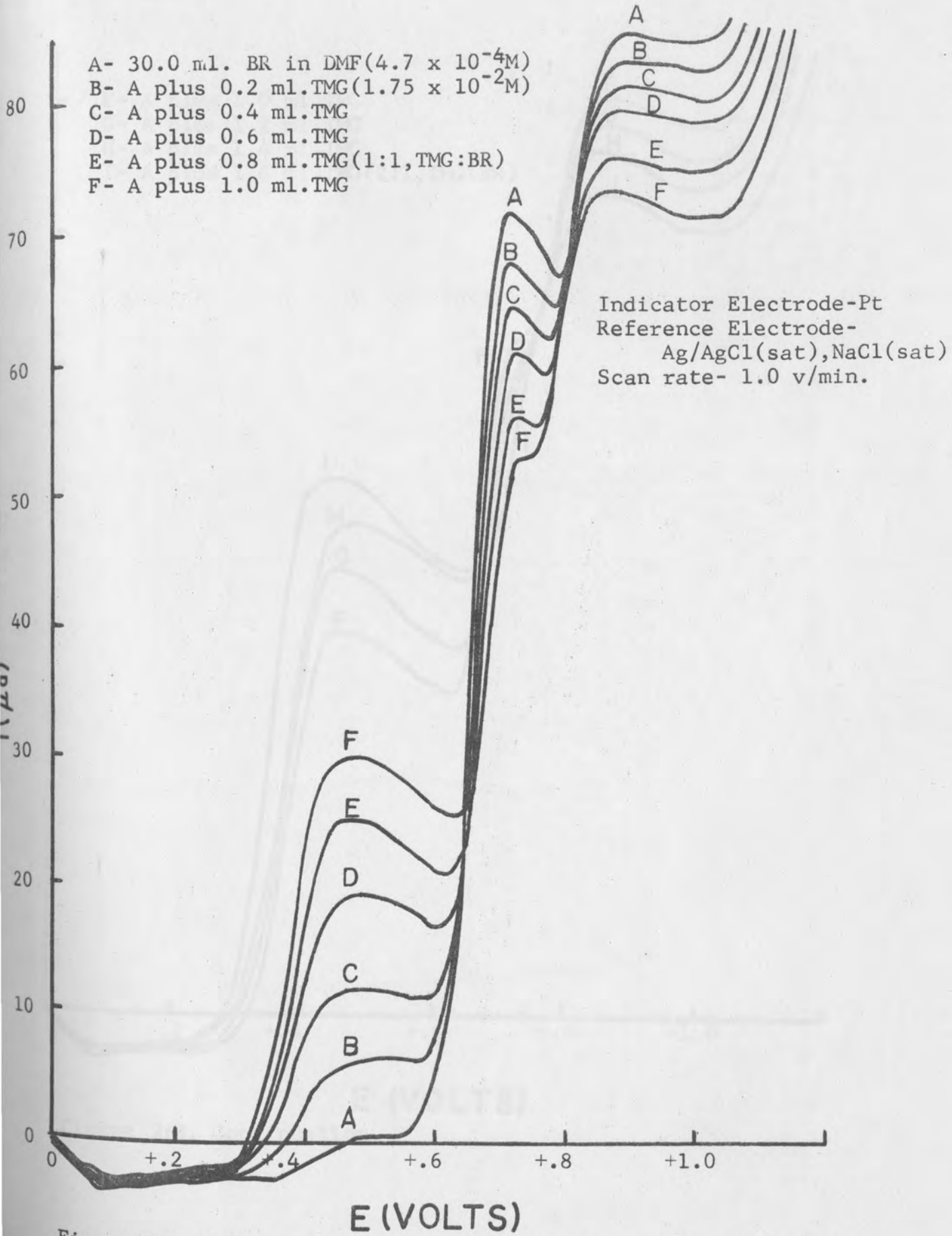


Figure 26A. The change in the oxidative voltammetry of bilirubin

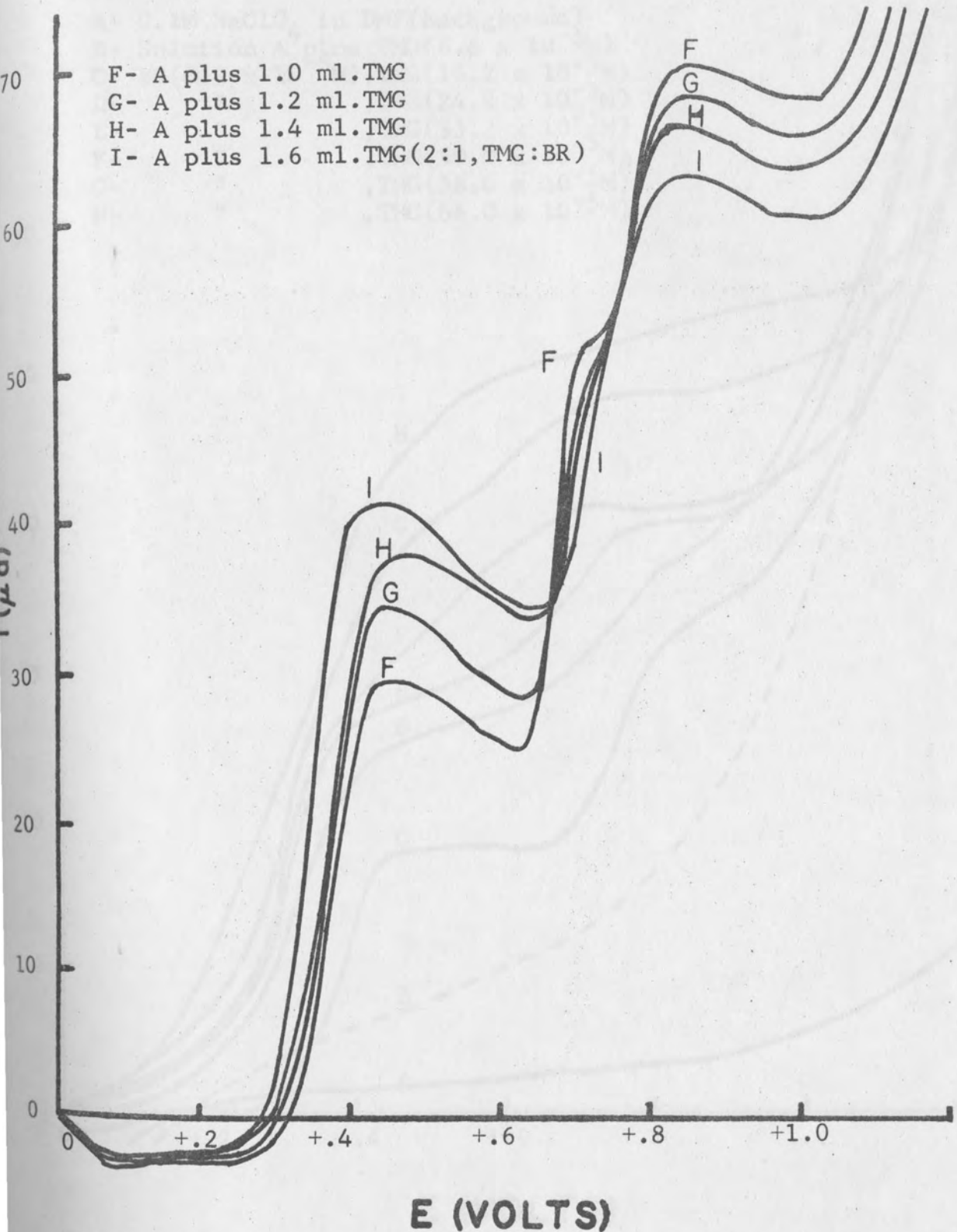


Figure 26B. Continuation.

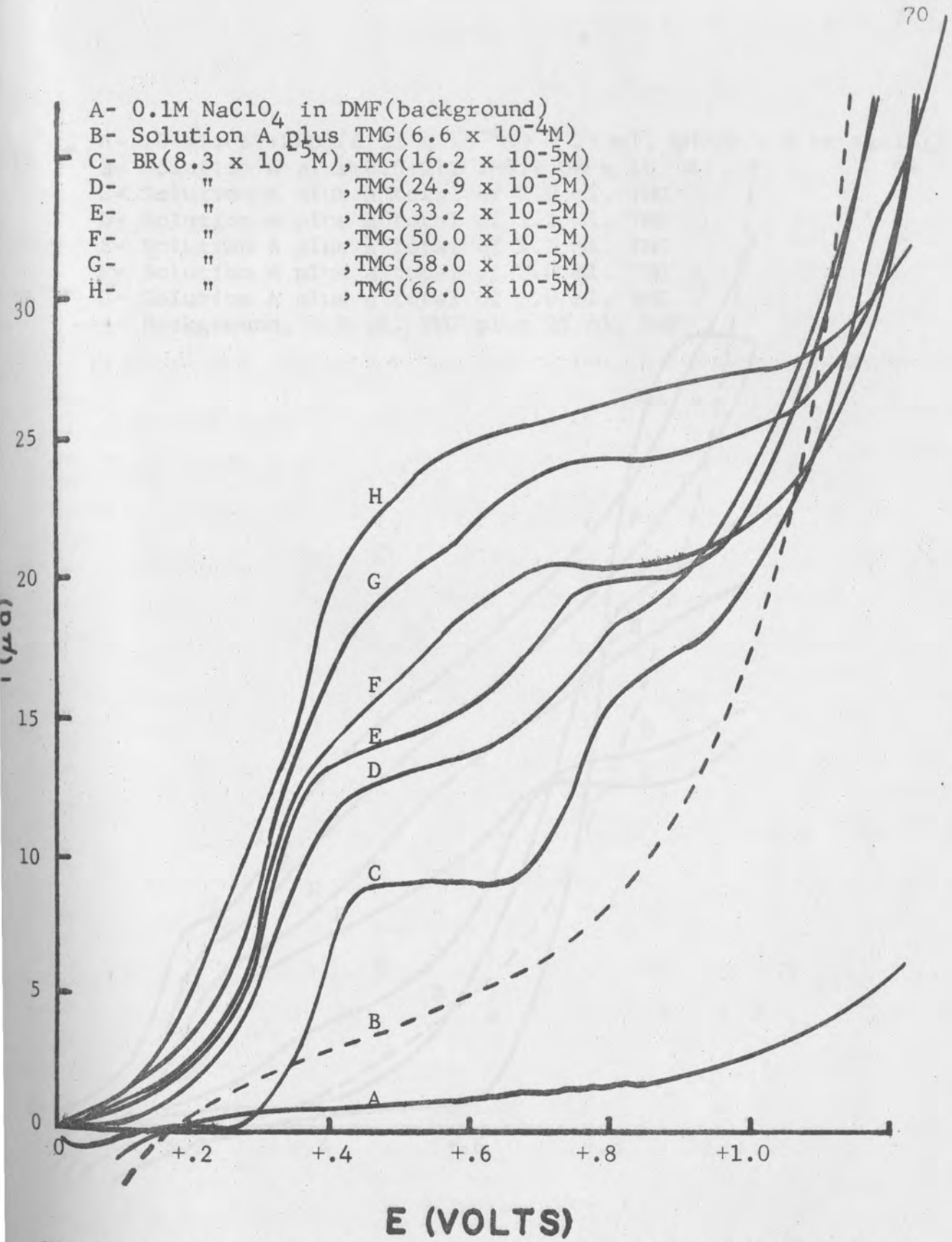


Figure 27. Oxidation of Bilirubin under Basic Conditions in DMF-Indicator Electrode, Pt: Reference Electrode, Ag/AgCl(sat),NaCl(sat); Scan rate, 1 v/min.

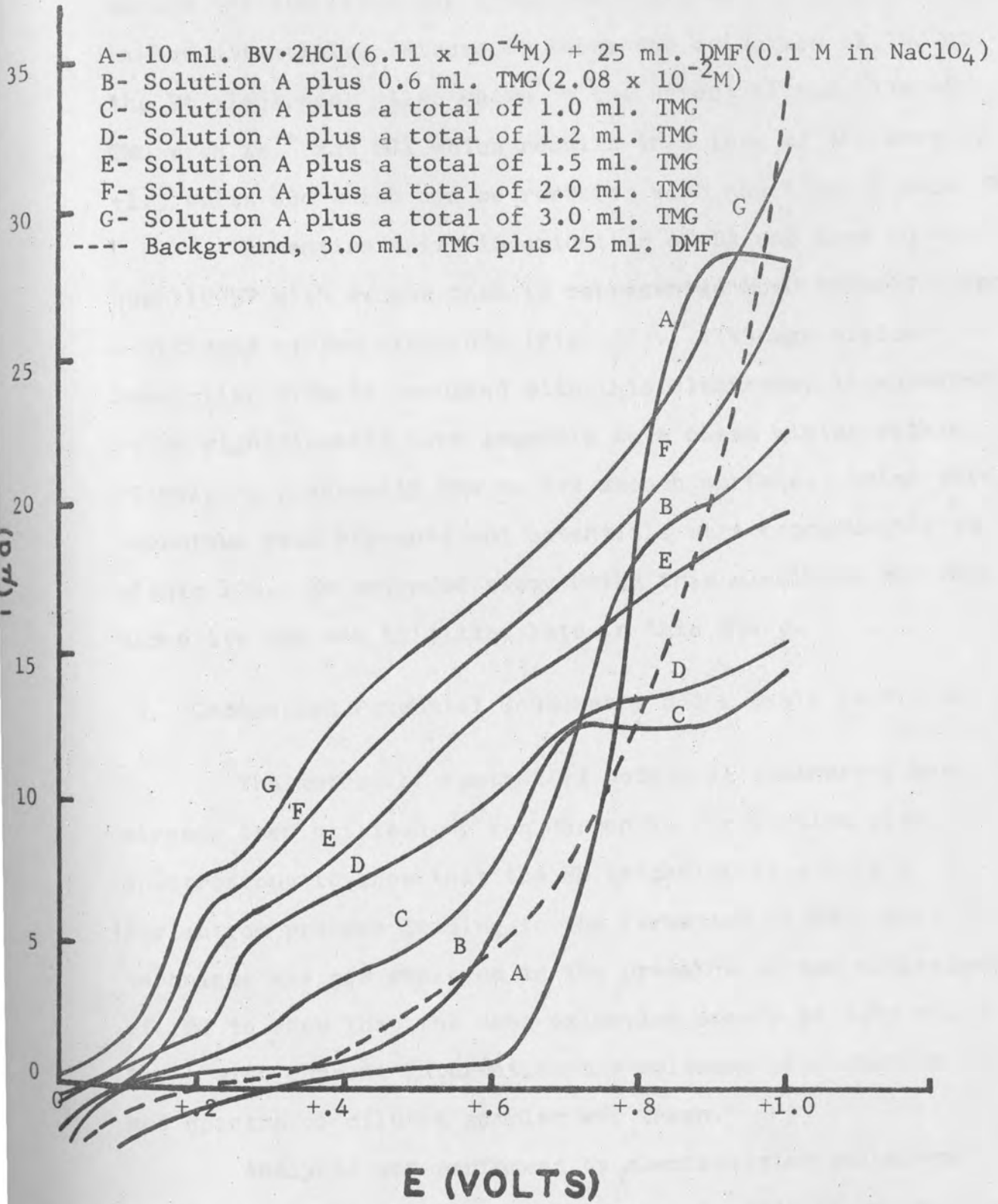


Figure 28. Oxidation of Biliverdin·2HCl under Basic Conditions in DMF- Indicator Electrode, Pt; Reference Electrode, Ag/AgCl(sat), NaCl(sat); scan rate, 1 v/min.

should not significantly alter the voltammetric wave profiles at low potentials. Figure 29 shows the oxidation of TMG at the Pt electrode. Also shown is the effect of reaction of TMG with Zn^{++} and HCl which results in a loss of the wave at +1.3 volts and which can be restored with addition of more TMG.

The entire shift in oxidation of BR and then subsequently BV with excess base is represented more clearly using a vitreous carbon electrode (Fig. 30). Although similar adsorption effects occurred with this electrode, it appeared to be significantly more amenable to a clean wiping with a "Kimwipe", presumably due to its smooth surface. Using this technique peak currents and potentials were reproducible to within 10%. No extended study using this electrode was made since its use was initiated late in this study.

3. Controlled Potential Coulometry Under Basic Conditions

The method of controlled potential coulometry has already been utilized by Van Norman in conjunction with spectroscopy to show that the BR oxidation at +.6 is a 2-electron process leading to the formation of BV. This technique was now employed in the presence of two equivalents of TMG to show that the same oxidation occurs at +.45 volts. Along with current integration the voltammetric behavior and spectra of diluted samples was taken.

Analysis was performed by electrolyzing solutions of BR at a large platinum foil in the presence of two equivalents of TMG at a potential of +.5 volts vs. the Ag/AgCl(sat),

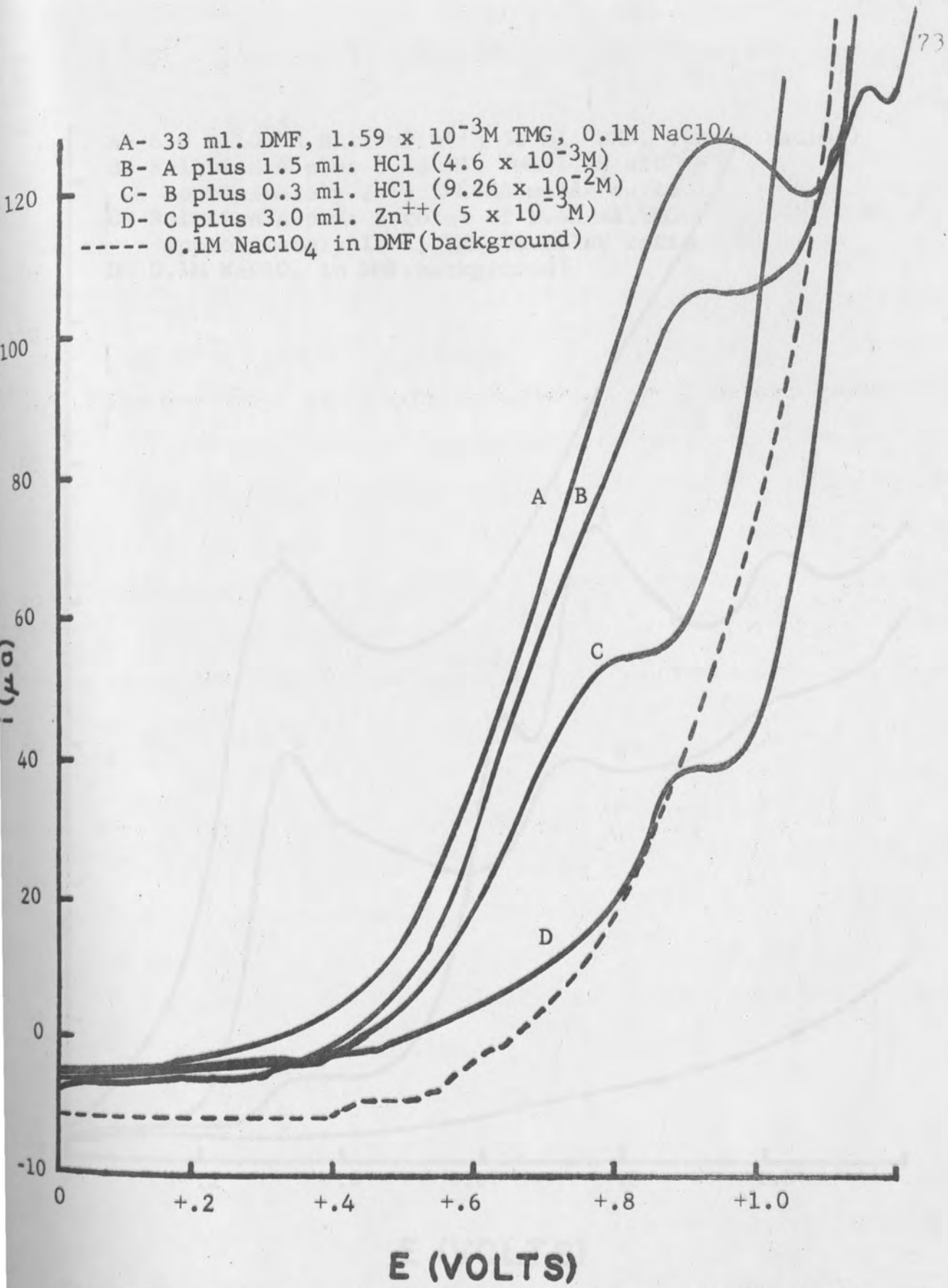


Figure 29. Oxidation of TMG in DMF- Indicator Electrode, Pt; Reference Electrode, Ag/AgCl(sat), NaCl(sat); Scan rate, 1 v/min.

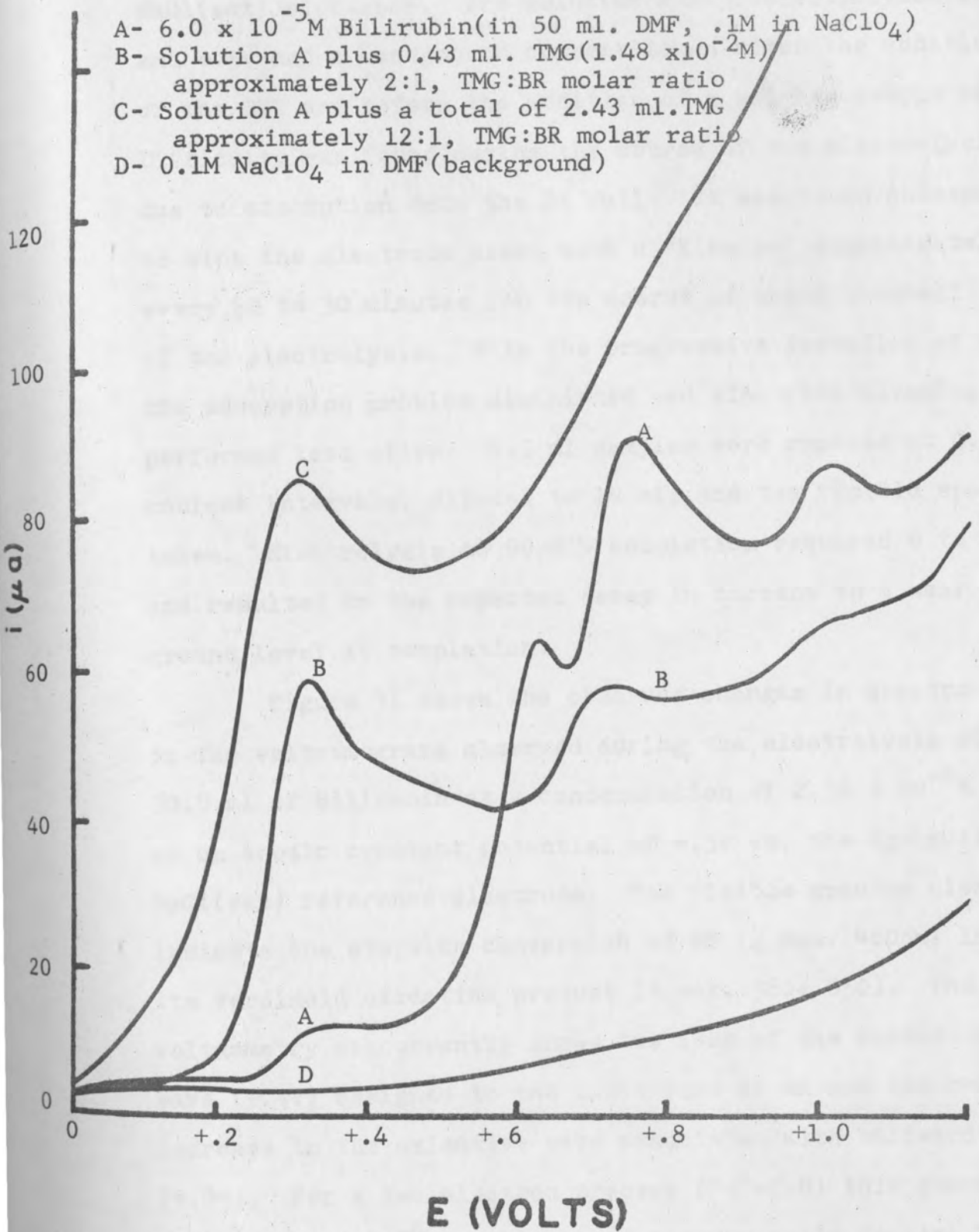


Figure 30 . Oxidation of Bilirubin under Basic Conditions in DMF-Indicator Electrode, vitreous carbon; Reference Electrode, Ag/AgCl(sat),NaCl(sat),0.1M $NaClO_4$; scan rate, 20 mv/min.

NaCl(sat) electrode. The solution was preelectrolyzed at +.8v and +.5v and a background current taken after the addition of the TMG and before the addition of a weighed sample of BR. Difficulty was found during the course of the electrolysis due to adsorption onto the Pt foil. It was found necessary to wipe the electrode clean with a "Kimwipe" approximately every 20 to 30 minutes for the course of about one-half of the electrolysis. With the progressive formation of BV the adsorption problem diminished and electrode cleaning was performed less often. 0.5 ml samples were removed at 0.2 coulomb intervals, diluted to 10 ml, and the visible spectra taken. Electrolysis to 90-95% completion required 6 to 8 hrs. and resulted in the expected decay in current to a near background level at completion.

Figure 31 shows the observed changes in spectra and in the voltammograms observed during the electrolysis of 30.8 ml of bilirubin at a concentration of 2.36×10^{-4}M and at an anodic constant potential of +.5v vs. the Ag/AgCl(sat), NaCl(sat) reference electrode. The visible spectra clearly indicate the stepwise conversion of BR (ε max. 460nm) into its verdinoid oxidation product (ε max. 380; 650). The voltammetry concurrently shows the loss of the oxidative wave (+.4v) assigned to the basic form of BR and the relative increase in the oxidative wave associated with biliverdin (+.8v). For a two electron process ("n"=2.0) this quantity of BR (7.08×10^{-6} moles) should theoretically require 1.37 coulombs for complete oxidation. However, 0.09 coulombs must be subtracted as a correction for the calculated

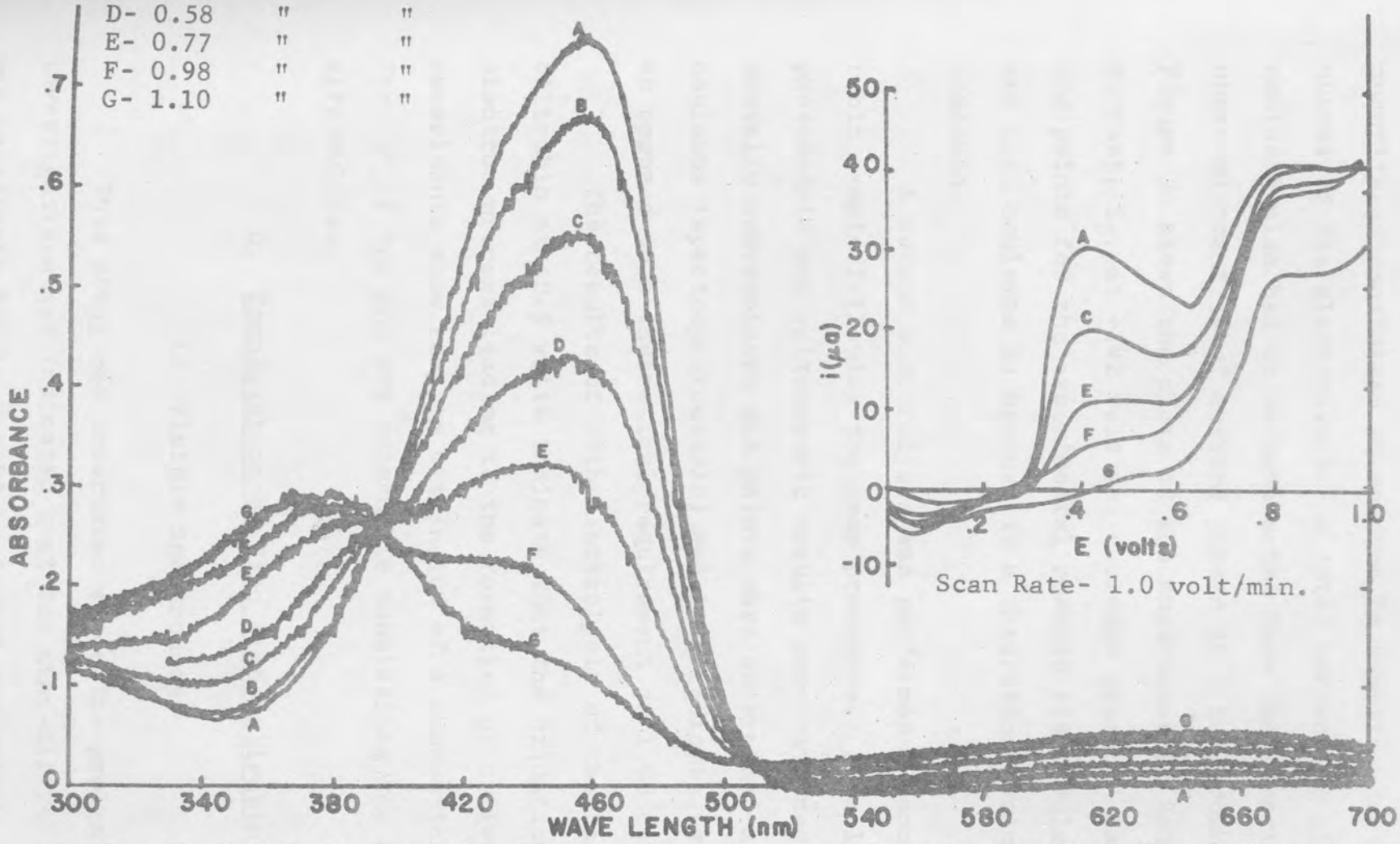


Figure 31- Spectral and Voltammetric Changes in a Bilirubin Solution during Electrolysis at +0.5 volts vs. Ag/AgCl(sat),NaCl(sat) in the presence of 2 equivalents of TMG.

quantity of unoxidized BR removed in sampling during the course of the electrolysis. A total correction of 0.07 coulombs also had to be subtracted from the experimentally observed quantity of current passed as a background correction. Figure 32 shows the plots of the absorbance at 460nm and peak current, i_p, at +.42 volt vs. coulombs passed. Extrapolated end points for the experimental results yield values of 1.20 and 1.25 coulombs as opposed to a theoretical value of 1.28 coulombs.

A second electrolysis was performed on another bilirubin sample following the same procedure. Parallel spectrophotometric and voltammetric results were obtained. Experimentally extrapolated end points were determined at 1.10 coulombs (spectrophotometric) and 1.05 coulombs (voltammetric) as opposed to a theoretical requirement of 1.08 coulombs.

The results of both electrolysis of the basic form of bilirubin at +0.5 volts indicate that the oxidation is a 2 electron process leading to the formation of biliverdin. Both experiments show results within 10% of a theoretical value for "n" of two and are reasonable considering the experimental difficulties.

D. Complexation of Zinc with Bilirubin

1. Visible Spectroscopy

This study was undertaken since the preliminary investigations had indicated that the zinc-bilirubin complex was relatively stable, could be formed reversibly, and

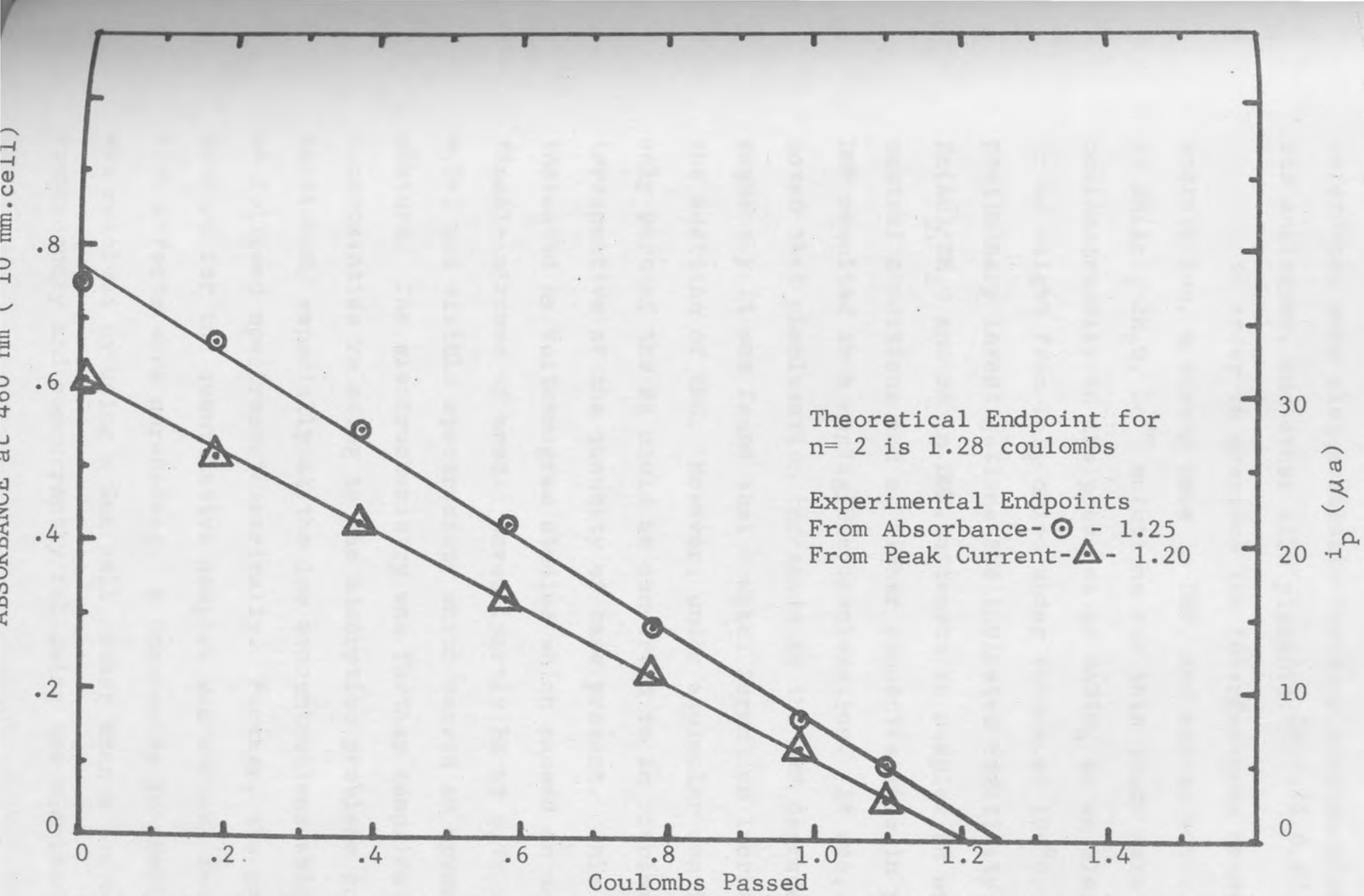


Figure 32. Plots of Absorbance at 460 nm(left) and peak current at +.42 v (right) versus coulombs passed (corrected for background)foroxidation of Bilirubin at +.5 volts in the presence of 2:1,TMG:BR-Indicator Electrode, Pt; Reference electrode, Ag/AgCl(sat),NaCl(sat).

appeared spectrophotometrically discernable. A number of references were also available for zinc complexation with BR, its analogues, and other bile pigments.[29,5,26,6,42]

In order to overcome the interferences caused by the acetate ion, a strong base in DMF, and excess water present in $Zn(Ac)_2 \cdot 2H_2O$, Zn^{++} solutions for this study were prepared coulometrically in the presence of $NaClO_4$ as an electrolyte or by weight from ZnI_2 dried under vacuum at 100°C. Although preliminary investigations had indicated reactivity between $Zn(Ac)_2 \cdot 2H_2O$ and BR in DMF, attempts to complex BR under neutral conditions and at higher concentrations in purified DMF resulted in a negligible complexation. It was, however, noted that complexation increased as the DMF degraded. Subsequently it was found that complex formation increased with the addition of TMG. However, under equimolar conditions only part of the BR could be converted to Zn complex irrespective of the quantity of base present. This was indicated by voltammogram studies which showed an undefinable mixture of anodic waves (partly BR at +.4v and +.7v) and visible spectroscopy which showed an apparent mixture. The electrochemistry was further complicated by uncertainties relating to the adsorption problems previously mentioned, especially at the low concentrations which could be followed spectrophotometrically. Further, the exact spectra for the quantitative complex was unknown and dilution effects were unresolved. A compromise in precision was resolved by using a 2mm cell rather than a 1cm cell for spectroscopy and concurrently following the electrochemistry

at a five-fold increase in concentration.

In order to resolve the conditions for complex formation a systematic study was undertaken which involved varyingthe initial molar ratio of Zn/BR, titrating the solution with tetramethylguanidine, and following the course of the reaction spectrophotometrically. Figure 33 shows the relationships obtained between Zn/BR ratio, complex formation (530nm), and relative base concentration. Figures 34, 35, 36, and 37 show the corresponding spectral changes occurring during these titrations. Three significant species appear predominant; neutral BR (453nm), basic BR (464nm), and Zn complex (530-540nm). In each case addition of excess TMG leads to a slow reversion of complex back to basic BR. This type of behavior is graphically represented in Figure 33, by the leveling of complex concentration as a function of base concentration. Maximum complex formation, and perhaps near complete complex formation always appeared at BR:Zn:TMG ratios of 1:2:8. Excess TMG subsequently resulted in a slight loss of complex. In no cases were any appreciable oxidations observed as verdinoid absorbance in the regions 600-700nm and 380-400nm during the day of the titrations. In addition, the addition of acid (HCl) resulted in a complete quantitative reversal of the system to neutral BR.

Maximum complex formation at the ratio 1:2:8 was also found by starting with a BR/TMG mixture of molar ratio 1:8 and titrating with Zn^{++}. Excess Zn^{++} also resulted in a slow reversion of the process. However, this reversion

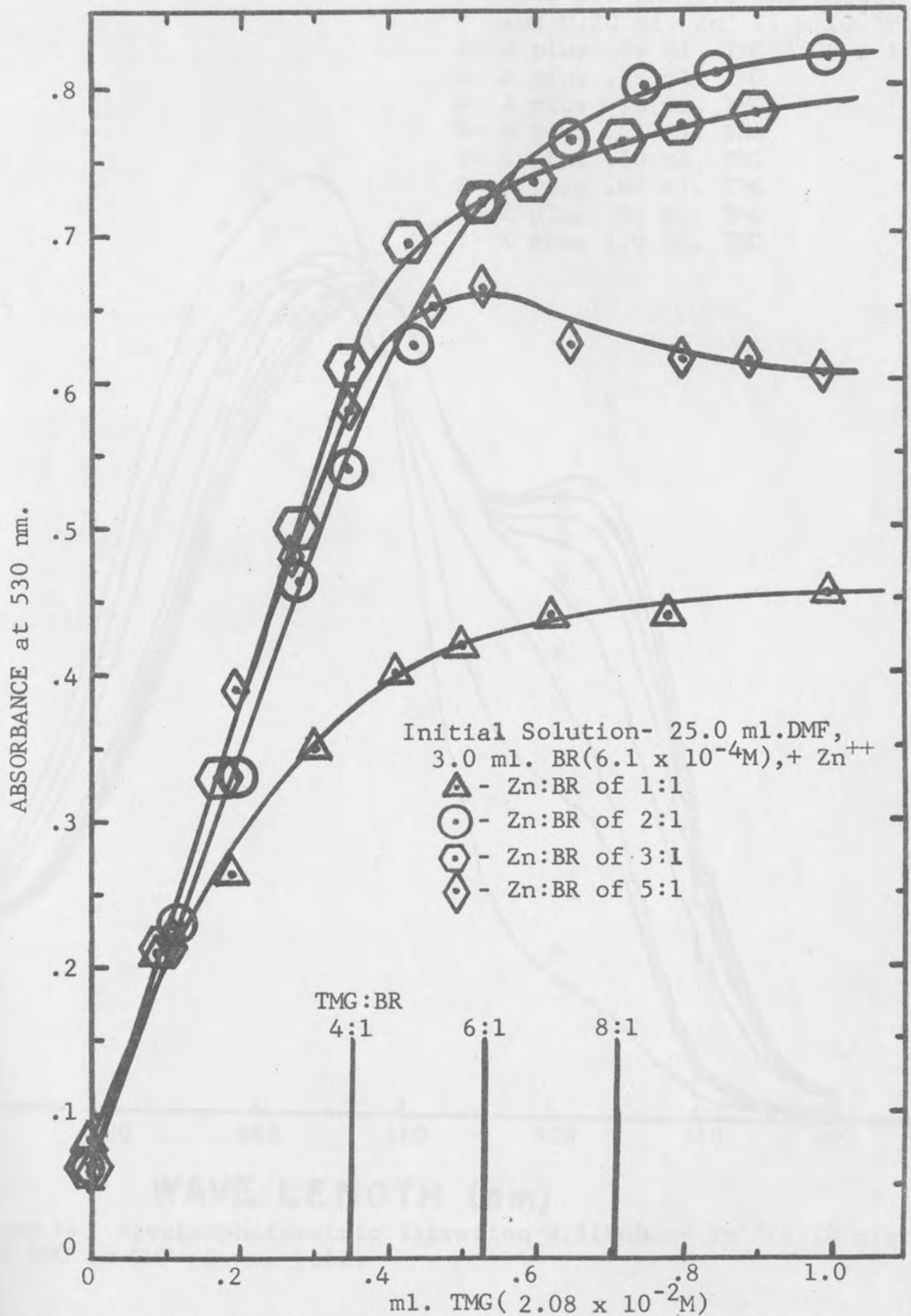


Figure 33. Spectrophotometric Titrations of Zn^{++}-Bilirubin Mixtures with TMG- The absorbing species is Zn_2-BR complex at 530 nm-2 mm.cell.

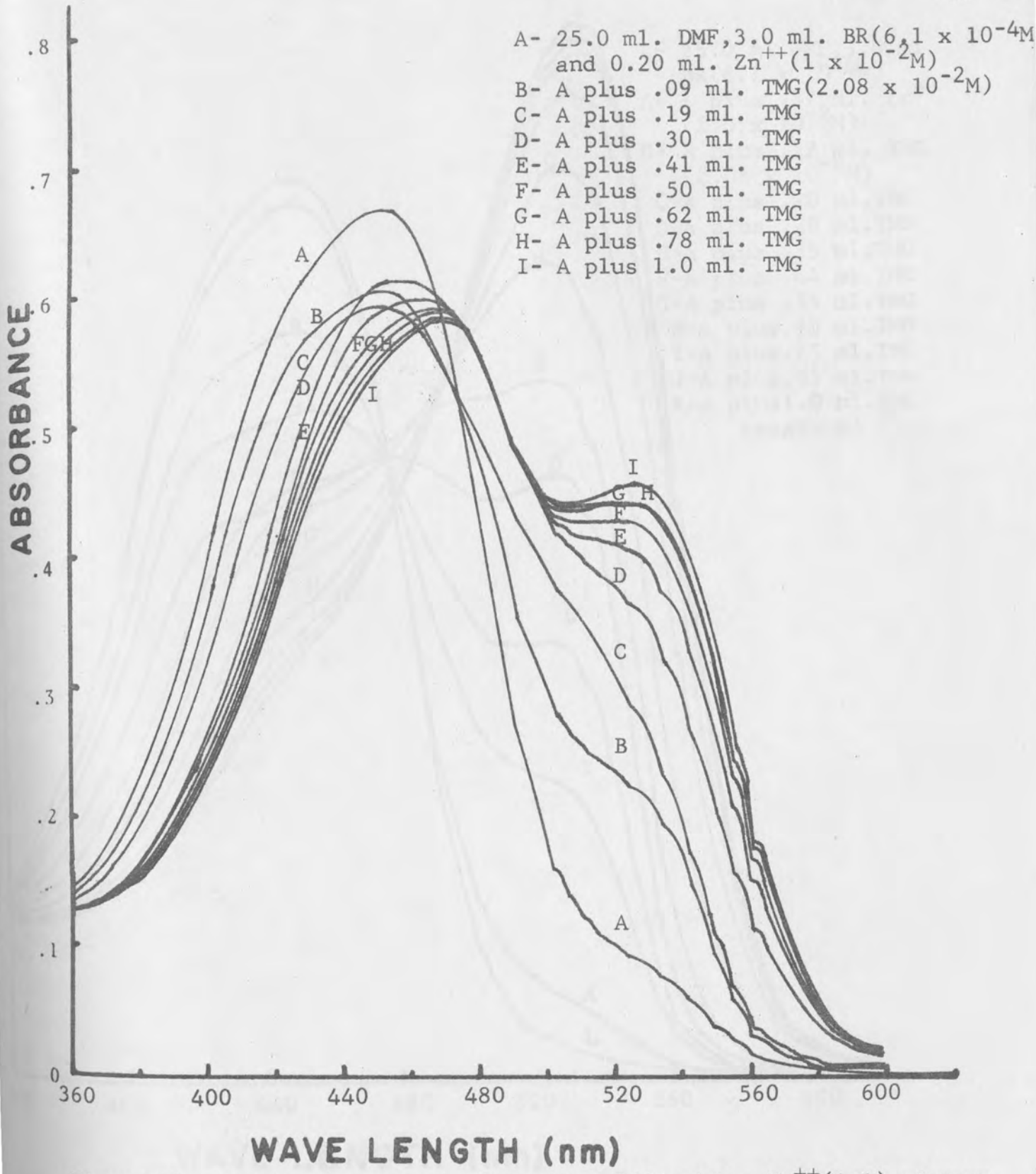


Figure 34 . Spectrophotometric Titration Bilirubin- Zn^{++}(1:1) mixture with TMG in DMF- 2 mm. Cell.

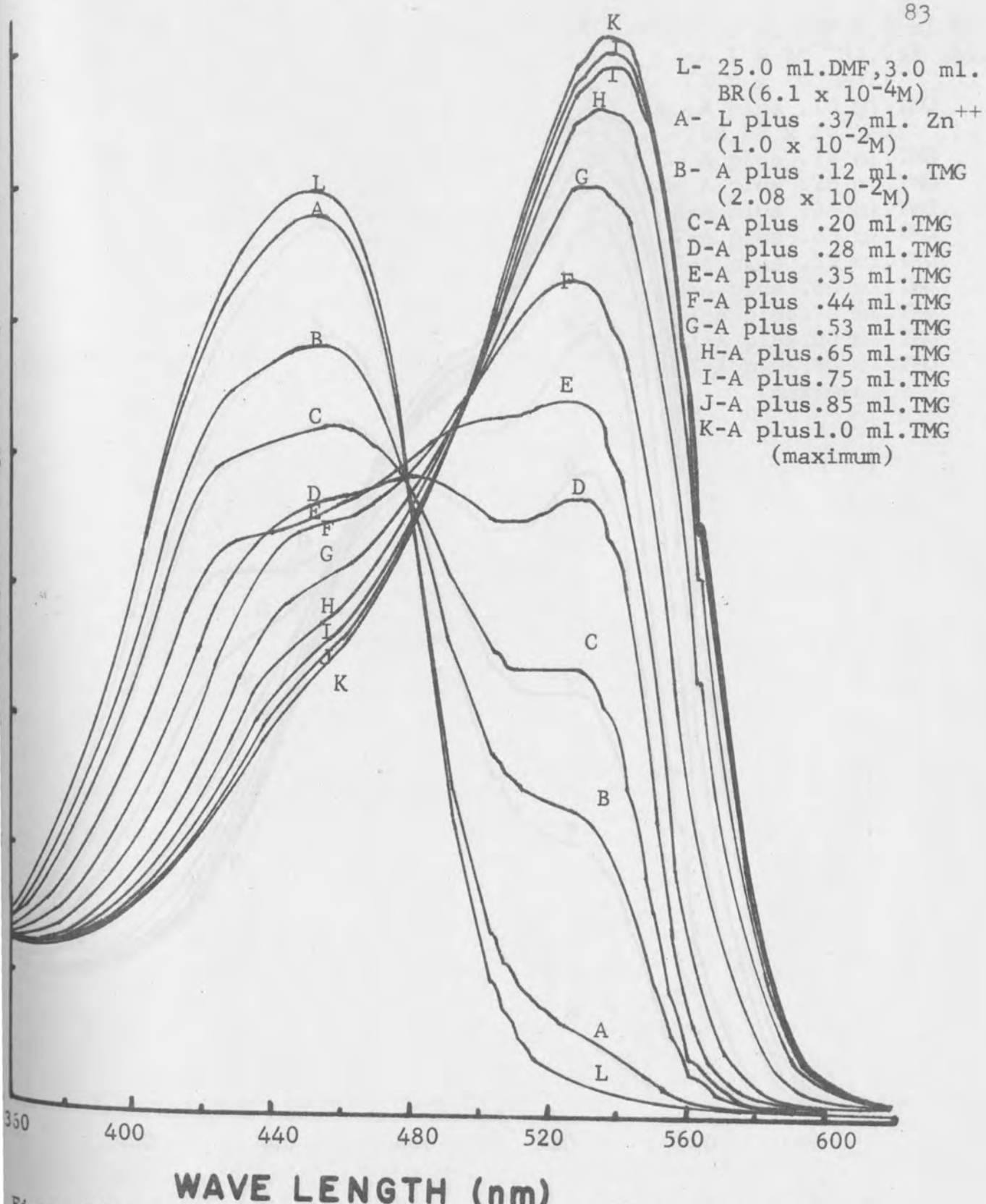


Figure 35.Spectrophotometric Titration of Zn^{++}-bilirubin solution, 2:1 molar ratio Zn^{++}:BR, in DMF- 2 mm. cell.

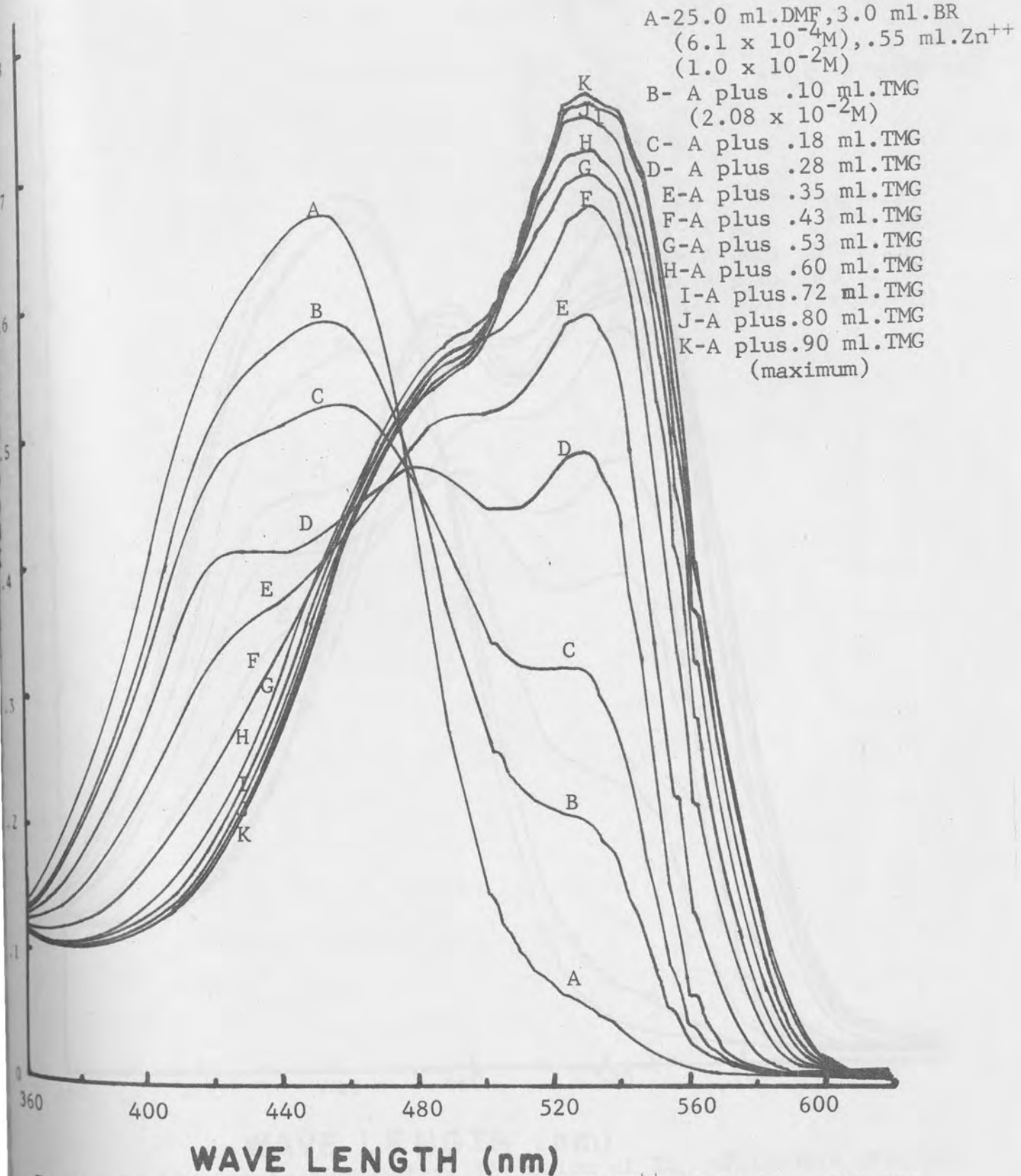


Figure 36. Spectrophotometric Titration of Zn^{++}-Bilirubin solution, 3:1 molar ratio Zn^{++}:BR, in DMF- 2 mm.cell.

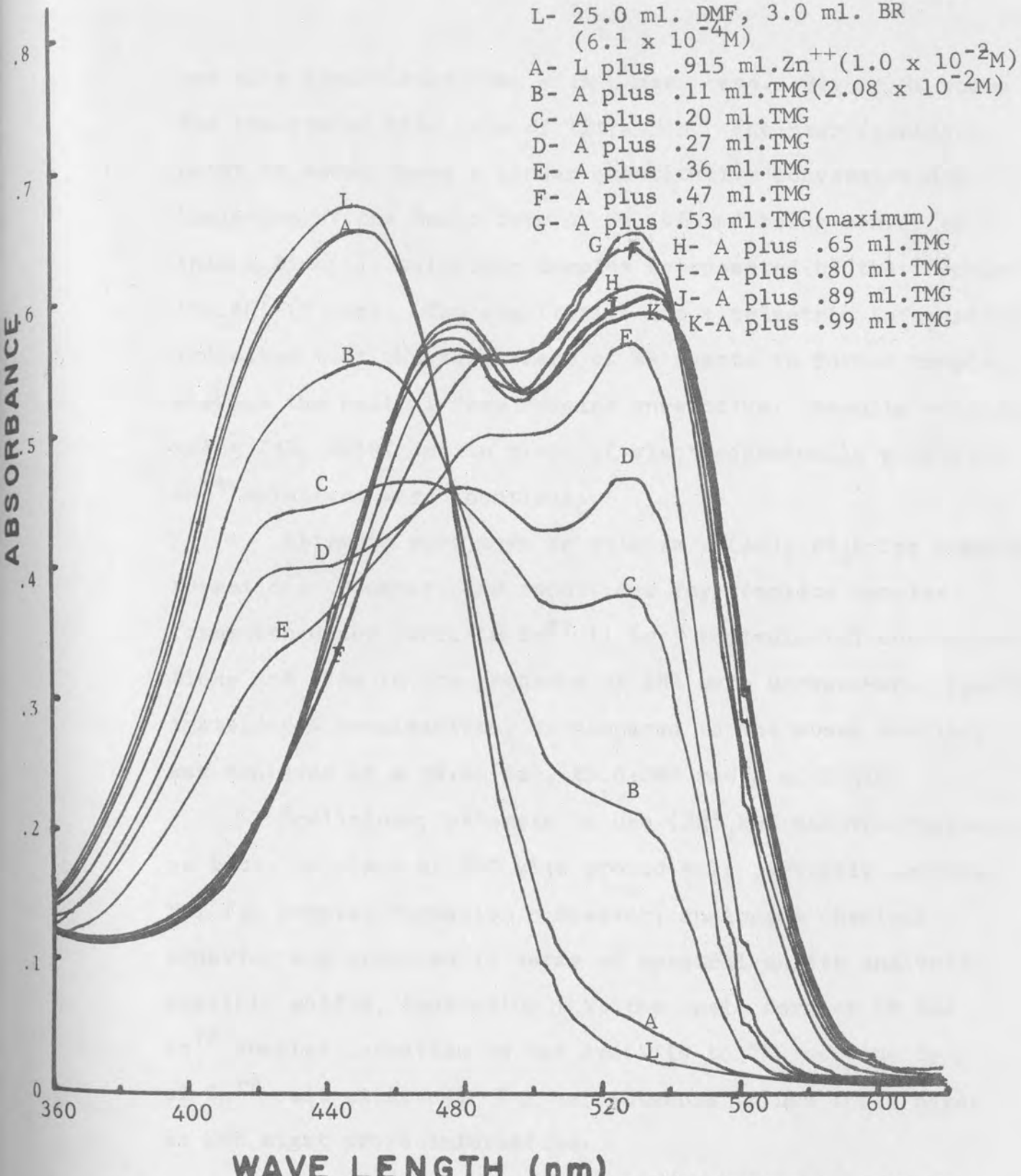


Figure 37. Spectrophotometric Titration of Zn^{++}-Bilirubin solution, 5:1 molar ratio Zn^{++}:BR, in DMF- 2mm. cell.

was more significant than with excess base. Figure 38 shows the results of this type of titration. The near isosbestic point at 490nm shows a linear quantitative conversion and isolation of the basic form of BR (464nm) being converted into a Zinc(II)-bilirubin complex represented by the formula 'Zn_2BR' (530nm). The qualitative and titrimetric information indicates that the basic form of BR reacts to form a complex, whereas the neutral form remains unreactive. Results obtained using ZnI_2 solutions in place of electrochemically generated Zn^{++} solutions were identical.

Attempts were made to utilize $Zn(Ac)_2 \cdot 2H_2O$ for complex formation. However, the conditions for complete complex formation under variable Zn^{++} (1 to 5 equivalents) concentrations and also in the presence of TMG were unresolved. Approximately 80% complexation, as compared to the above results, was achieved at a BR:$Zn(Ac)_2 \cdot 2H_2O$:TMG ratio of 1:5:8.

Preliminary attempts to use $(Bu)_4NOH$ and triethylamine as bases in place of TMG also proved only partially successful for complex formation. However, analogous chemical behavior was observed in terms of spectral shifts and voltammetric shifts, indicating that the basic form of BR and Zn^{++} complex formation is not specific to TMG nor the type of Zn^{++} salt utilized. Further attempts to use these bases in DMF might prove informative.

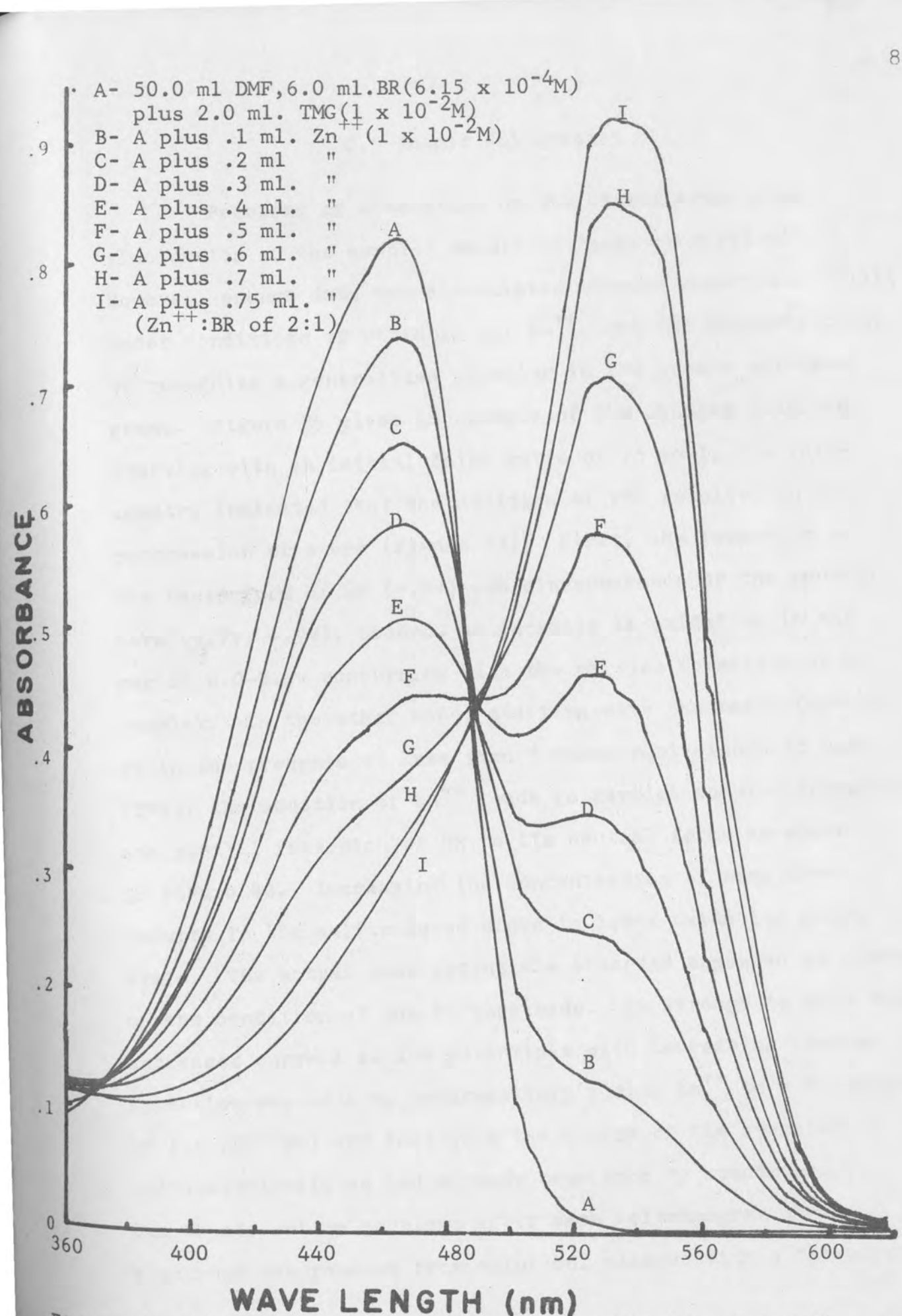


Figure 38. Spectrophotometric Titration of Bilirubin with Zn^{++}

2. Anodic Voltammetry

Problems of adsorption on the Pt electrode made elucidation of the complex anodic voltammetry difficult. However, enough data was accumulated through repetitive trials under conditions of variable BR, Zn^{++}, and TMG concentrations to recognize a generalized behavior in the anodic voltammograms. Figure 39 gives an example of the changes observed. Starting with an initial Zn:BR ratio of .5 to 1, the voltammetry indicated that the addition of TMG resulted in a progression of steps (Figure 33): First, the formation of the basic form of BR (+.4v) and disappearance of the neutral form (+.7v, +.9v); second, an increase in oxidation in the region 0.0-0.2v concurring with the partial formation of Zn complex. On the other hand, starting with the basic form of BR in the presence of less than 8 molar equivalents of base (TMG), the addition of Zn^{++} leads to partial complex formation and partial reversion of BR to its neutral forms as shown in Figure 40. Increasing the concentration of base then results in the shifts noted above to lower oxidation potentials. The actual peak potentials observed appeared to depend on the condition of the Pt electrode. An attempt to show this increased current at low potentials with increasing complex formation was made by progressively adding Zn^{++} to a solution of 1:8 (BR:TMG) and following the course of the reaction voltammetrically as had already been done by spectroscopy. Due to adsorption problems after each voltammogram the Pt electrode was removed from solution, cleaned with a "Kimwipe",

	BR x 10^4	Zn^{++} x 10^4	TMG x 10^4
A-	2.39	-	-
B-	2.34	1.17	-
C-	2.29	1.15	3.35
D-	2.25	1.13	6.58
E-	2.21	1.11	9.69

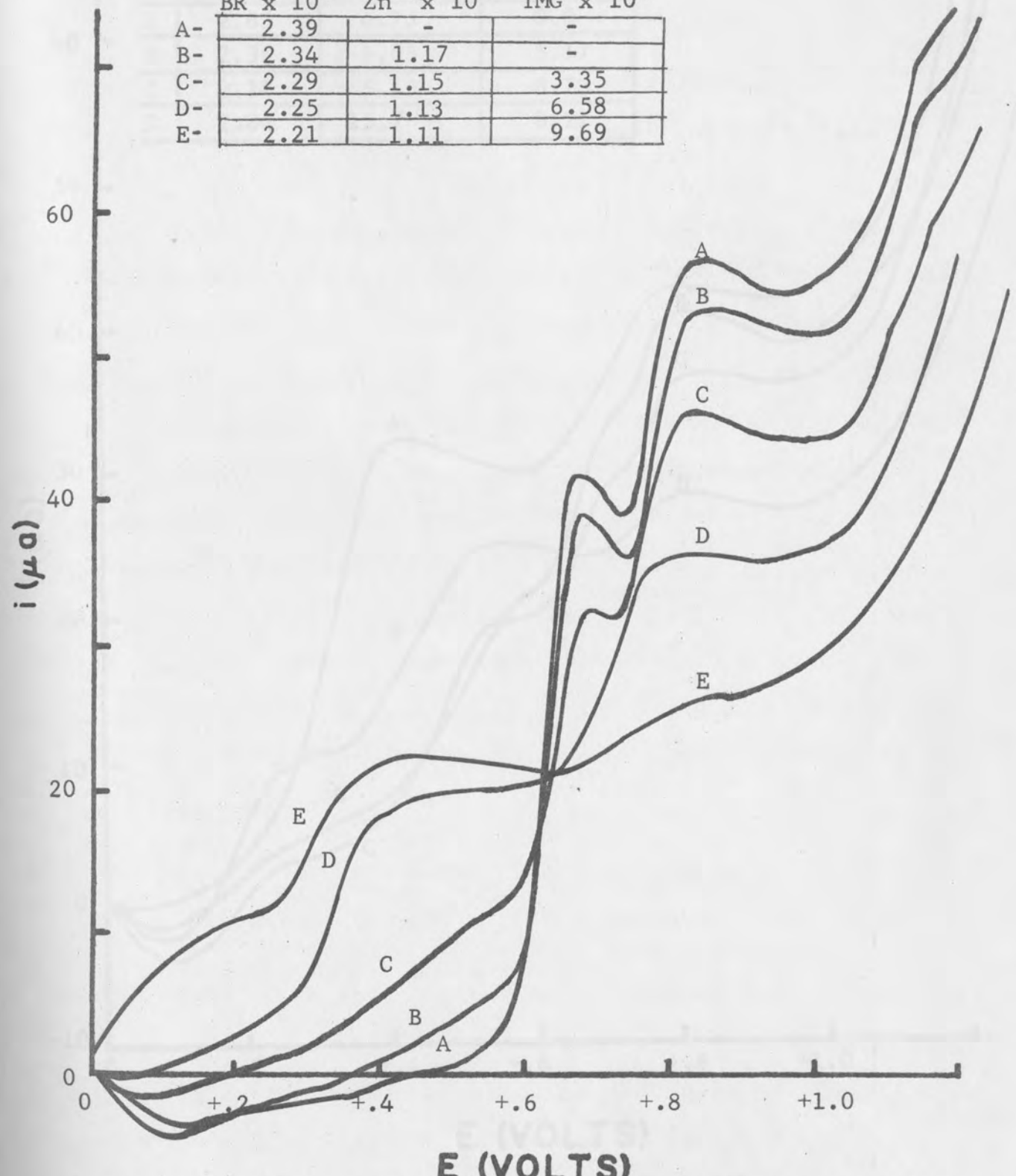


Figure 39 . Changes in Anodic Voltammograms of Zinc-Bilirubin Solutions with addition of TMG in DMF- Indicator Electrode,Pt; Reference Electrode,Ag/AgCl(sat),NaCl(sat); Scan rate, 1.0 v/min.

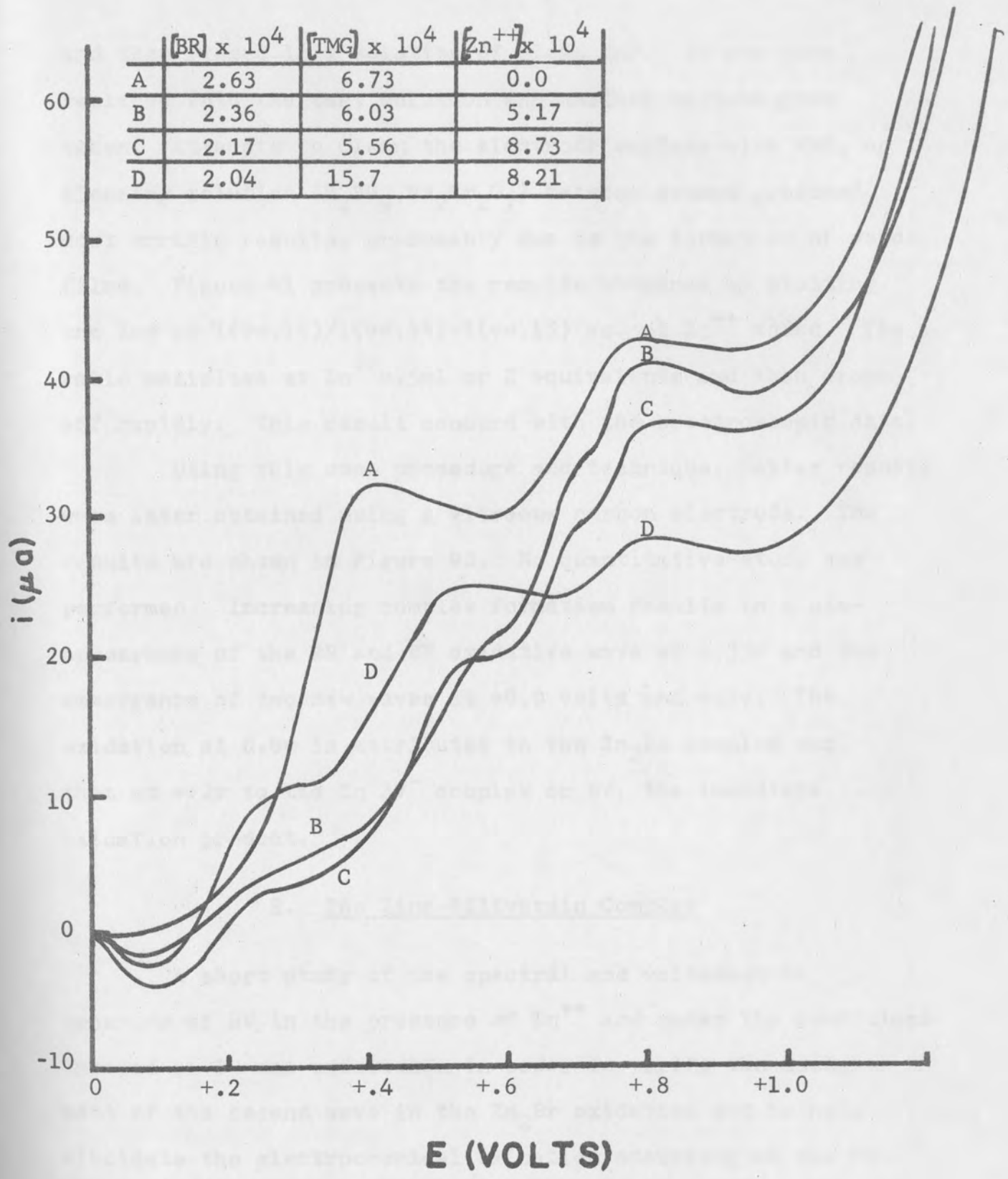


Figure 40. Addition of Zn^{++} to Bilirubin under Basic Conditions-Indicator Electrode, Pt; Reference Electrode, Ag/AgCl(sat), NaCl (sat); Scan rate 1 v/min.

and then rinsed in a solution of clean DMF. It was then replaced into the test solution and another voltammogram taken. Attempts to clean the electrode surface with HNO_3 or cleaning solution (H_2SO_4,$Na_2Cr_2O_7$) between sweeps produced most erratic results, presumably due to the formation of oxide films. Figure 41 presents the results obtained by plotting the log of i(v=.15)/i(v=.45)-i(v=.15) vs. ml Zn^{++} added. The ratio maximizes at Zn^{++}=.5ml or 2 equivalents and then drops off rapidly. This result concurs with the spectroscopic data.

Using this same procedure and technique, better results were later obtained using a vitreous carbon electrode. The results are shown in Figure 42. No quantitative study was performed. Increasing complex formation results in a disappearance of the BR and BV oxidative wave at +.35v and the emmergence of two new waves at +0.0 volts and +.2v. The oxidation at 0.0v is attributed to the Zn_2BR complex and that at +.2v to the Zn BV^- complex or BV, the immediate oxidation product.

E. The Zinc-Biliverdin Complex

A short study of the spectral and voltammetric behavior of BV in the presence of Zn^{++} and under the conditions imposed on BR was undertaken in order to verify the assignment of the second wave in the Zn_2Br oxidation and to help elucidate the electrochemical oxidation occurring at the Pt electrode under conditions of constant potential coulometry.

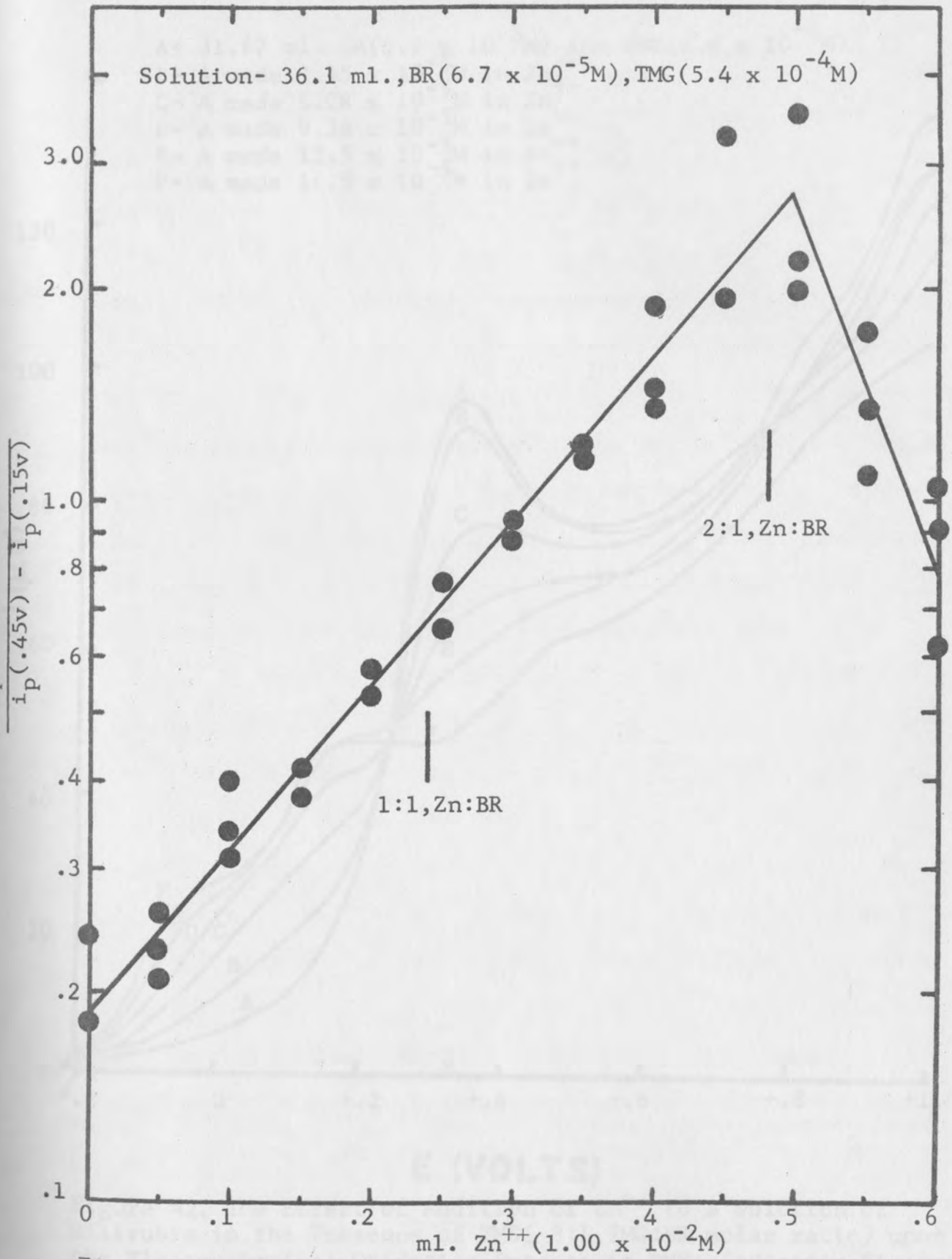


Figure 41. Plot of Voltammetric Changes occurring during the Titration of Bilirubin with Zn^{++} under basic conditions in DMF-Indicator Electrode, Pt; Reference Electrode, Ag/AgCl(sat),NaCl (sat); Scan rate, 1.0 v/min.

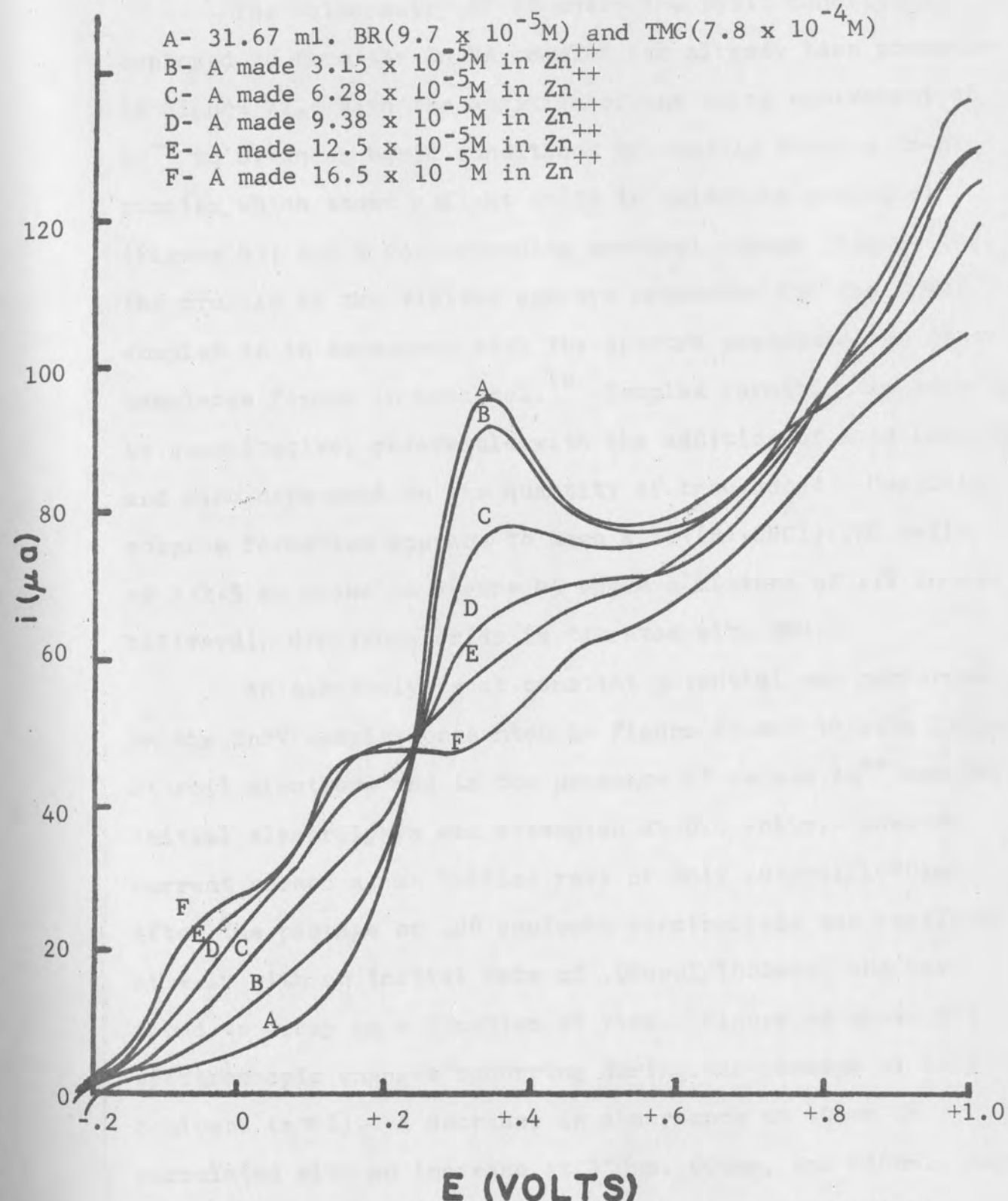


Figure 42. The Effect of Addition of Zn^{++} to a Solution of Bilirubin in the Presence of TMG(8:1,TMG:BR molar ratio) upon the Electrochemical Oxidation Process in DMF- Indicator Electrode, Vitreous Carbon; Reference Electrode, Ag/AgCl(sat),NaCl(sat); Scan rate, 2.5 v/min.

The voltammetry of BV under the basic conditions employed to form the Zn_2BR complex has already been presented in Figure 22. With the addition of one molar equivalent of Zn^{++} to BV under basic conditions BV readily forms a Zn-BV complex which shows a slight shift in oxidative potential (Figure 43) and a corresponding spectral change (Figure 44). The profile of the visible spectra presented for the Zn-BV complex is in agreement with the spectra presented for Zn-BV complexes formed in methanol.[10] Complex formation appears to be quantitative, reversible with the addition of acid (HCl-DMF), and also dependent on the quantity of base added. Complete complex formation appears to have a Zn:(BV·2HCl):TMG ratio of 1:1:5 as shown in Figure 45 where a mixture of 1:1 Zn and biliverdin dihydrochloride is titrated with TMG.

An electrolysis at constant potential was performed on the $ZnBV^-$ complex presented in Figure 43 and 44 at a large Pt foil electrode and in the presence of excess Zn^{++} and TMG. Initial electrolysis was attempted at 0.0 volts. However, current passed at an initial rate of only .02coul/1000sec. After the passage of .08 coulombs electrolysis was continued at +.1v with an initial rate of .08coul/1000sec. and was found to decay as a function of time. Figure 46 shows the spectroscopic changes occurring during the passage of 1.15 coulombs ($n \cong 2$). A decrease in absorbance at 400nm is correlated with an increase at 350nm, 600nm, and 660nm. Background currents under these conditions were found to be negligible. However, a significant rate of electrolysis was

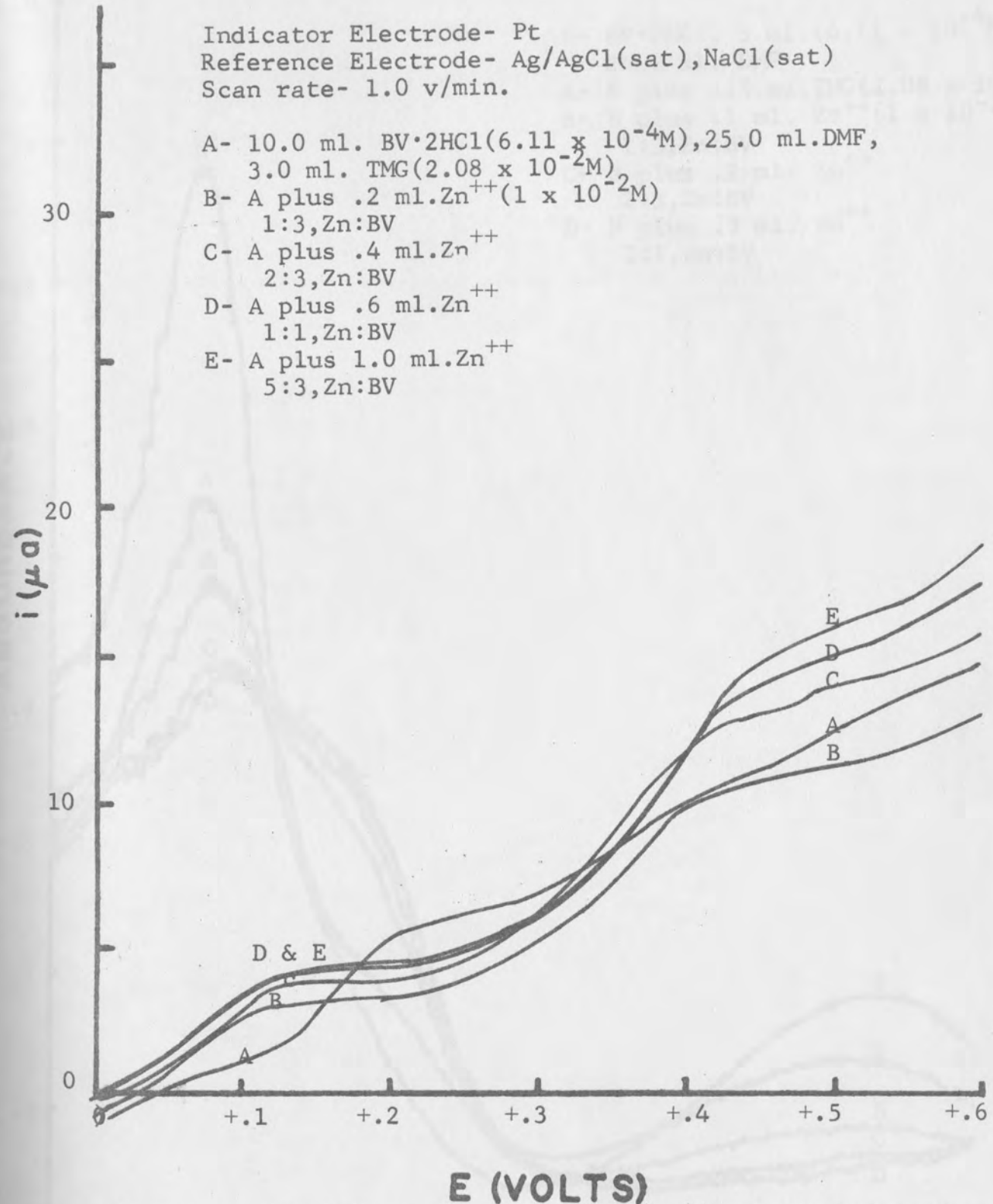


Figure 43 . The Effect of Addition of Zn^{++} to a solution of Biliverdin in the presence of TMG(10:1,TMG:BV) upon the anodic voltammograms in DMF.

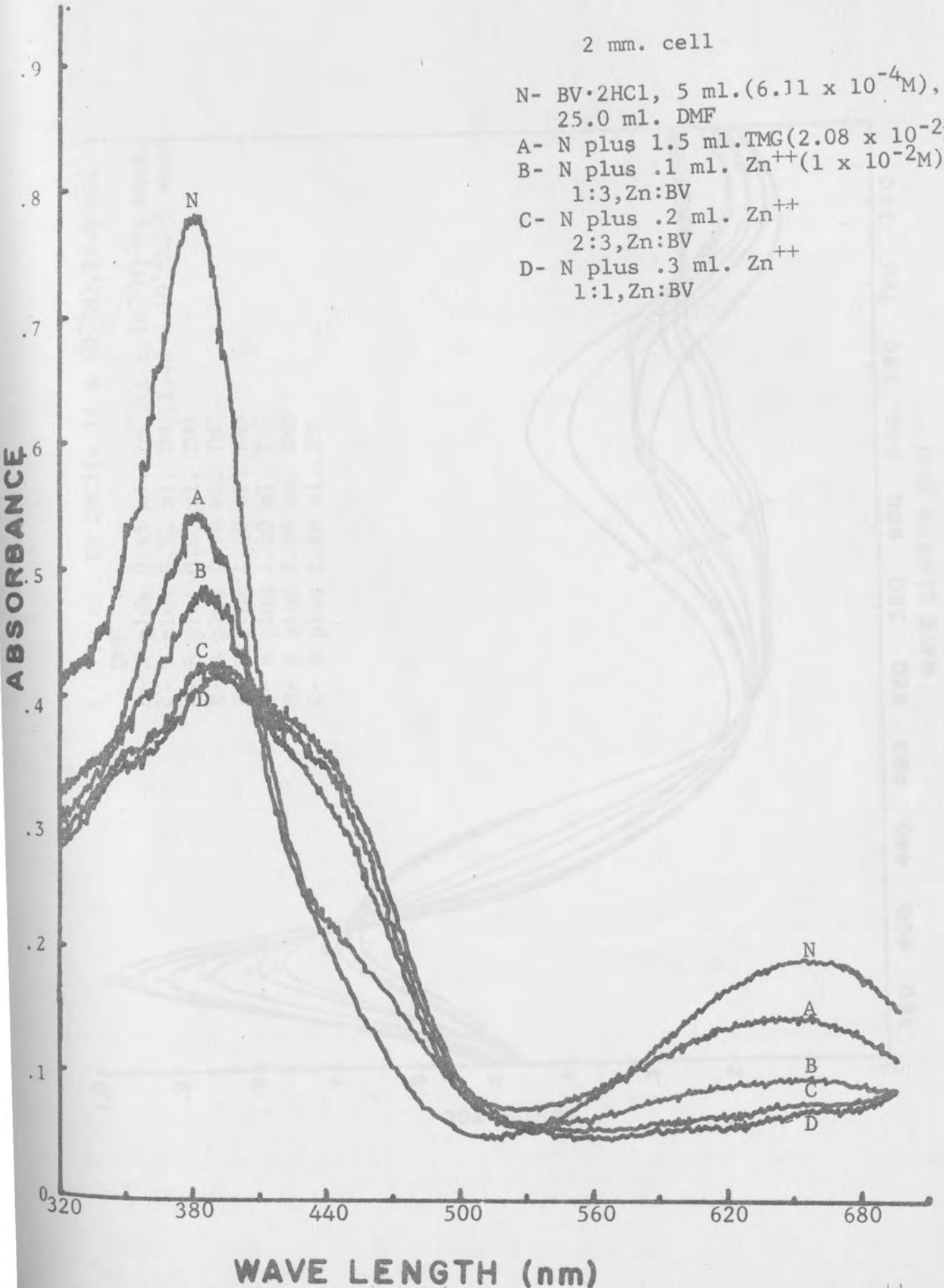


Figure 44 . Spectrophotometric Titration of Biliverdin with Zn^{++} in

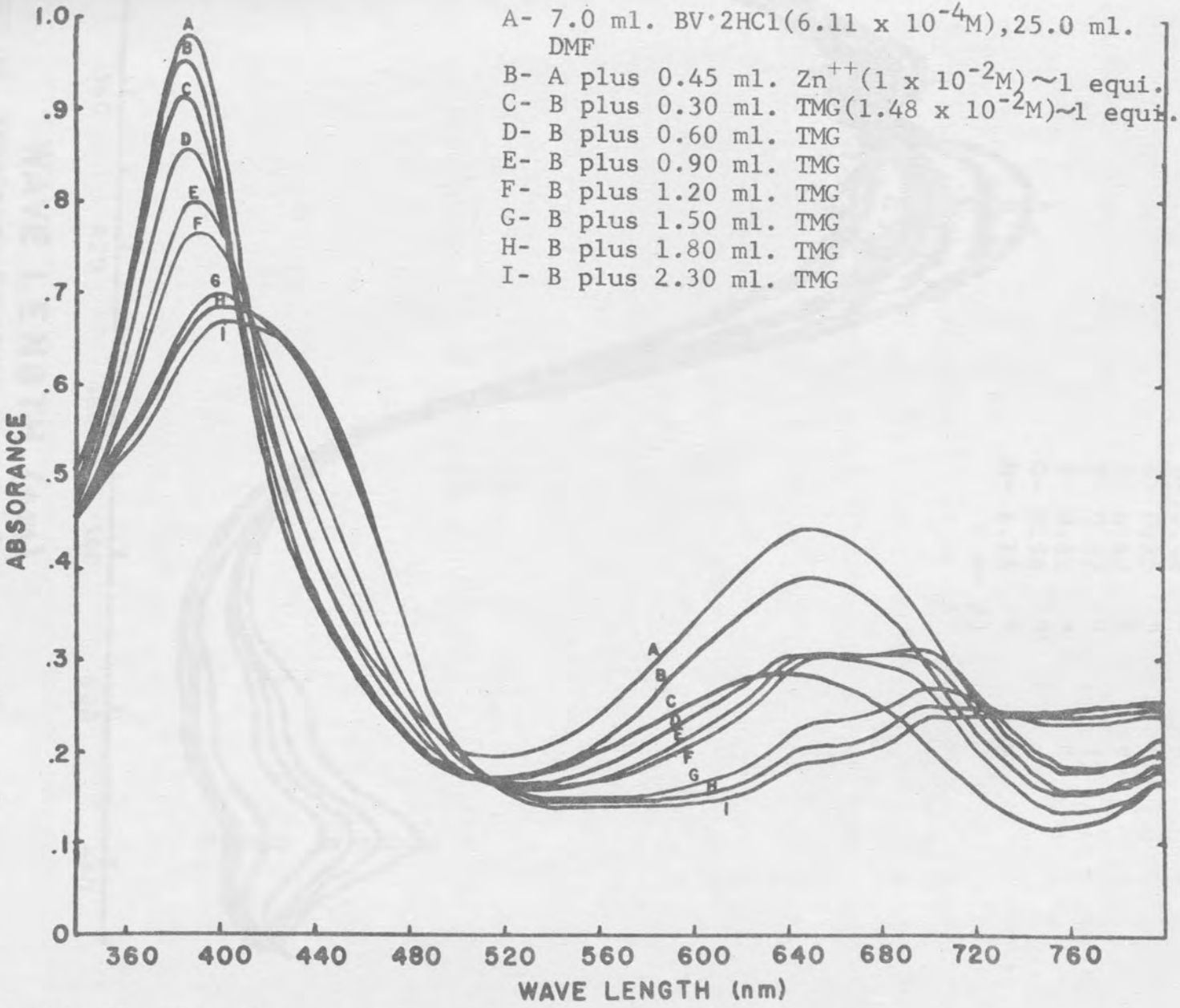


Figure 45- Titration of Biliverdin Dihydrochloride(1.32×10^{-4} M) and Zn^{++} (1.39×10^{-4}M) with TMG to Form $ZnBV^{=}$ Complex.

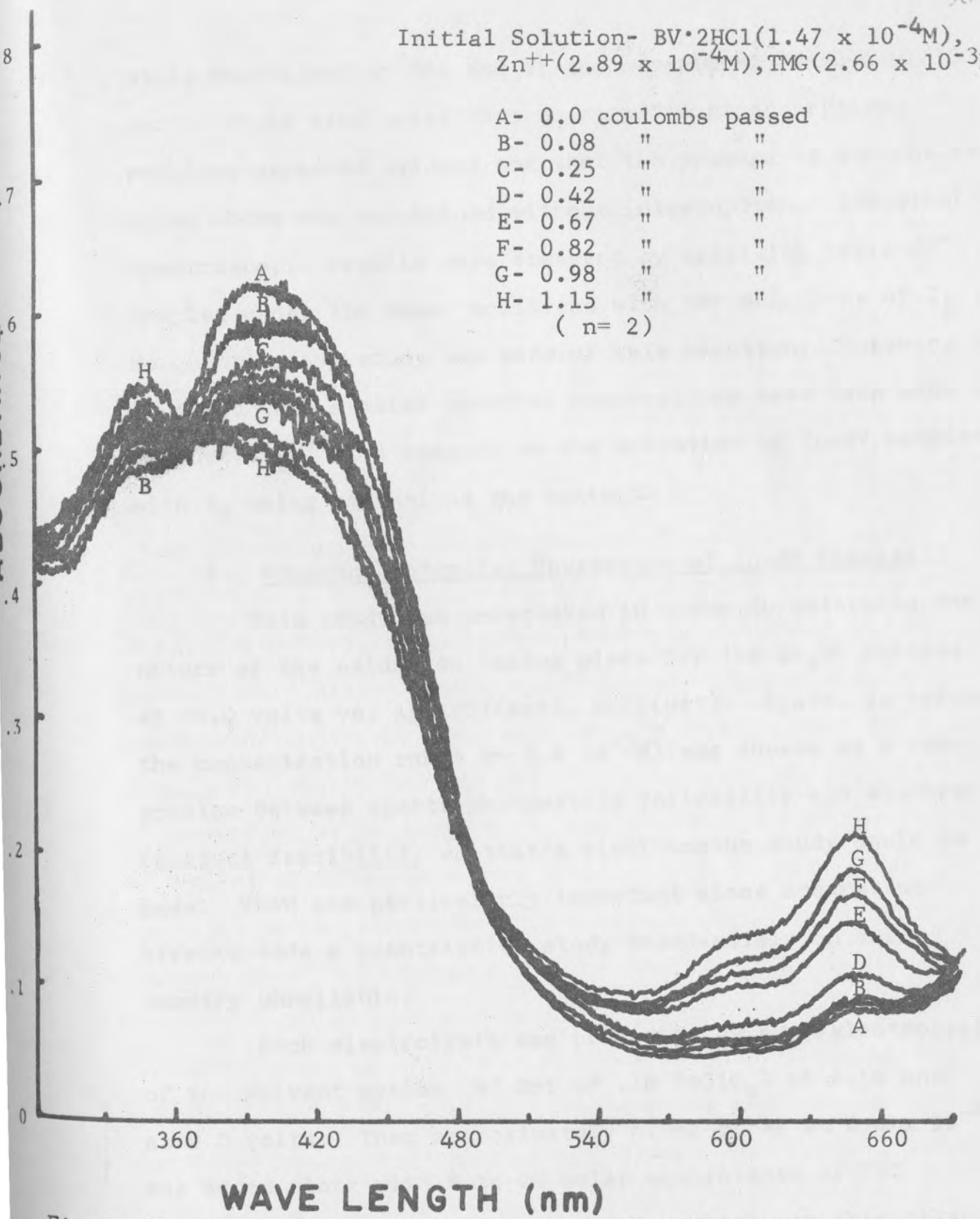


Figure 46. Spectrophotometric Changes During the Electrolysis of Zn-BV at +.10 volts vs Ag/AgCl(sat),NaCl(sat) electrode- Pt electrode, 2 mm. cell.

still maintained at the end of the experiment (~ .04coul/1000 sec). It is also noted that no significant adsorption problems appeared evident and that the passage of current as noted above was maintained without interruption. Identical spectroscopic results were observed by oxidizing the $ZnBV^-$ complex under the same conditions with DMF solutions of I_2. No quantitative study was made of this reaction. However, it is noted that similar spectral observations have been made by Gray et al.[10] with respect to the oxidation of Zn-BV complexes with I_2 using ethanol as the solvent.

F. Constant Potential Coulometry of Zn-BR Complex

This study was undertaken in order to determine the nature of the oxidation taking place for the Zn_2BR complex at +0.0 volts vs. Ag/AgCl(sat), NaCl(sat). Again, as before, the concentration range (~ 8×10^{-5}M) was chosen as a compromise between spectrophotometric reliability and electrochemical feasibility so that a simultaneous study could be made. This was particularly important since adsorption effects made a quantitative study based solely on voltammetry unreliable.

Each electrolysis was preceeded by a preelectrolysis of the solvent system (45.0ml of .1M $NaClO_4$) at +.5v and at 0.0 volts. Then approximately 6.0ml of BR at 6.0×10^{-4}M was added along with 8 to 24 molar equivalents of TMG (~ 2×10^{-2}M). A background of this system was then taken at 0.0 volts and proved to be negligible (~ .002coul/1000sec). This was followed by a spectrophotometric titration of the

solution with Zn^{++} ($\sim 1 \times 10^{-2}M$) which resulted in a maximum complex formation each time between 2 to 2.5 calculated molar equivalents of Zn^{++} to BR. The absorbance for the complex (530nm) always exceeded the BR absorbance (464nm) by 5-15%. Independent background electrolysis of Zn-TMG mixtures at 0.0v showed a negligible background 'cathodic' current. Initial voltammograms started at -.2v and swept positively always showed two weakly defined waves for the Zn_2BR solution, one maximized at between -.5 to 0 volts and another at +.1 to .2v.

Electrolysis was then initiated using a large Pt foil electrode at 0.0v and the progress of the electrolysis followed spectrophotometrically at intervals of .1 to .2 coulombs passed. At these intervals approximately .4ml samples were removed and the spectra taken (2mm path length) over the visible region. After the initial spectra, approximately 10-20ul of a decomplexing agent was added (2,4-pentanedione, diethylenetriamine, or ethylenediamine) directly to the cell and the spectra taken of the uncomplexed BR. 2,4-Pentanedione appeared to give the best results since the other two reagents appeared to leave a turbidity or precipitate after addition. In some of the experiments enough sample was removed to fill a 1cm cell and follow the region 600-700nm under magnified conditions to observe changes in the region of the weakly absorbing verdinoid product. Thus, three parameters were concurrently followed in order to elucidate the chemical changes taking place. Although adsorption did occur at the

Pt electrode and did reduce the current to about 1/2 its optimum value, the effect did not appear nearly as prohibitive as noted earlier in the electrolysis with BR in the presence of base and improved considerably during the course of the electrolysis. The Pt electrode was wiped with a "Kimwipe" after each sample was taken.

Figure 47Ashows the spectroscopic changes observed during the course of a typical electrolysis. Significant oxidation of Zn_2BR complex is noted and verdinoid formation with near isosbestic behavior is observed in the region 580-700nm., and increased absorbance at 400nm. However, near exhaustive electrolysis at 0.0 volts does not lead to the spectra expected for Zn-BV complex (the dotted line in Figure 41 shows the spectra of Zn-BV complex at 6.0×10^{-5}M) in the region 360 to 580nm even though nearly enough current has been passed ($.7 \times 10^{-6}$ faradays) for a two-electron oxidation. The current at this point is passing too slowly to be carried further at 0.0 volts ($\sim 10^{-8}$ faradays per 1000 seconds). It should be noted that if any significant quantity of BR is present at this point that its absorbance under these basic conditions should virtually bury any features attributable to $Zn\text{-}BV^-$ absorbance in the region 400nm to 580nm. Also, it should be noted that BR will not oxidize at this low potential even in the presence of excess base. Further complicating the situation is the fact that the Zn_2BR complex will revert to uncomplexed BR readily if the proper BR:Zn:TMG ratios are not maintained. This of course is impossible under the conditions of electrolysis.

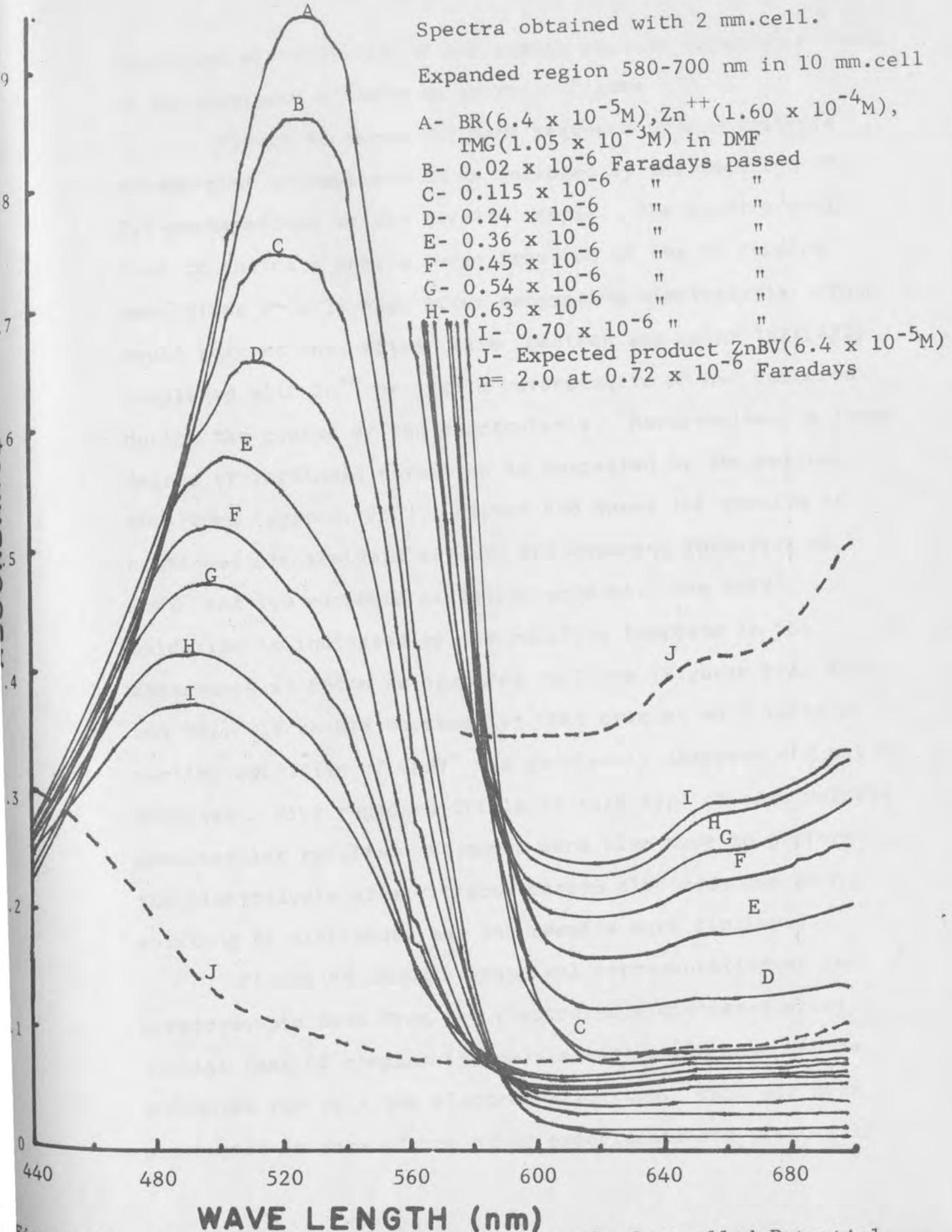


Figure 47A. Spectral Changes Occurring during the Controlled Potential

Continued electrolysis of the sample at +.5v apparently leads to an undefined mixture as shown in Figure 47B.

Figure 48 shows the same sequence of electrolysis except that uncomplexed BR is followed by the addition of 2,4-pentanedione to the cuvette sample. The spectra would tend to indicate that a large fraction of the BR remains unoxidized ($\sim 1/3$) even after exhaustive electrolysis. This would suggest that either this fraction was never initially complexed with Zn^{++} or that a reversion to BR had occurred during the course of the electrolysis. Nevertheless, a large degree of verdinoid formation is suggested by the region 600-700nm (approx. 2/3). Figure 48B shows the results of continued electrolysis at +.5v and apparent formation of $ZnBV^-$ and its purpurin oxidation product. The $ZnBV^-$ oxidation is indicated by the relative increase in the absorbance at 650nm as compared to 700nm (Figures 47A, 47B, and 46). It should be recalled that even at +0.0 volts a partial oxidation of $ZnBV^-$ was previously observed and may be expected. Five repeated trials at this type of electrolysis gave similar results. Attempts were also made to perform the electrolysis at a vitreous carbon electrode and at a rotating Pt electrode, but the results were similar.

Figure 49 shows a graphical representation of the spectroscopic data from the electrolysis discussed above. Initial loss of complex (530nm) is more rapid than can be accounted for by a two electron oxidation. This was more pronounced in some of the other experiments and presumably

K- 0.73 x 10^{-6} Faradays passed
L- 0.83 x 10^{-6} " "
M- 0.96 x 10^{-6} " "
N- 1.11 x 10^{-6} " "
O- 1.55 x 10^{-6} " "

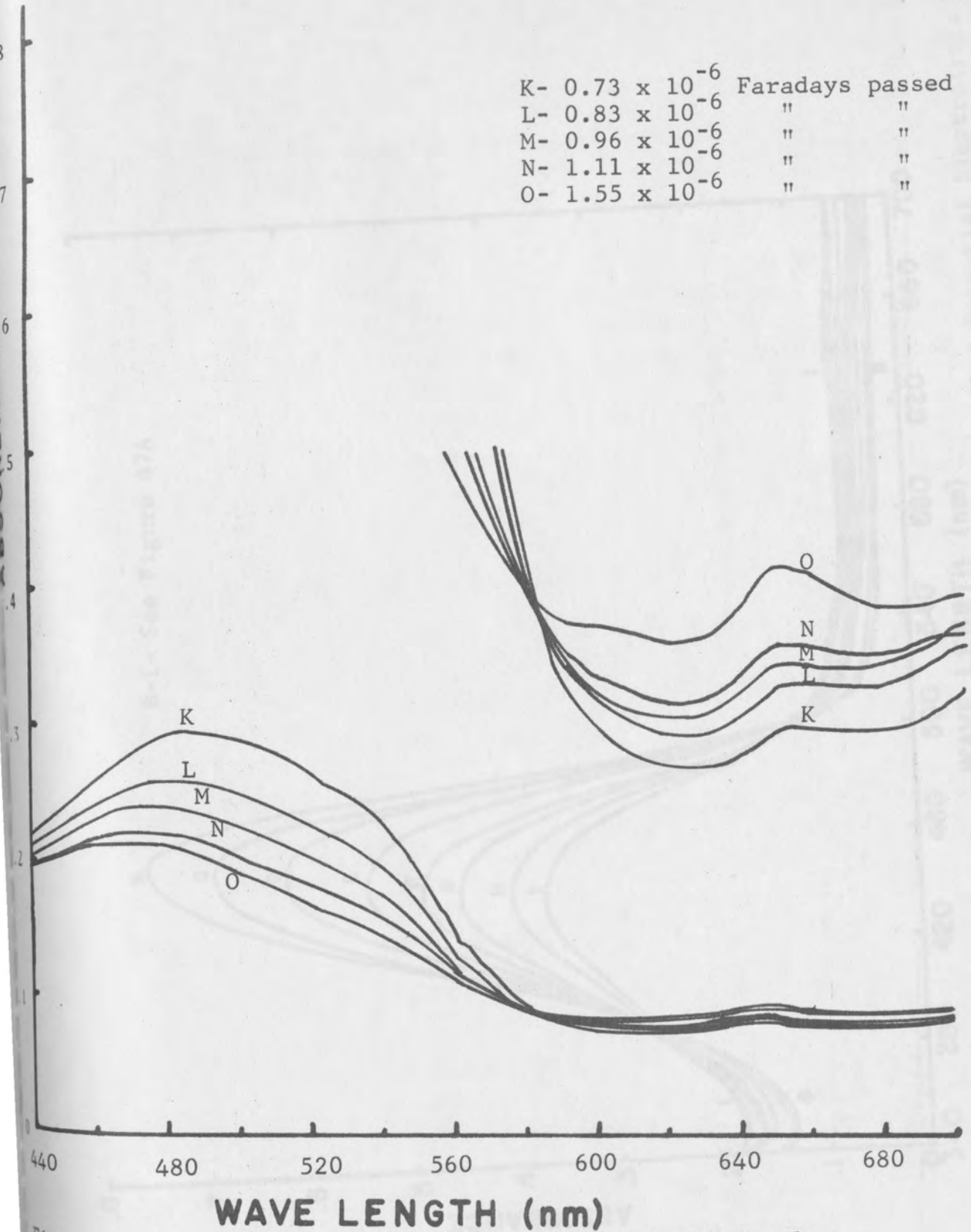


Figure 47 B. Continuation, Potential Changed to +0.5 volts.

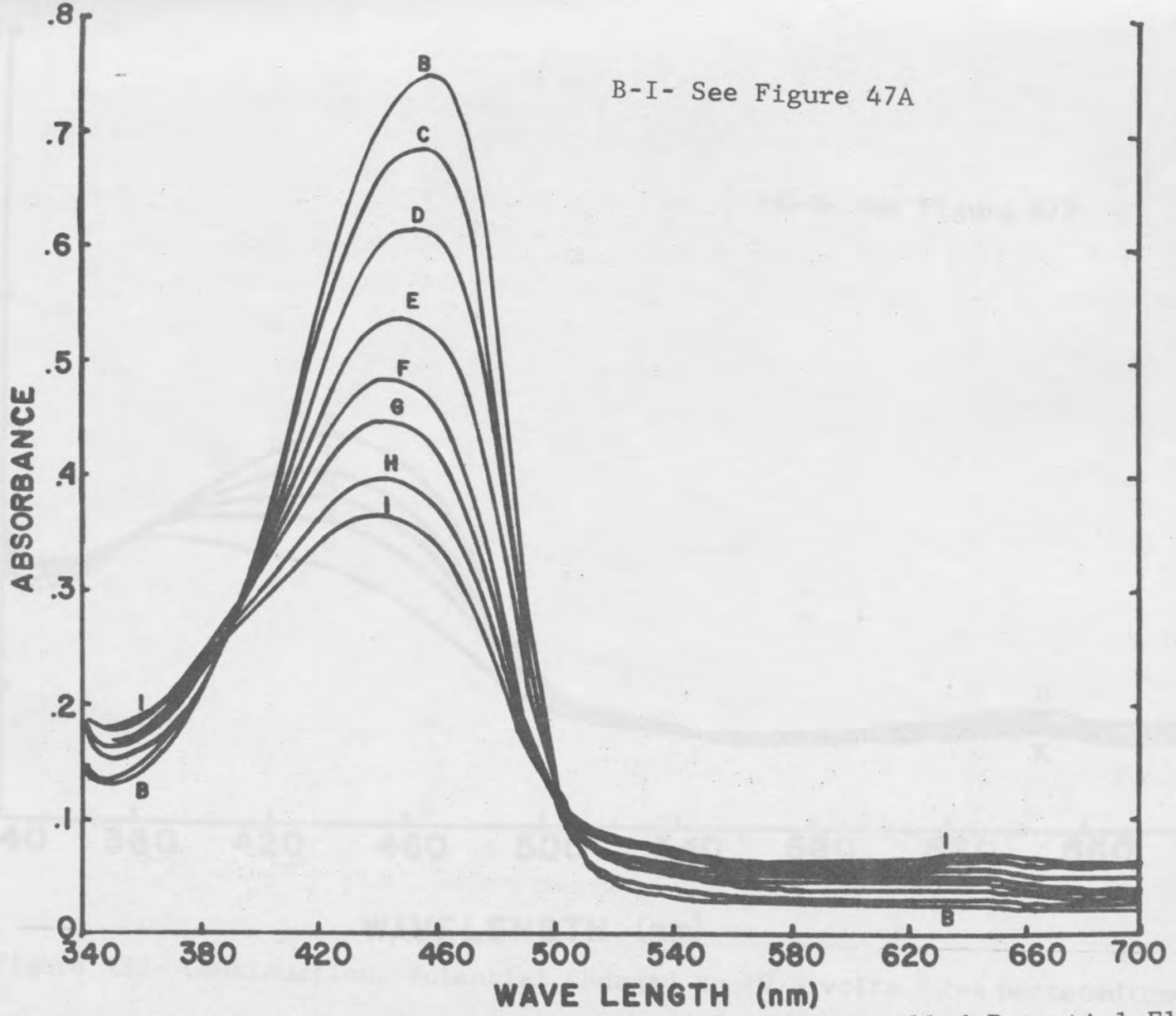


Figure 48A- Spectral Changes Occurring during the Controlled Potential Electrolysis of Zn_2BR at 0.00 volts, after addition of 2-4pentanedione.

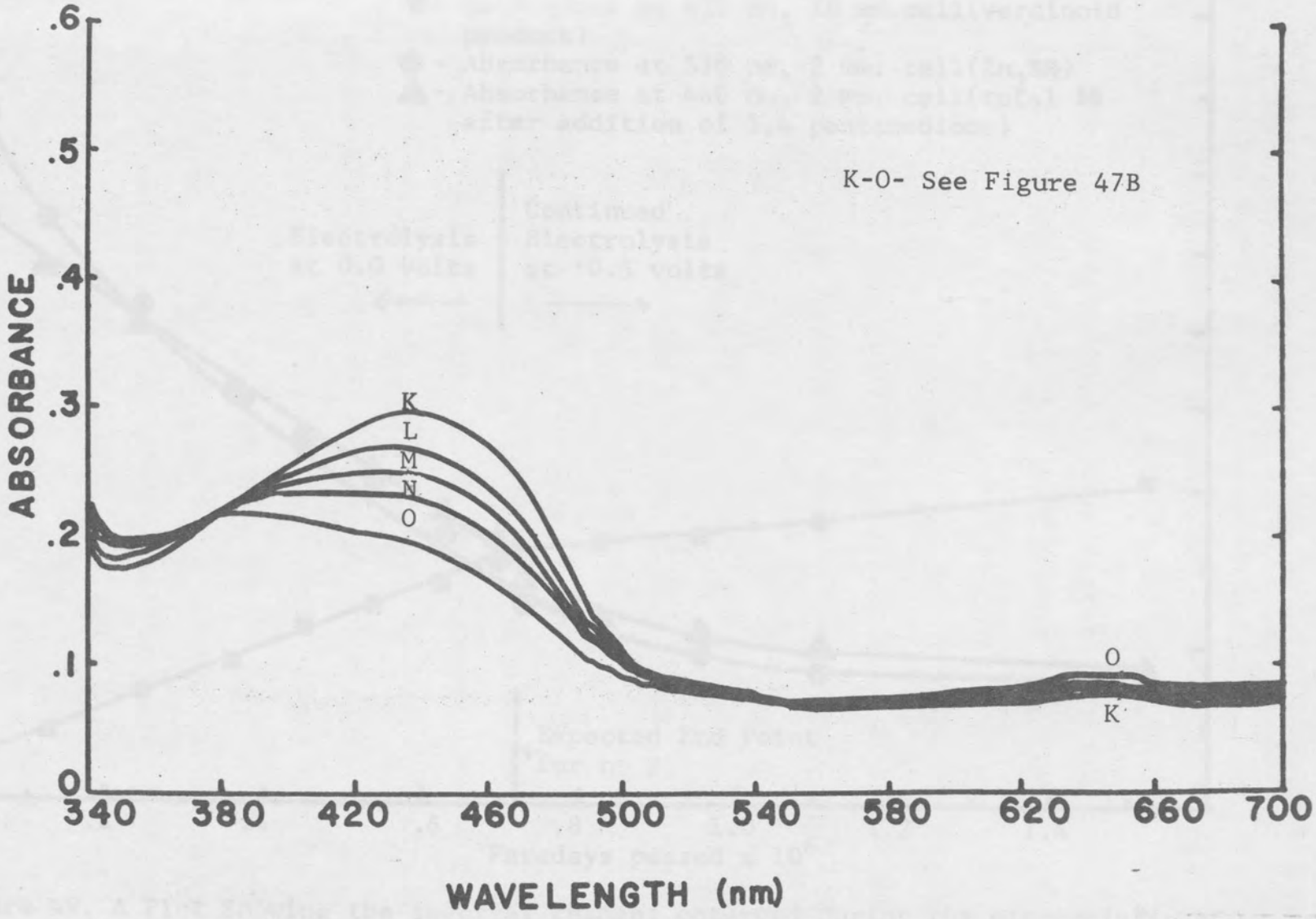


Figure 48B- Continuation, Potential Changed to +0.5 volts, 2-4 pentanedione added.

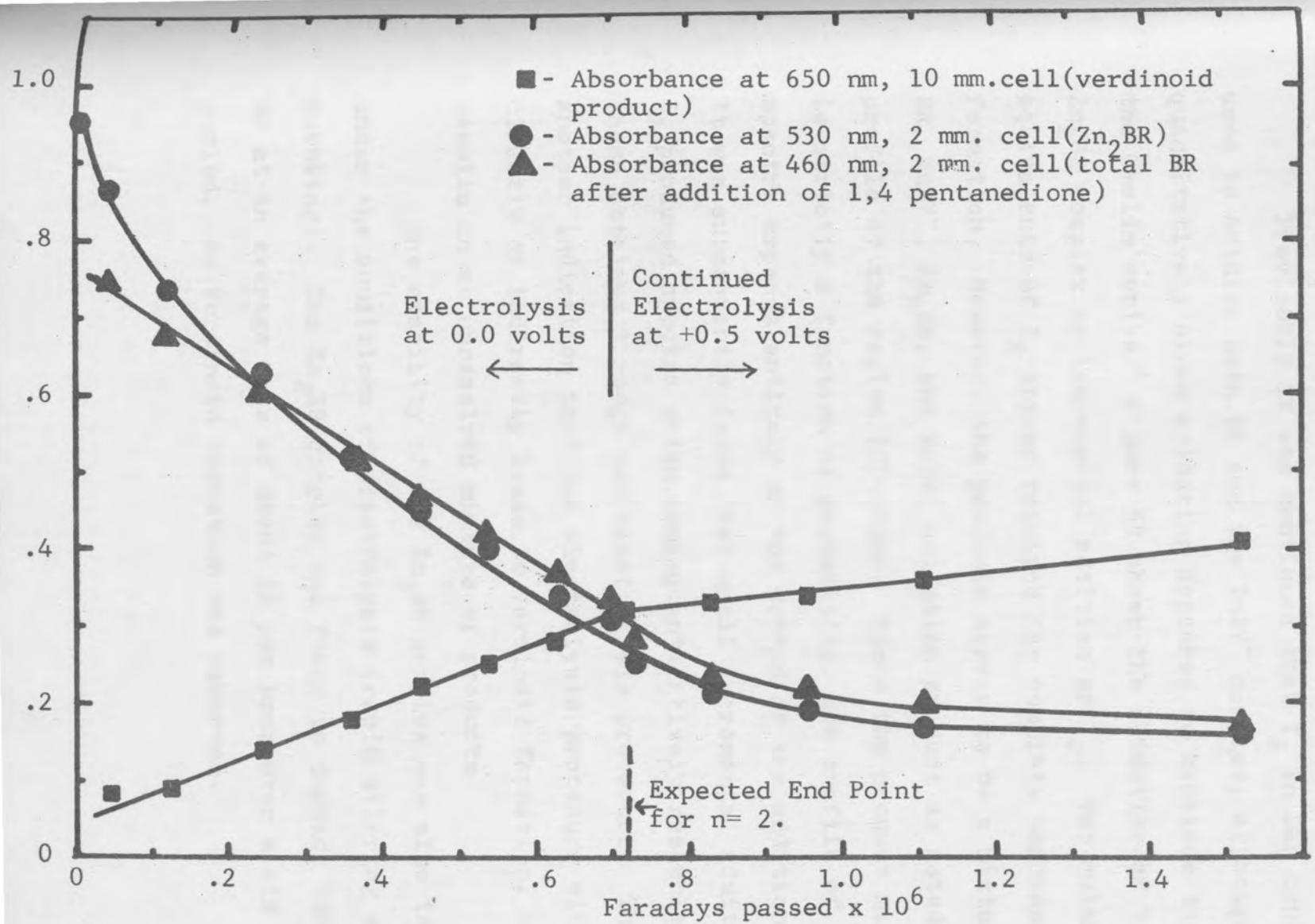


Figure 49. A Plot Showing the spectral changes observed during the electrolysis of the Zn_2BR complex as shown in Figures 47A,47B,48A and 48B.

represents a reversion of complex to BR. Concurrently, total BR and BV appear to linearly decrease and increase respectively during the course of the electrolysis.

Previously it was mentioned that I_2 in DMF could be used to oxidize both BR and the $ZnBV^-$ complex, although non-quantitatively since oxidation appeared to continue through the Gmelin series. Figure 50 shows the oxidation of the Zn_2BR complex by incremental addition of I_2. Two molar equivalents of I_2 appear required for complete verdinoid formation. However, the products appear to be a mixture of BR, $ZnBV^-$, Zn_2BR, and $ZnBV^-$ oxidation product as noted by the profile of the region 600-700nm. Since the product mixture is strictly a function of probability, the profile of the spectra depends entirely on the method of the addition of I_2. It was subsequently found that small incremental additions of I_2 produced spectra which semi-quantitatively resembled those obtained through the electrolysis procedure. This is another indication that the electrolysis procedure either directly or indirectly leads to verdinoid formation, but also results in an unresolved mixture of products.

The stability of the Zn_2BR complex was also tested under the conditions of electrolysis (rapid stirring and bubbling). The Zn_2BR complex was found to degrade back to BR at an average rate of about 2% per hour over a six hour period. No verdinoid formation was observed.

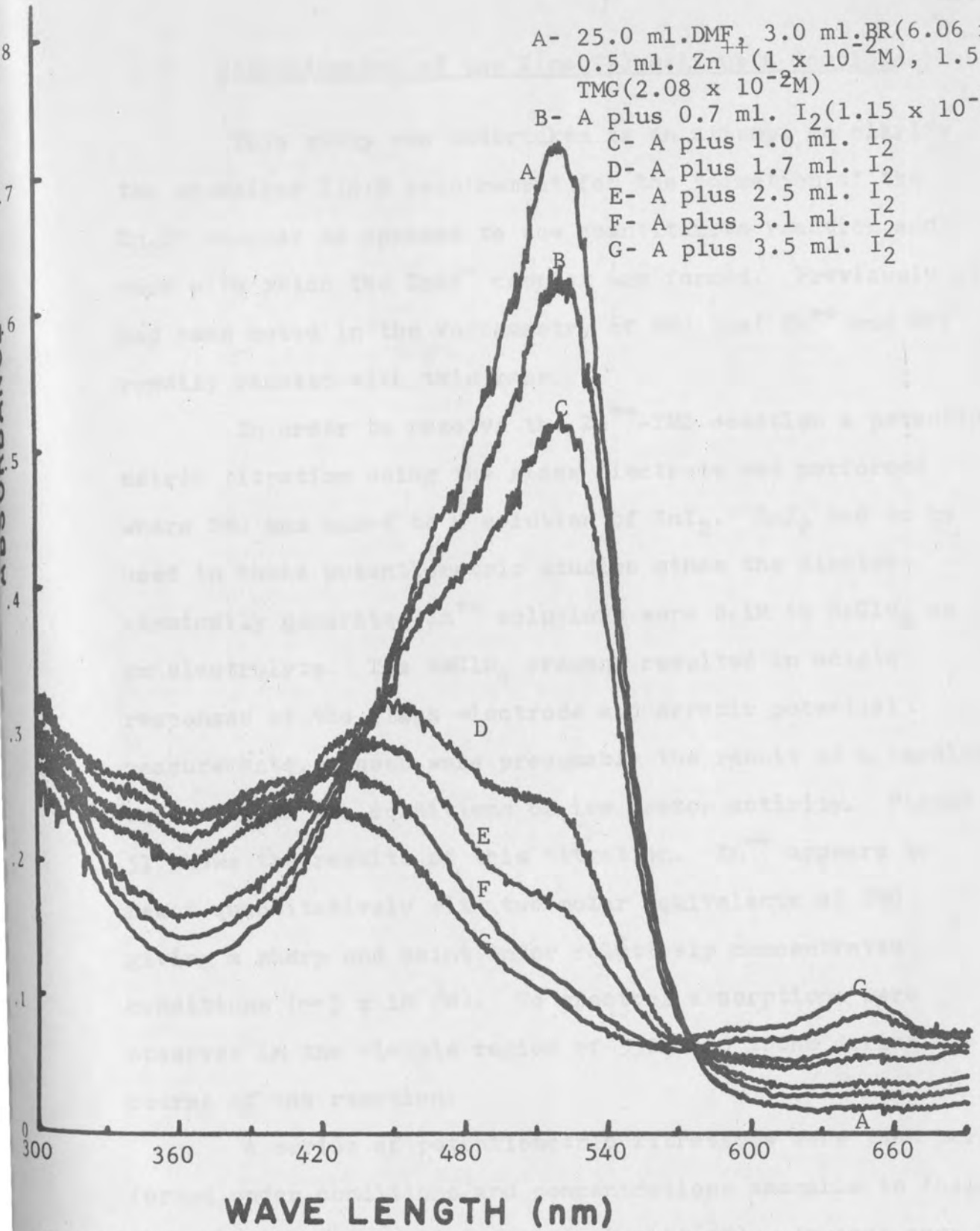


Figure 50. Spectrophotometric Titration of zinc-bilirubin complex with I_2 in DMF- 2 mm. cell.

G. Potentiometry of the Zinc(II)-Bilirubin Complex

This study was undertaken as an attempt to clarify the anomalous 1:2:8 requirement for the formation of the Zn_2BR complex as opposed to the quantitative reaction and ease with which the $ZnBV^-$ complex was formed. Previously it had been noted in the voltammetry of TMG that Zn^{++} and HCl readily reacted with this base.

In order to resolve the Zn^{++}-TMG reaction a potentiometric titration using the glass electrode was performed where TMG was added to a solution of ZnI_2. ZnI_2 had to be used in these potentiometric studies since the electrochemically generated Zn^{++} solutions were 0.1M in $NaClO_4$ as an electrolyte. The $NaClO_4$ present resulted in acidic responses of the glass electrode and erratic potential measurements. These were presumably the result of a 'sodium error' under the conditions of low proton activity. Figure 51 shows the results of this titration. Zn^{++} appears to react quantitatively with two molar equivalents of TMG giving a sharp end point under relatively concentrated conditions ($\sim 5 \times 10^{-3}$M). No spectral absorptions were observed in the visible region of 350nm to 700nm during the course of the reaction.

A series of potentiometric titrations were then performed under conditions and concentrations amenable to following BR spectrophotometrically ($\sim 8 \times 10^{-5}$M). In each case 3.0 ml of BR (6.0×10^{-4}M) was added to 25.0ml of freshly distilled DMF followed by the addition of varying molar equivalents of

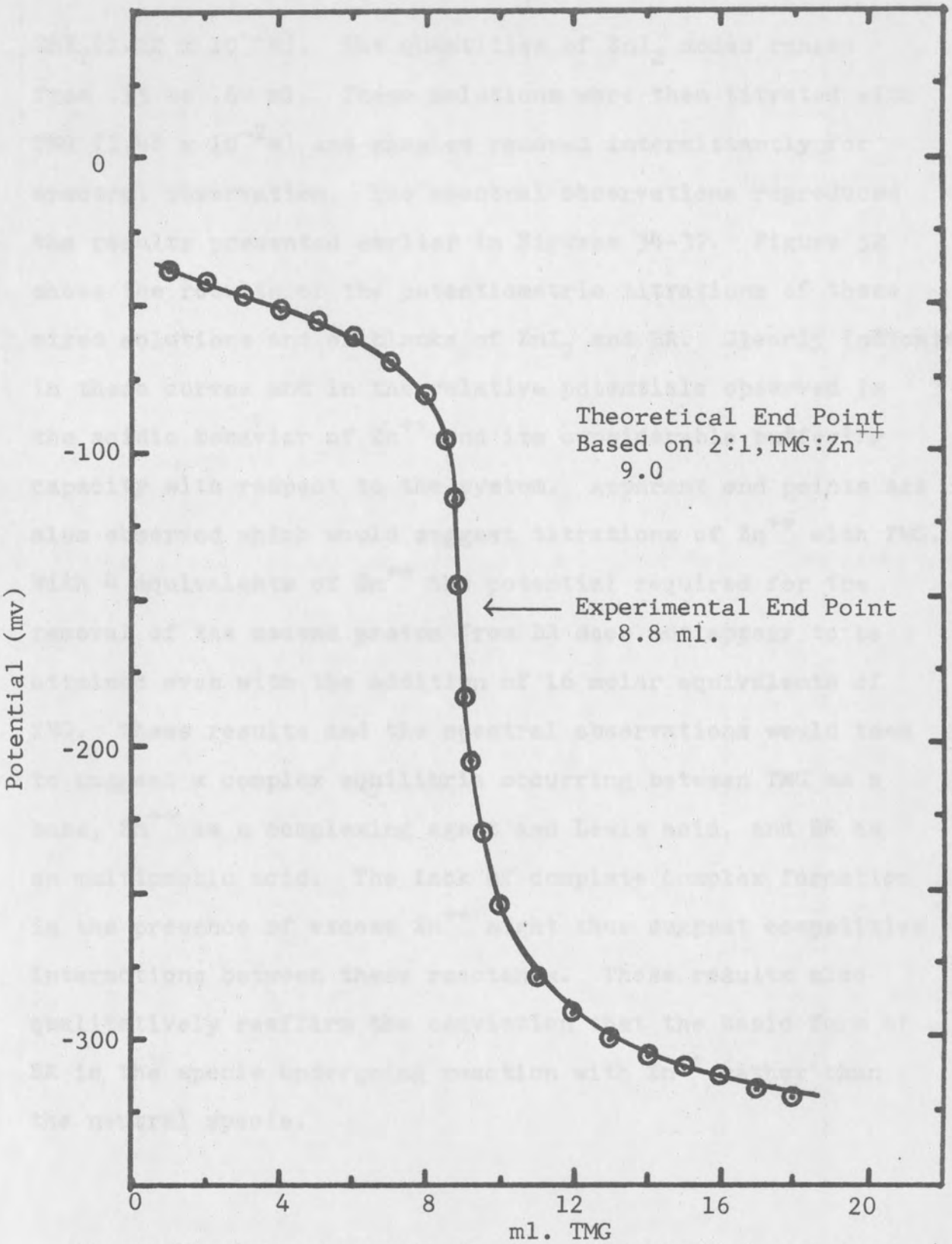


Figure 51. Potentiometric Titration of 20.0 ml. ZnI_2(4.7×10^{-3}M) with TMG(2.08×10^{-2}M)-Indicator Electrode, Glass Electrode; - - - Reference Electrode, Ag/AgCl(sat);NaCl(sat).

ZnI_2(1.22×10^{-2}M). The quantities of ZnI_2 added ranged from .15 to .60 ml. These solutions were then titrated with TMG (1.48×10^{-2}M) and samples removed intermittantly for spectral observation. The spectral observations reproduced the results presented earlier in Figures 34-37. Figure 52 shows the results of the potentiometric titrations of these mixed solutions and of blanks of ZnI_2 and BR. Clearly indicated in these curves and in the relative potentials observed is the acidic behavior of Zn^{++} and its considerable buffering capacity with respect to the system. Apparent end points are also observed which would suggest titrations of Zn^{++} with TMG. With 4 equivalents of Zn^{++} the potential required for the removal of the second proton from BR does not appear to be attained even with the addition of 16 molar equivalents of TMG. These results and the spectral observations would tend to suggest a complex equilibria occurring between TMG as a base, Zn^{++} as a complexing agent and Lewis acid, and BR as an multiprotic acid. The lack of complete complex formation in the presence of excess Zn^{++} might thus suggest competitive interactions between these reactants. These results also qualitatively reaffirm the conviction that the basic form of BR is the specie undergoing reaction with Zn^{++} rather than the neutral specie.

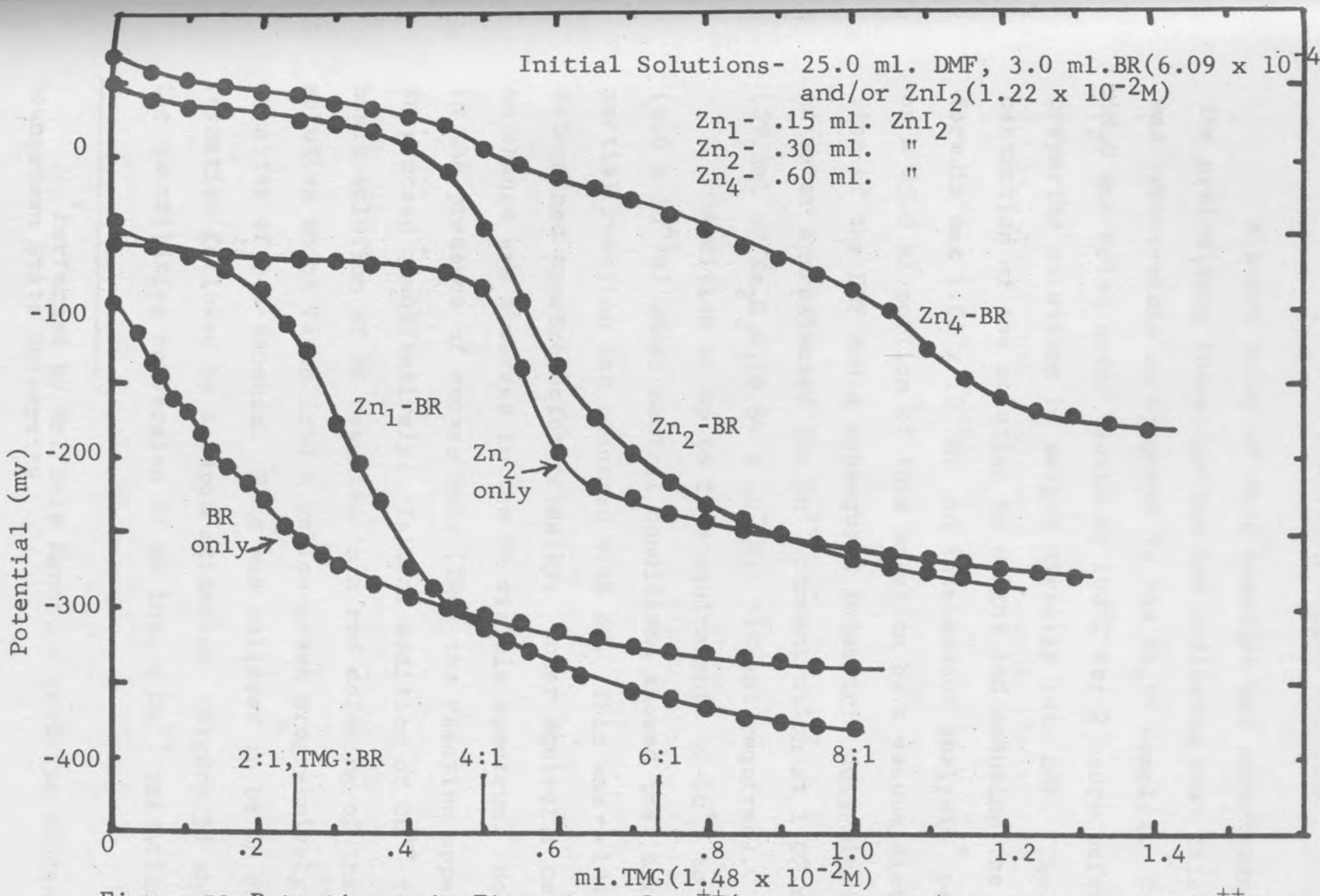


Figure 52. Potentiometric Titration of Zn^{++}(added as ZnI_2), bilirubin, and Zn^{++}/BR solutions with TMG in DMF-Indicator Electrode, Glass Electrode; Reference Electrode, Ag/AgCl(sat), NaCl(sat).

H. The Bilirubin-Copper Reaction

A short study of this reaction was undertaken since the preliminary investigation had indicated that this reaction was irreversible as compared to the Zn_2BR complex. $Cu(ClO_4)_2 \cdot 6H_2O$ was dried under vacuum at 100°C for 2 hours before preparing solutions by weight directly into DMF. The concentration of the solution by weight and assuming the above formula was 1.01×10^{-2}M. An independent analysis* performed on a 25.0 ml portion of this solution by a vacuum distillation of the DMF and a subsequent iodometric determination[43] in water approximated the Cu^{++} concentration at 1.07×10^{-2}M (27.2ml of $Na_2S_2O_3$(9.84×10^{-3}M) titrant required).

Addition of up to five equivalents of Cu^{++} to BR (6.0×10^{-5}M) under neutral conditions showed that only a partial reaction had occurred with BR. This was ~10% as determined spectrophotometrically. Under equimolar conditions no change was observed in the BR visible spectrum. However, in the presence of excess base (TMG) the reaction appeared to proceed quantitatively. Initial addition of Cu^{++} to the basic solution of BR resulted in a red coloring of the solution which faded into a yellow-green progressively within a matter of 3-4 seconds. This was believed to be BR complex formation followed by a rapid oxidation. Figure 53 shows the quantitative conversion of BR into a Cu^{++} oxidation

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*Performed by Mr. Dale Manos, a graduate student at Youngstown State University.

product at approximately 435nm and a shoulder at 360nm. The absorbance at 435nm appears to be minimized at a Cu^{++}/BR ratio of 1:1. Addition of excess Cu^{++} results in a slight increase of the absorbance at 435nm and of the shoulder at 360 nm ($\sim$ .03 absorbance units). Thus an end point for the reaction is difficult to ascertain spectrophotometrically. Decomplexation of the product with excess 2,4-pentanedione or diethylenetriamine was unsuccessful. However, neutralization with HCl resulted in the apparent purpurin spectra as shown in Figure 48. That the reaction of Cu^{++} with BR indeed represents an oxidation is suggested by the reaction of BV with Cu^{++} under identical conditions. This reaction is shown in Figure 54 and indicates that the same product is formed. However, the anomolous result that only about .5 to .75 molar equivalents of Cu^{++} are required for an apparent spectrophotometric end point remains unresolved.

Attempts were made to determine a potentiometric end point for the reaction using a Pt indicator electrode for the Cu(II)-Cu(I) redox system.[44] However, no conclusive results were obtained. Qualitatively, it appears Cu^{++} undergoes a complexation with TMG as was the case with Zn^{++}. No further investigations were made into this system.

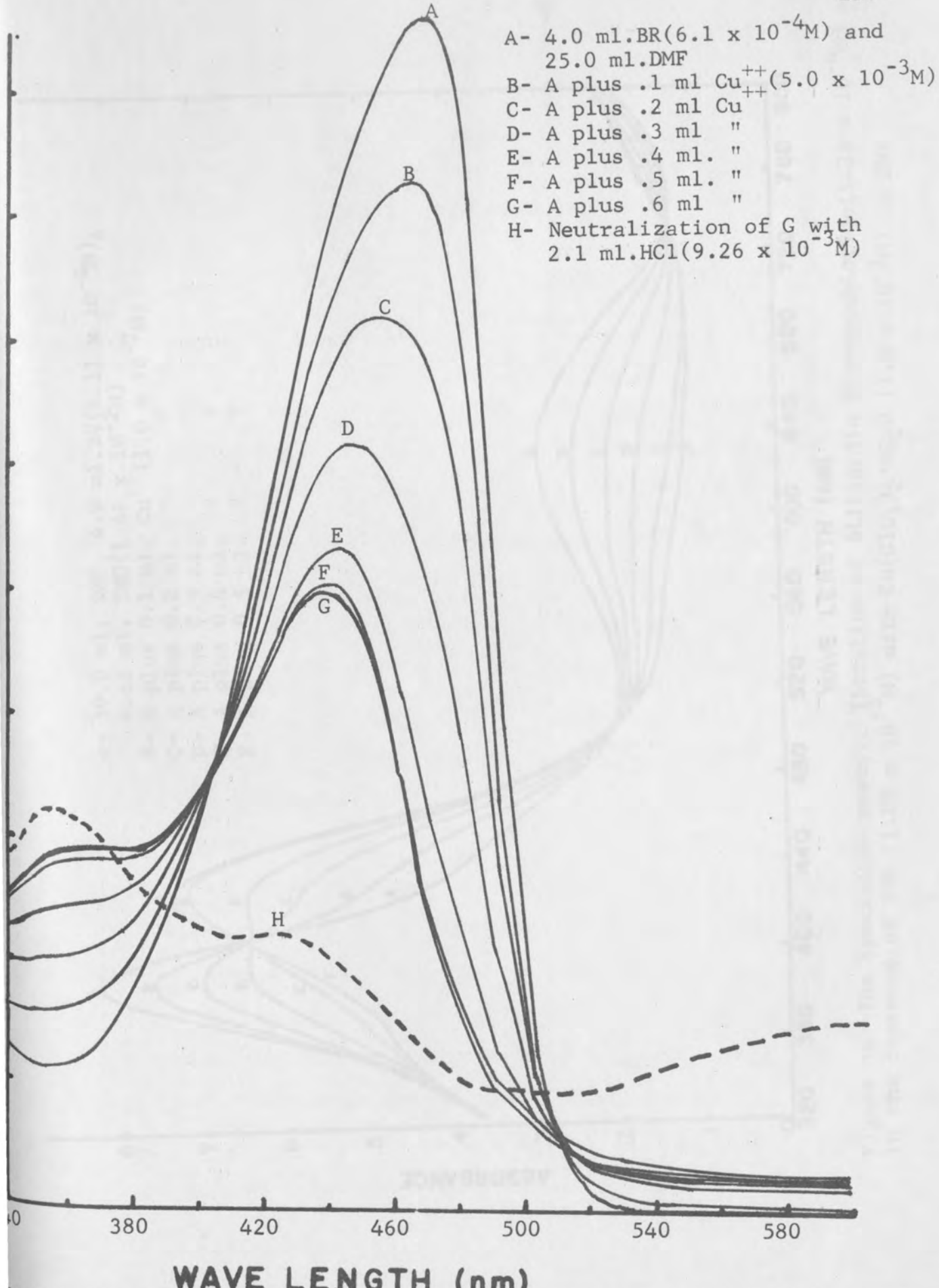

Figure 53. Spectrophotometric Titration of Bilirubin with Cu^{++}.

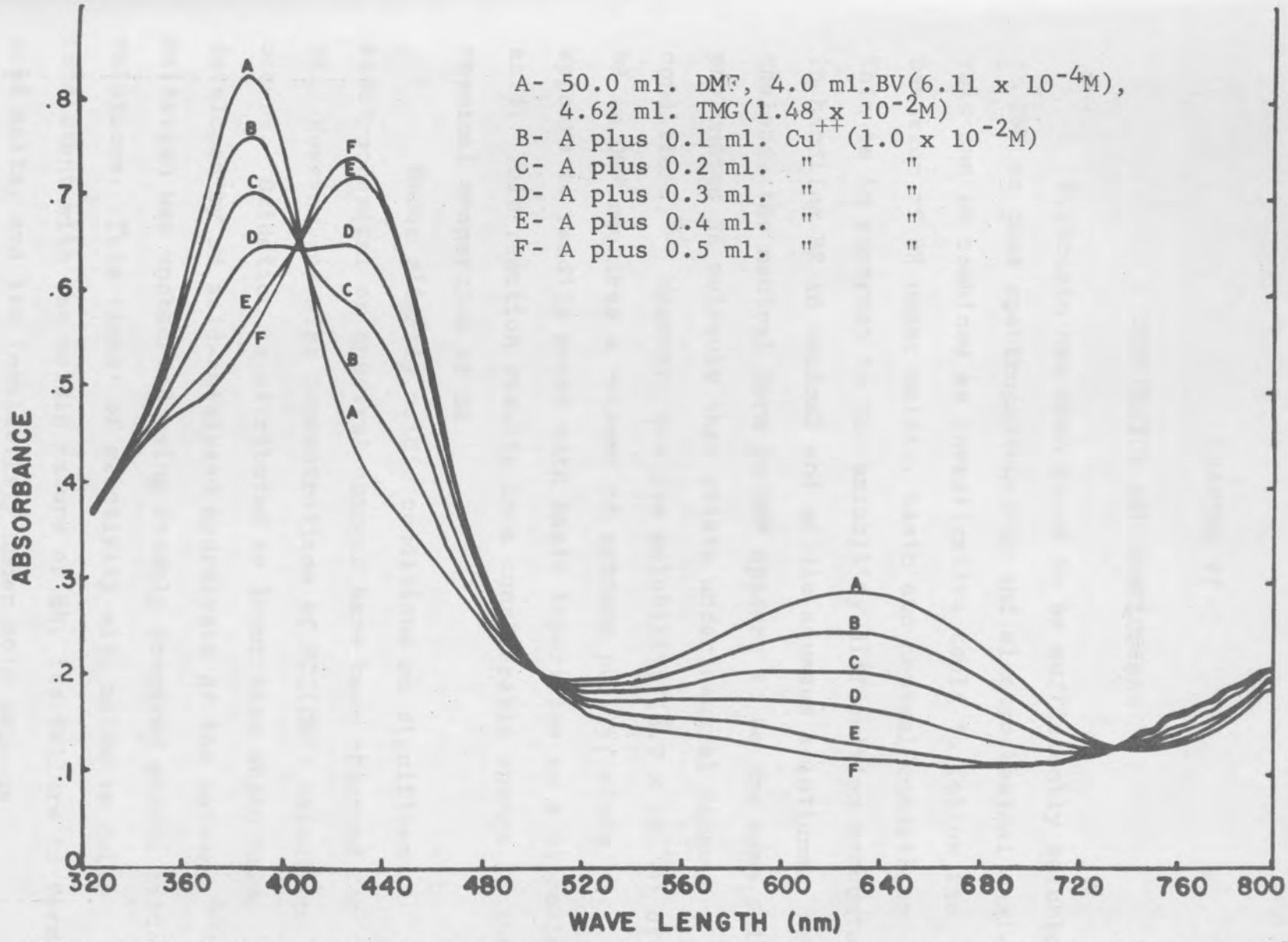

Figure 54- The Spectrophotometric Titration of Biliverdin Dihydrochloride(1.25 x 10^{-4}M) in the Presence of TMG (1.00 x 10^{-3}M) with $Cu(ClO_4)_2 \cdot 6H_2O$ (1.0 x 10^{-2}M) in DMF

CHAPTER VI

DISCUSSION AND CONCLUSIONS

Bilirubin has been found to be sufficiently soluble in DMF so that spectrophotometric and electrochemical analysis can be combined as investigative tools to follow the behavior of BR under acidic, basic and neutral conditions. This is in contrast to the solubility difficulties encountered in handling BR in neutral and acidic aqueous solutions. Nevertheless, the neutral form in DMF appears to be the same fully protonated BR molecule that exists under neutral aqueous conditions.[45] However, the low solubility (6.7×10^{-4}M) of BR in DMF requires a solvent of extreme purity, since BR appears to readily react with basic impurities as a diprotic acid. This reaction results in a considerable change in the chemical properties of BR.

Under slightly acidic conditions no significant electrochemical or spectral changes have been observed for BR. However, at high concentrations of HCl(DMF) oxidation occurs. Oxidation is attributed to impurities which have developed by an acid-catalyzed hydrolysis of the solvent.[40,41] Oxidation was unobserved using freshly prepared picric acid solutions. This 'lack' of reactivity with acids is fully consistent with the acidic nature of BR, its failure to form acid salts, and its insolubility under acid aqueous conditions.[42,46]

The quantitative behavior of BR as a diprotic acid has been followed potentiometrically, spectrophotometrically, and using oxidative voltammetry. These results suggest that under neutral solvent conditions BR exists in solution in its neutral form containing six protons and maintaining intramolecular hydrogen-bonding. The neutral form of BR appears inert to complexation with Zinc(II) or Copper(II) and maintains a relatively high oxidative potential of +.6v vs. the S.C.E. With the quantitative removal of two protons, presumed to be carboxylic acid protons, the oxidative potential drops to approximately +.3v vs. the S.C.E. However, the point of oxidation has been shown to still persist at the central methene bridge which oxidizes in a two-electron process to form the verdinoid product. The removal of two protons apparently disrupts intramolecular hydrogen-bonding (see Figure 3) and leads to steric changes and or inductive effects which leave the methene bridge more prone to oxidation. Removal of two protons also leads to the formation of a basic form of BR which readily reacts with Zinc(II) or Copper(II). The preliminary investigations indicated that BR can also react in DMF with Fe(III), Ni(II), and Co(II). It is believed that quantitative reactions of these metals with BR will also be dependent on the presence of base and the production of the basic form of BR.

The complexation of Zn^{++} with BR has been found to maximize at a BR:Zn:TMG ratio of 1:2:8. The large change in absorbance and wavelength and its isosbestic nature

suggests that complexation does indeed occur with the pyrrole nitrogens rather than simply due to a salt formation. This type of spectral behavior is similar, although not analogous, to the incorporation of Zn^{++} into the porphyrin molecule.[47] The ratio of 1:2:8 might be explained either as the formation of a Zn-BR complex of 1:1 ratio in competitive equilibrium with a $Zn(TMG)_2^{++}$ complex, or as a Zn_2BR complex which might be either a distinct complex or the result of molecular aggregation or polymerization. The effects of molecular aggregation of bilirubin molecules have been documented[48,46] and might be expected to occur in DMF. The following speculations might be presented in view of the experimental results.

1. Two protons must be removed from BR before it can react in complexation. These protons presumably come from the carboxylic acid groups.

$$BRH_2 \text{(neutral form)} + 2\ TMG = BR \text{ (basic form)} + 2TMGH^+$$

2. Reaction of Zn^{++} with BR results in the liberation of at least two more pyrrole protons and a complex formation which is analogous to metalloporphyrins. This might in part explain the acidic behavior of Zn^{++} and the reversion of a large fraction of free $BR^=$ to the neutral form.

$$2TMG + BR^= + Zn^{++} = ZnBR^= \text{ (bis-lactim complex)} + 2TMGH^+$$

or

$$4TMG + BR^= + Zn^{++} = ZnBR^{4-} \text{(bis-lactam complex)} + 4TMGH^+$$

$$2TMGH^+ + BR^= = BRH_2 + 2TMG$$

The anion complexes might be stabilized by anion-cation pair formation.

3. The acidic behavior of Zn^{++} might also be explained in terms of complexation of Zn^{++} with TMG.

$$BR^{=} + Zn^{++} + 2TMGH^{+} = Zn(TMG)_2^{++} + BRH_2$$

4. The Zn-BR complex may actually be a molecular aggregate of BR:Zn:TMG in a ratio of 1:2:4. In this complex one Zn^{++} might complex as a metalloporphyrin, another as the carboxylate salt complex, and four TMG may fullfill the octahedral requirements of both Zn^{++} ions in DMF, thus forming a neutral complex.

$$2\ Zn^{++} + 8TMG + BRH_2 = Zn_2BR\cdot 4TMG + 4TMGH^{+}$$

More information is required before the exact nature of the complex can be established.

The oxidative potential of the Zn_2BR complex is approximately -0.1v vs. the S.C.E. and shows a considerable change from that for the neutral and basic forms of BR. The experimental results suggest that oxidation proceeds by the same two-electron mechanism which leads to verdinoid formation as in the other cases. However, this conjecture has still not been firmly established. This progressive ease in oxidation of the neutral, basic, and complex forms of BR is fully consistent with the chemical reactivity observed for the above species in various solvents and is analogous to their reactivity in water.

same methods used in this work to the bilirubin esters, methoxides, glucuronides and mesobilirubins. Although NMR work in DMF might be precluded due to the low solubility of BR, dimethylsulfoxide has already been shown to be a viable solvent for this type of study. The neutral form of BR in this solvent system has also been shown to be the fully protonated form. Infrared work also would be most informative. However, the problems of crystallization and purification of the compound and complexes from DMF would have to be resolved before such work could be initiated. A great deal of information concerning the reductive processes involved in the systems studied in this work might also be investigated utilizing the polarographic techniques already well established in this solvent system.

Zinc(II) complexation with biliverdin dihydrochloride is quantitative and appears to be near complete at a Zn:(BV·2HCl):TMG ratio of 1:1:5. This ratio suggests that complexation follows with the removal of two hydrochloride protons, two acid protons, and one pyrrole proton to form a stable bis-lactim BV complex.

$$Zn^{++} + BV\cdot 2HCl + 5TMG = 5TMGH^{+} + 2Cl^{-} + ZnBV^{-}$$

The spectra and relative ease of $ZnBV^{-}$ complex formation are fully consistent with previous observations by Gray et al. in ethanolic zinc acetate.[10] The electrochemical behavior of BV and its complex is qualitatively similar to that of BR although it has not been quantitatively elucidated.

Copper appears to complex with BR under basic conditions and catalyze an immediate oxidation to form a complex which is either the Cu-BV complex or a purpurin complex. The behavior observed spectroscopically in DMF is analogous to the observations of Velapoldi and Menis[5] for the reaction of BR with Copper (II) in a chloroform: methanol (1:1) mixture. Their explanation for this type of oxidation relates to the formation of a square planar BR complex which results in a strain at the already labile methene bridge. This argument may also be qualitatively extended to explain the relative ease of oxidation of the Zn_2BR complex.

The elucidation of the structural changes occurring with BR under conditions of base and complexation with Zn^{++} and Cu^{++} could be further developed by a study of BR analogues in DMF. This might involve the application of the

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