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I am submitting herewith a thesis written by Gina Ann Stanley entitled "The Electrochemical Characterization of Ethidium Bromide." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.

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and Dean of the Graduate School

The Electrochemical Characterization of Ethidium Bromide

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Gina Ann Stanley

December 1991

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. James Q. Chambers, for his guidance and patience. I would like to express gratitude to my parents for their love and support throughout my college education. I would like to thank everyone at the University of Tennessee for their support: Beverly and Dave Swaile, the Sepaniak group, Patti Kreke, Thomas Blanchard, Yingbo Guo, and Costas Tsintavis. A special thanks to Rikki Waterhouse is extended for giving me a place to reside and to my husband Bill for understanding during trying times.

ABSTRACT

Electrochemical Characterization of ethidium bromide (2,7-diamino-9-phenyl-10-ethylphenanthridinium bromide) is described. The voltammetric and polarographic behavior of ethidium bromide was compared with that of a similar compound, triphosphopyridine nucleotide sodium salt hydrate.

Voltammetric and spectroelectrochemical studies have been employed on the oxidation of ethidium bromide by utilizing the polymer Nafion as a thin film on the surface of a platinum electrode. Nafion was to be used as an easy less dangerous method of dealing with this harmful mutagen. The ethidium cation was found to be electroinactive through spectroelectrochemistry under the experimental conditions employed.

Polarographic (normal pulse and differential pulse) and voltammetric studies have been carried out on the reduction of ethidium bromide with the use of mercury electrodes. The effects of pH and concentration were observed with ethidium and NAD+ (under the same experimental parameters). Upon comparison with NAD+, a two-electron, irreversible redox scheme was suggested to exist. When dealing with voltammetry, some adsorption controlled phenomenon was observed that was not seen with polarography.

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I. INTRODUCTION

A. Research Purpose.

Ethidium bromide is an aromatic intercalating dye (intercalation as used here is binding to DNA by insertion between base pairs); the display of intercalation has been the subject of many studies to explain biological processes of interest(1-5). Ethidium bromide has not been researched to the fullest capacity. Characterization of the electrochemistry of ethidium bromide to develop a better understanding of ethidium bromide and to increase possible applications of ethidium bromide were the goals of the present study.

Nafion, employed in this work to minimize the dangers of ethidium, is a perfluorinated ion-exchange polymer which can be applied as a thin film to an electrode. The one disadvantage of Nafion is its expense. However, Nafion films have proven effective for several compounds with similar characteristics to ethidium bromide (6). The use of Nafion could possibly make any previous work with ethidium more applicable.

Triphosphopyridine nucleotide sodium salt hydrate, NAD+ (an analogous compound of ethidium bromide in structure and ionic form), was used to suggest a possible redox scheme by comparing the known initial one-electron

reduction of NAD+ to the reduction of the ethidium ion. Several studies of NAD+ have analyzed the redox process by examining differential pulse polarograms (7-10). As a result of these studies, the initial one-electron transfer results in a radical ($NAD^{\cdot}$) which dimerizes to result in an overall two-electron exchange(11). The redox process of this nontoxic derivative of the alkaloid nicotine occurs in the nicotinamide ring(8).

Electrochemical characterization of ethidium bromide can be helpful in understanding the biological effects of intercalation. Intercalation enhances the fluorescence of ethidium, to inhibit DNA reproduction and to block viral replication, all of which are very interesting biologically.

B. Electrochemical Techniques

1. Cyclic Voltammetry

Perhaps the most effective electroanalytical tool available for the mechanistic study of redox systems (12), cyclic voltammetry, employs a triangular pulse which yields information on current-potential dependence, Figure 1. Parameters of interest in a cyclic voltammogram include: (1) the peak potentials, Ep anodic and Ep cathodic, and (2) the peak currents, Ip anodic and Ip cathodic, as illustrated in Figure 2. These parameters can be used to predict the reversibility and irreversibility of a system, Figure 3.

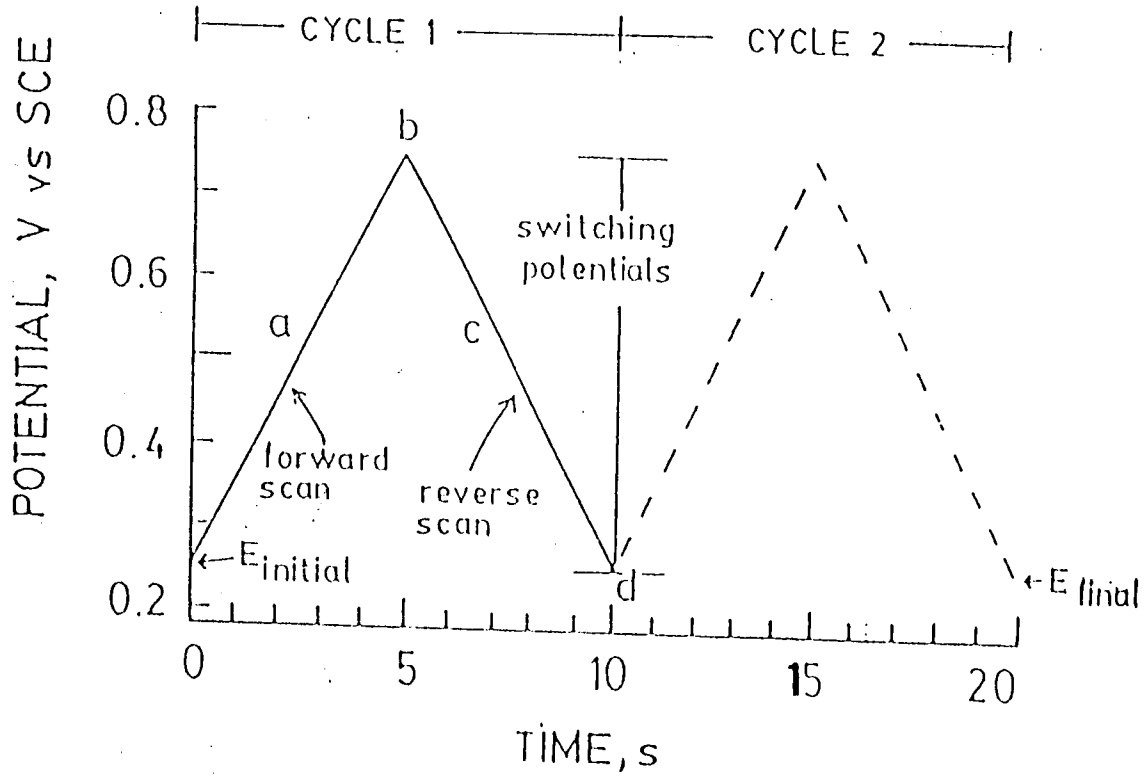


Figure 1. Typical potential-time excitation signal for cyclic voltammetry. (From Peter T. Kissinger and William R. Heineman Laboratory Techniques in Electroanalytical chemistry, Allen J. Baird,Ed., Marcel Dekker, INC., New York and Basel, 1984.)

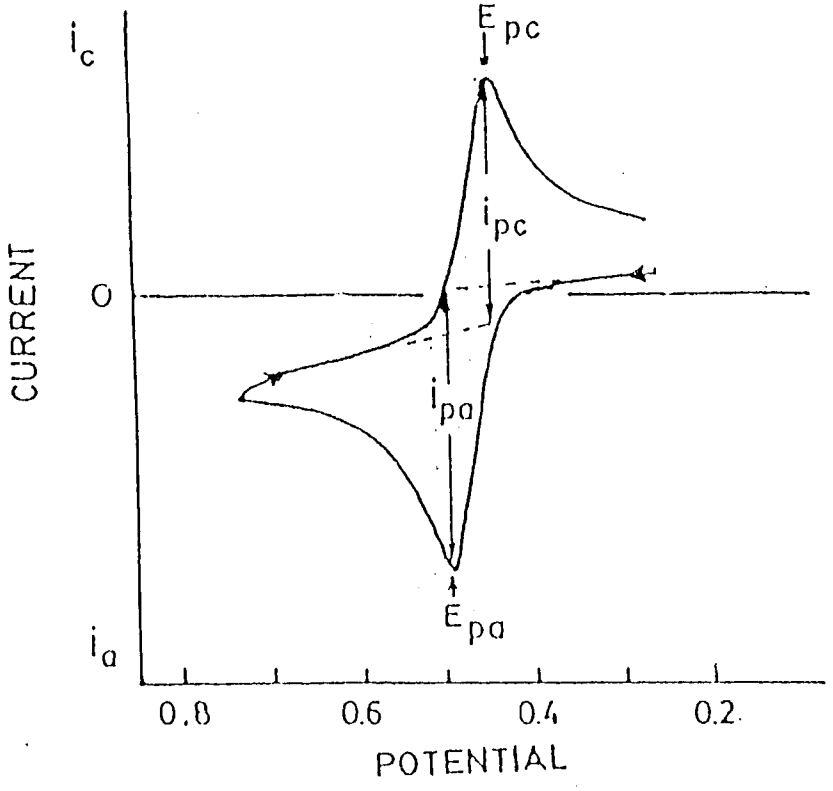


Figure 2. A typical cyclic voltammogram illustrating the potentials and current peaks.

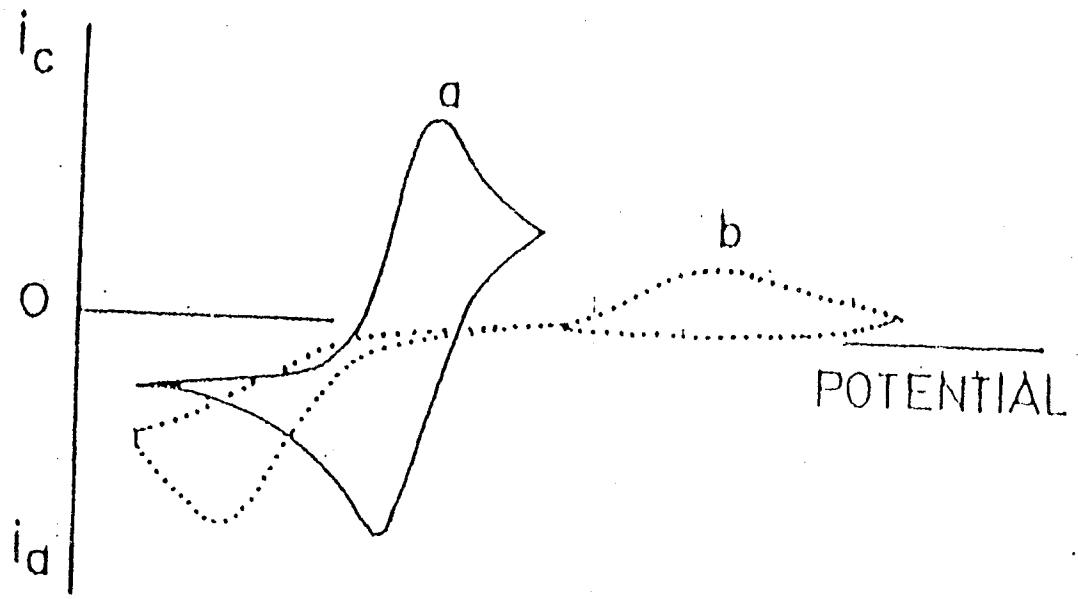


Figure 3. Cyclic Voltammogram illustrating the potential and current peaks of (a) a reversible system and (b) an irreversible system.

Easily obtainable peak potentials can be used to predict the reversibility of a system by making use of Equation 1.

$$\Delta Ep = Epa - Epc \qquad \text{Equation 1.}$$

If the system is reversible, the separation of the peak potentials is approximately .059 volts per number of electrons transferred and is independent of the scan rate. If the change in Ep is greater than .059 volts per number of electrons transferred and dependent on the scan rate, the system is thought to be irreversible.

Difficulty in measuring accurate peak currents is possibly the biggest liability of cyclic voltammetry. This difficulty arises because measurement involves extrapolation of the base line. For a reversible system, plots of Ip cathodic and Ip anodic vs.$(\text{scan rate})^{1/2}$ should be linear with the intercept at the origin. This occurs if there are no kinetic complications because under these conditions Ip anodic and Ip cathodic are equal, as seen in equation 2.

$$Ipa/Ipc = 1 \qquad \text{Equation 2.}$$

For an irreversible system, it is often true that current peaks on the reverse scan decrease due to the diffusion of the product from the electrode surface. At high scan rates, an irreversible system will appear to become

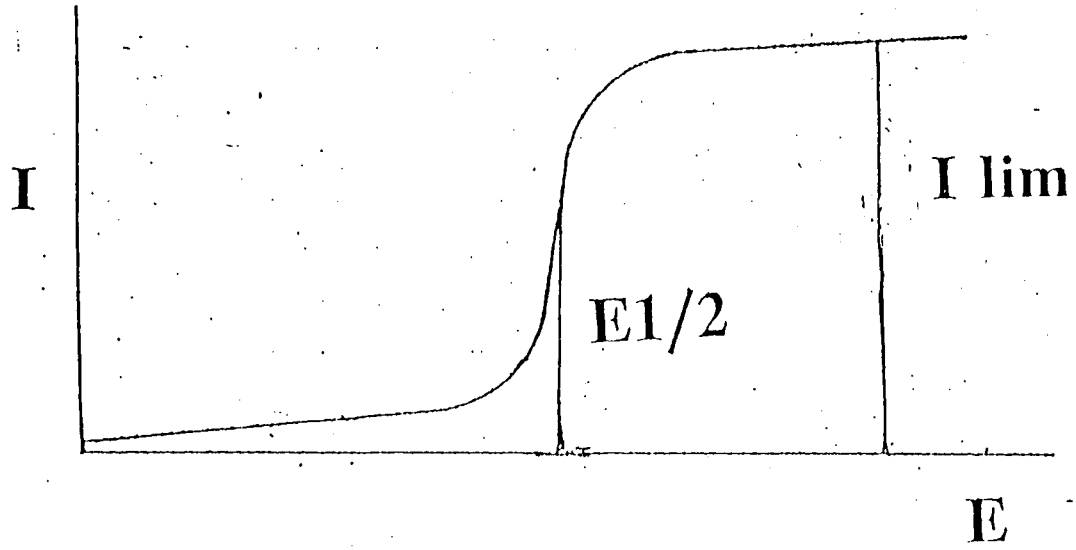
more irreversible by spreading the Epc and Epa values farther apart and/or by increasing or decreasing the Ipa and Ipc ratio value. Because of these and other complications it is dangerous to infer quantitative information from Ip ratios for extremely irreversible couples.

Cyclic voltammetry enables a wide potential range to be rapidly scanned for reducible and oxidizable species. Along with good sensitivity and a variable time scale, this capability makes it one of the most versatile electroanalytical techniques thus far developed.

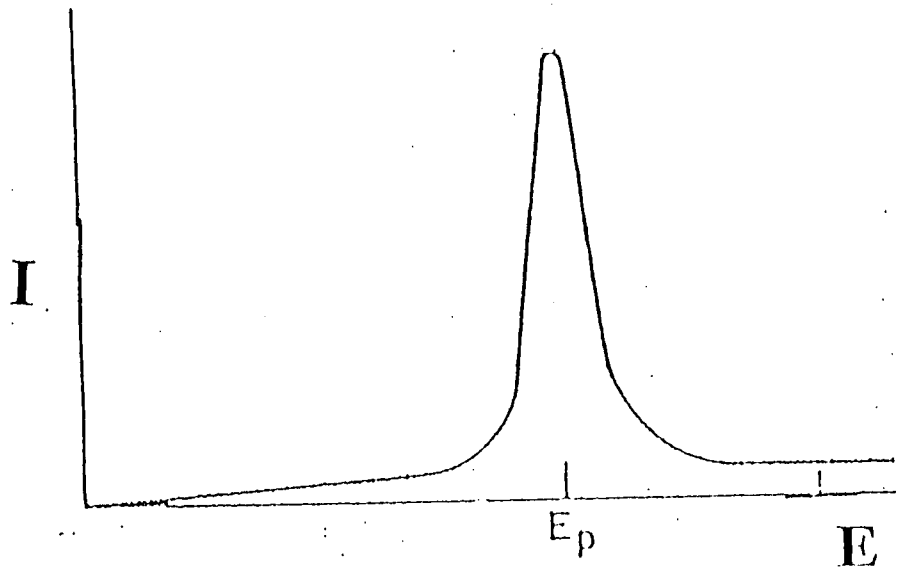
2. Pulse Polarography

The electrochemical technique, polarography, was described by Heyrovsky and Shikata(13); the term pulse polarography was initially used by Geoffrey Barker to describe the techniques of normal (NPP) and differential (DPP) pulse polarography (13,14). Much information can be extrapolated from each of these techniques.

From a normal pulse polarogram, the measurement of $E_{1/2}$, half potential, and I_{lim}, limiting current are obtainable, Figure 4. The measurement of the peak potential, Ep, can be obtained from differential pulse polarography, also seen in Figure 4.



A.



B.

Figure 4. Parameters seen with (a) Normal Pulse Polarography and (b) Differential Pulse Polarography.

The half potential, E1/2, is related to the formal potential as seen in equation 3: where n is the number of

$$E1/2 = Eo' - .059/n \log(Do/Dr)^{1/2} \quad \text{Equation 3.}$$

electrons transferred; Do and Dr are the diffusion coefficients of oxidation and reduction, respectfully; and Eo' is the formal potential. E 1/2 is useful for quantitative analysis of electroactive species because of its relationship with the formal potential of the redox couple.

The limiting current can be calculated using equation 4,

$$I_{lim} = 708\ n\ D^{1/2}Co^*\ m^{2/3}\ t^{1/6} \quad \text{Equation 4.}$$

the Ilkovic equation, where the variables are m, mass of the mercury drop, t, time in seconds, Co, concentration in mol/cm^3, n, number of electrons transferred and Do, diffusion coefficient, are the parameters of interest. However, it is easier to measure limiting currents using normal pulse polarography, by measuring the current at which the slope levels and remains essentially equal to zero.

The peak current for differential pulse polarography can be calculated using Equation 5, where v is the scan rate and all other values are defined as in the Ilkovic equation.

$$ip = 2.69 \times 10^5 n^{3/2} A D_o^{1/2} C_o^* v^{1/2} \quad \text{Equation 5}$$

this equation can be manipulated to obtain a current function, CF (Equation 6), which should be a constant if the reaction is

$$CF = ip/(v^{1/2} C_o) \qquad \text{Equation 6}$$

diffusion controlled, since the peak current is proportional to concentration in diffusion-controlled reactions.

Both NPP and DPP obtain a major advantage, use of the dropping mercury electrode,DME. The DME is an essential component of all polarographic experiments; in 1903, it was introduced by Kucera (15); and in 1921, Heyrovsky used the DME in his original polarographic studies (16). The DME possesses two major advantageous characteristics: uniformity and reproducibility of the electrode surface, if the mercury is clean, and a very high overpotential for hydrogen evolution. These advantages gave polarographic techniques a favored position among electroanalytical methods.

First, continuous renewal of the DME surface is an important advantage; mercury flows through a capillary at a constant rate and small, nearly-spherical drops form and drop at a regular interval. Since the electrode surface is continually renewed, most surface adsorption effects are not observed.

With most electrodes, hydrogen evolution,

$2H+ + 2e- = H_2$, results in a steep increase in current at slightly negative currents. The second major advantage of the DME is a very high hydrogen evolution overpotential. Unlike any other electrode, a relatively well defined polarographic wave at potentials more negative than -2.0V vs. SCE can be detected with use of a DME. However, the DME, like most mercury electrodes has serious limitations in applications at positive potentials; this occurs because the oxidation of mercury occurs at low potentials around 0.0V. The DME has allowed much information to be obtained through pulse polarography.

Polarography can be used to determine many important parameters in a chemical system. However, it is no longer a popular electrochemical technique; it may be considered an ancient technique because of the newer faster techniques such as square-wave voltammetry.

3. Spectroelectrochemistry

The development of optically transparent electrodes enabled spectral observations to be made directly through the electrode simultaneously with electrochemical perturbations (17,18,19). The combination of electrochemistry and spectroscopy has proven to be an effective approach for studying redox chemistry, Figure 5. Oxidation states are changed electrochemically by addition or removal of electrons at an electrode. The reduced species is controlled by the diffusion of the oxidized species diffuses at the electrode

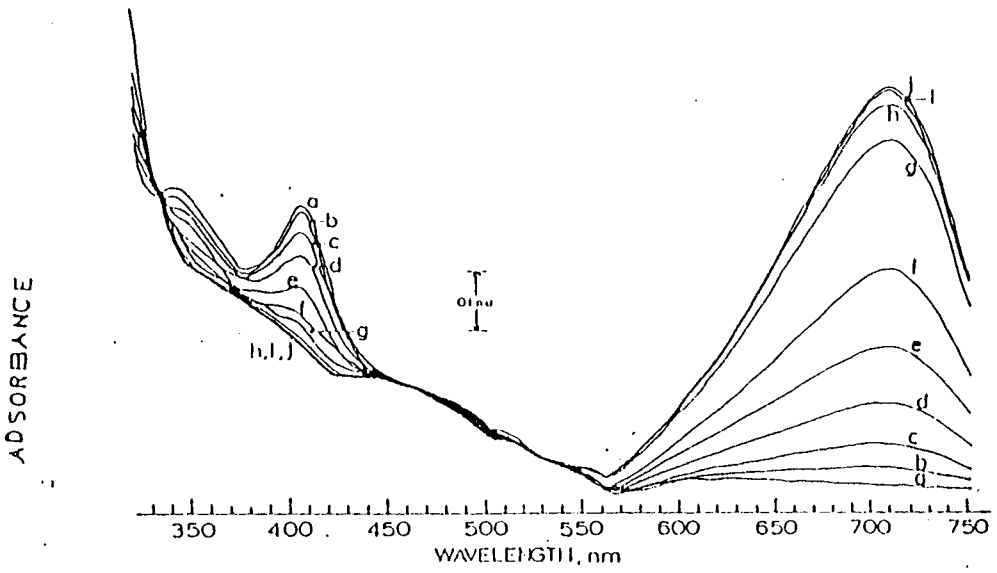


Figure 5. A typical redox system studied with spectroelectrochemistry. (Spectra of cobalt complex with ligand bis(salicylaldehyde) ethylene diimine, obtained at OTTLE. Applied potentials: (a) -.900, (b) -1.120, (c) -1.140, (d) -1.160, (e) -1.180, (f) -1.200, (g) -1.250, (h) -1.300, (i) -1.400, (j) -1.450V vs. SCE. (From D.F. Rohrbach, E. Deutsch, and W.R. Heineman in "Characterization of Aqueous Solvents" G. Mamantov, Ed., Plenum, New York, 1978.)).

surface, the increase in absorbance reflects the generation of R which increases till there is no more oxidized species. The generation of R will not exist if the system does not respond electrochemically. The light beam of a spectrometer is directed perpendicular the transparent electrode and the solution as seen in Figure 6. The electrode transparency enables changes in absorbance that occur in the solution adjacent to the electrode to be monitored during electrochemical perturbation. The actual change in absorbance is related to concentration (c), optical path length (b), and molar absorptivity (E) by Beer's law, Equation 7.

$$A = Ebc \qquad \text{Equation 7}$$

However, the continuous increase in the thickness of the diffusion layer at the OTE and the fact that the concentration of the absorbing species is not homogeneous within this layer must be taken into account and expressed mathematically as in Equation 8.

$$A_t = E_r \int_0^{\infty} C_r(x,t)\ dx \qquad \text{Equation 8.}$$

Er is the molar absorptivity(l/(mol) (cm)), and $C_r(x,t)$, which is changing constantly during electrogeneration, is the concentration distance profile of R. Equation 8 is equivalent to Beer's Law.

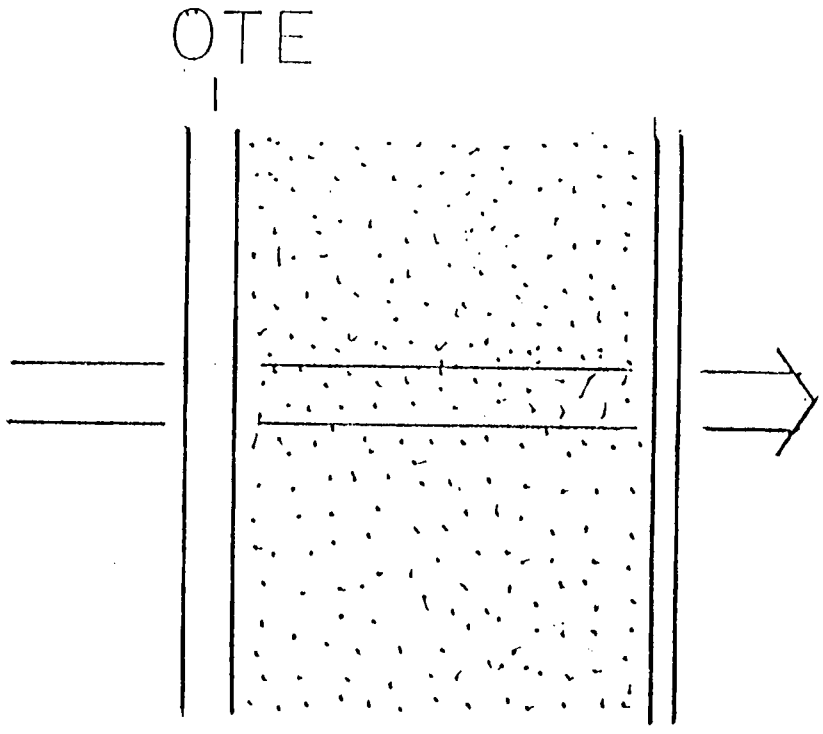


Figure 6. Transmission of Light by means of OTE.

Spectroelectrochemistry is a very useful technique when dealing with redox systems. Many new applications will be developed in the future.

C. ETHIDIUM AS AN INTERCALATING DYE

Ethidium bromide (Figure 7) has an aromatic structure which can bind to DNA by insertion between base pairs in a process called intercalation (20,21,22,23). Ethidium bromide is an intercalating dye which displays many biological processes. Two of these processes include intense fluorescence, which is enhanced through intercalation, (24,25). Intercalation of ethidium causes the DNA double helix to bend, which can cause inhibition of DNA polymerase and antiviral properties.

Ethidium bromide specifically binds to DNA located in nucleic acid and mitochondria of the mammalian cell. Upon binding of ethidium cation to the DNA double helix, the ethidium fluorescence shows up to fifty-fold enhancement. This phenomenon has been attributed to the removal of quenching by polar solvent as the dye is absorbed in the hydrophobic region of the nucleic acid duplex (26,27).

Intercalation of ethidium with DNA can cause bending or unwinding of the double helix of classical and non classical DNA, Figure 8. Ethidium and other phenanthridine's have the largest unwinding or bending

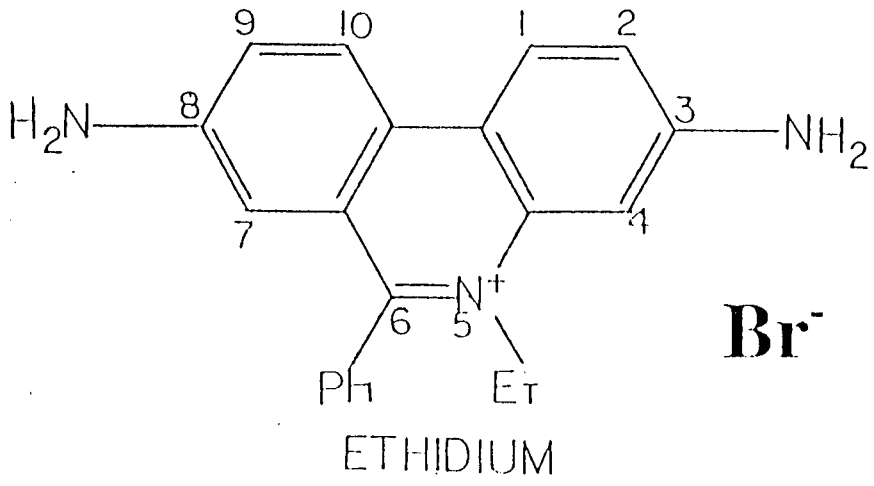


Figure 7. The Structure of ethidium bromide.

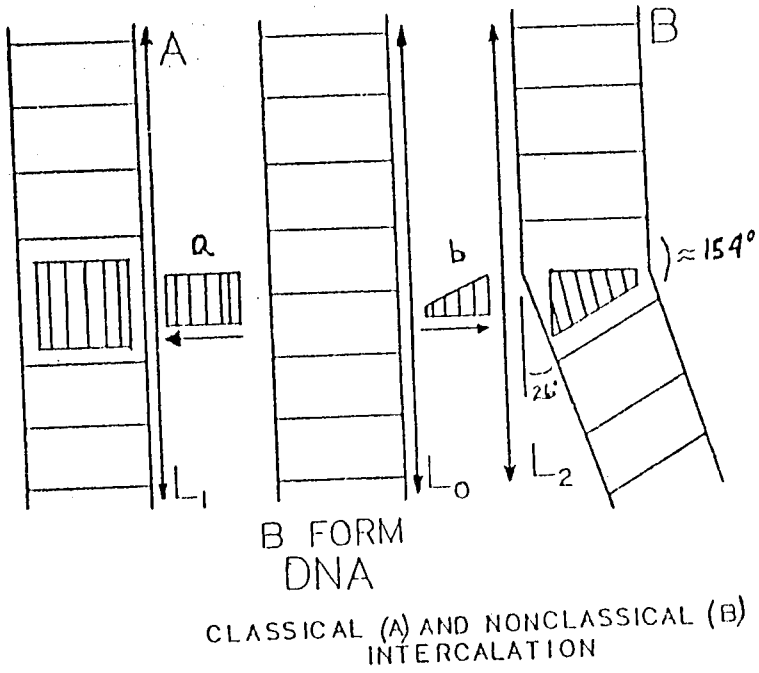


Figure 8. Bending of the double helix cause by intercalation (20).

angles found for any intercalating monomer ligand, 26°, which is 8° more than a large number of intercalating molecules which seem to have angles near 8° because of very specific hydrogen bonding. The denatured DNA resulting from the bending of the double helix could lead to conformational changes in the nucleosome, and an increased binding constant for the first molecule of ethidium which binds. This strong binding could lead to direct inhibition of replication and transcription, recombination, self-splicing, ribosome function and nucleic acid synthesis in proteins. The inhibition of these processes is termed mutagenation. A mutagen causes alterations in the characteristics of particular organisms which are passed from generation to generation in living systems.

Ethidium, like many other intercalating agents, could become a valuable drug. Mutagenation caused by intercalation with ethidium bromide, has been shown to have antiviral properties by blocking viral replications.

D. COMPOUNDS WITH SIMILAR CHARACTERISTICS TO ETHIDIUM BROMIDE

Being a chemical of biological interest, ethidium has many interesting characteristics; including fluorescence and intercalating properties. There are many other compounds (Figure 9) which display some of the same characteristics.

A compound with similar fluorescence quality, is Hoechst

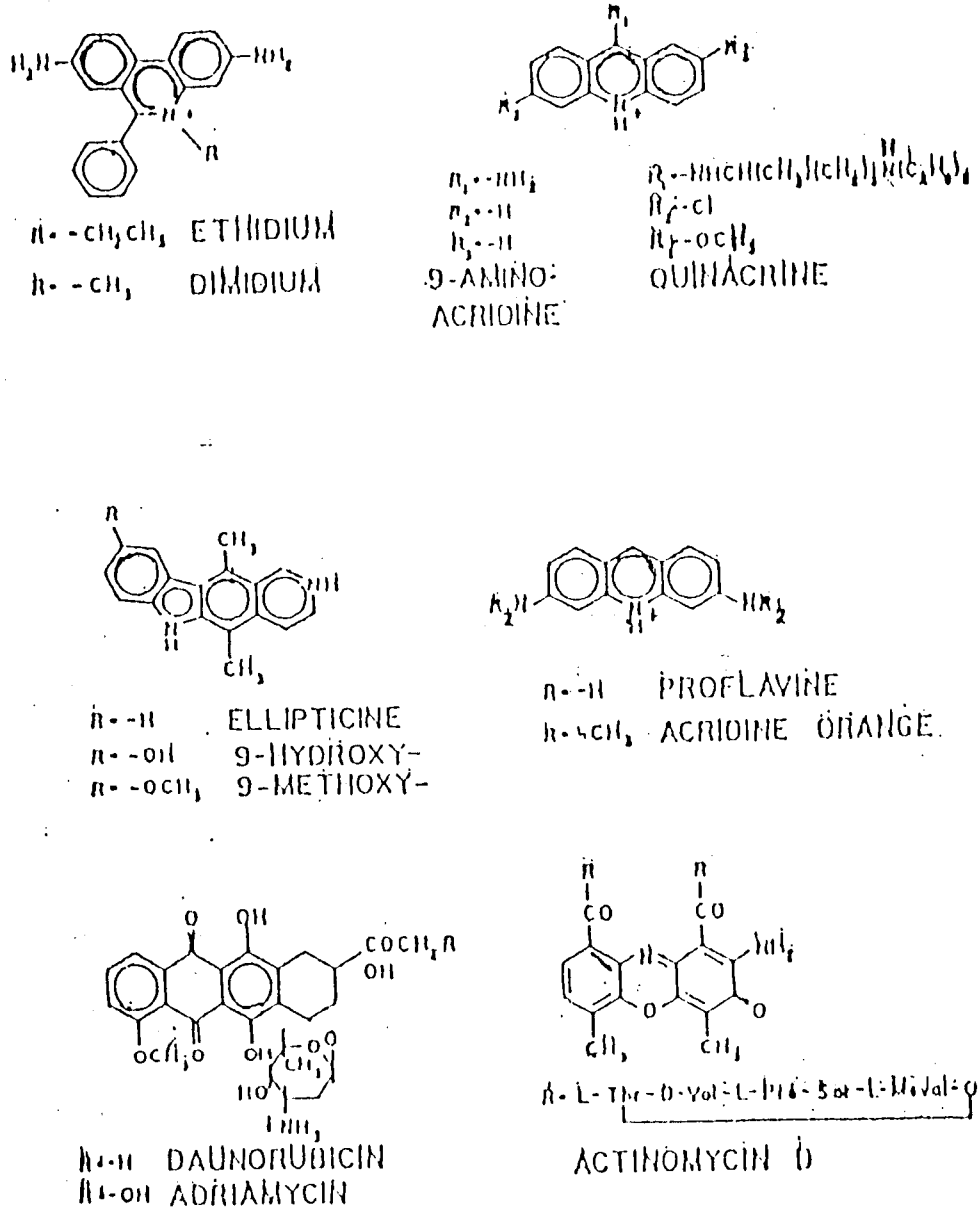


Figure 9. Compounds with similar characteristics to ethidium bromide (20).

33258 (bisbenzimidazole): Hoechst 33258 is particularly useful in quantitative analysis of DNA (28). Ethidium bromide will fluoresce and intercalate with RNA (29); a major advantage of Hoechst 33258 is the use of crude tissue without elimination of RNA; Hoechst is very inactive upon introduction to RNA.

Triphosphopyridine nucleotide, NAD+, (Figure 10), a compound of similar electrochemical characteristics to ethidium bromide, is an essential factor in mammals due to its importance in production of pyridine nucleotides NAD+/NADP; being a pyrimidine of great biological importance niotinamide has undergone many electrochemical studies (29-33). Cathodic reduction of NAD+ appears to occur in two discrete one electron steps; at approximately -1.0V a free radical (NAD·) is formed from NAD+ reduction. The free radical rapidly dimerizes to $(NAD)_2$, and the dimer is oxidized back to NAD+ at -.3V. If pulsed at approximately -1.6V, the second electron step occurs; and NAD· is reduced to NADH. Both 1,4 and 1,6 isomers of NADH are apparently observed. It has been observed that NAD· reduction appears to pass through an intermediate which rearranges to form the observed isomers, 1,4 and 1,6 NADH. The initial NAD· formation process (Figure 11) is the rate determining step of the overall redox scheme of Triphosphopyridine nucleotide sodium salt hydrate.

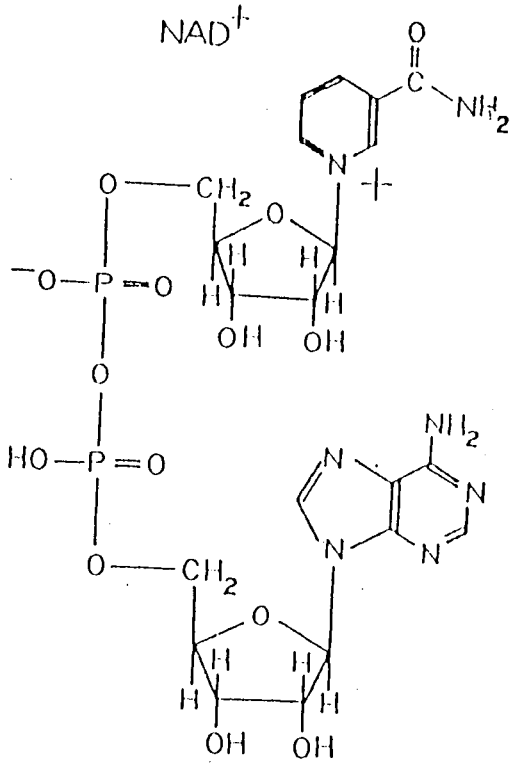
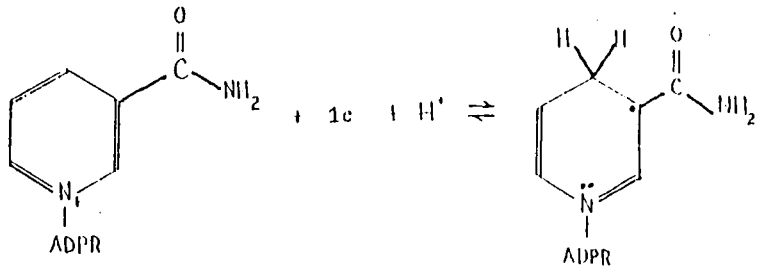


Figure 10. Triphosphpyridine nucleotide sodium salt hydrate, NAD+, in its enzymatically active B form.



NAD+

Figure 11. The initial one-electron reduction of NAD+ to which we are comparing ethidium, ADPR is adenosine diphosphoribose.

II. EXPERIMENTAL

A. Instrumental

Three instruments were employed throughout this research. These include a Bioanalytical Systems (BAS)-100 electrochemical analyzer, a Hewlett Packard (HP) 8452A diode array spectrophotometer, and a Princeton Applied Research (PAR) 174A polarographic analyzer.

The BAS-100,which exploited the power of a microprocessor, made available in one unit an extensive menu of electrochemical techniques. The electrochemical technique of primary interest to this thesis was cyclic voltammetry. Data received from the BAS-100 was plotted on a Houston Instruments plotter DMP 40 series.

The PAR-174A is an electronic polarographic instrument capable of performing more than one electrochemical technique; the two techniques of interest were normal pulse polarography, NPP, and differential pulse polarography,DPP. The Model 174A is supplied with a drop timer that dislodges the mercury drop with minimal perturbation of the solution. An Omnigraphics Houston 2000 recorder was used to record the data.

The HP 8452A diode array spectrophotometer is a single beam microprocessor controlled UV/VIS spectrophotometer with collimating optics. The output was plotted using a HP Color Pro Graphics plotter model 7440A.

B. Electrode Construction

A number of electrodes were constructed in order to complete experimentation. These included a platinum in Teflon electrode (Figure 12), a glassy carbon in Teflon electrode (which was not used because of the inconsistent results obtained), a dropping mercury electrode (DME), a hanging mercury drop electrode (HMDE), a gold optically transparent electrode and a thin Nafion film electrode.

Jacketed in Teflon, the platinum and glassy carbon were applied to the study of the oxidation of ethidium bromide. Platinum and glassy carbon rods were sealed in Teflon, a synthetic polymer obtained from Commercial Plastics in Knoxville and connected to a brass conductive rod as seen in Figure 12.

A hanging mercury drop was prepared by pulsing a platinum electrode, which had been sealed in glass, in a saturated mercurous chloride solution for approximately thirty minutes at a potential of -1.0V and attaching a drop or two of mercury trapped into a glass spoon from the DME onto the platinum surface. The hanging mercury drop electrode was used to produce a cyclic voltammogram of the reductive cycle of ethidium bromide; a fresh mercury drop was applied before each sweep.

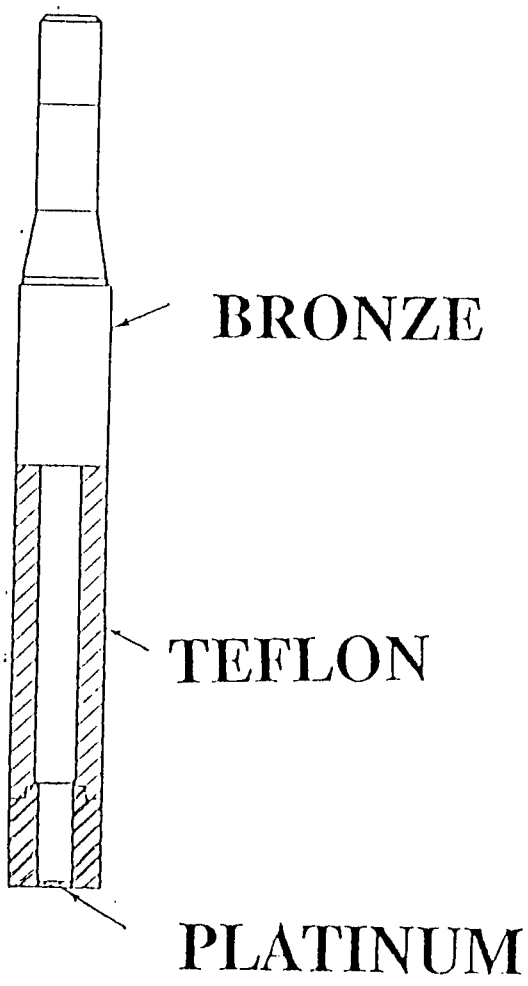


Figure 12. Diagram of Teflon platinum disk electrode.

The DME, dropping mercury electrode, was synchronized using an electrode drop timer ASSY 174/170 (PAR). The main disadvantage was capillary clogging. The main advantage was a clean, uniform, reproducible surface with each drop. The DME was used in polarographic measurement.

A Teflon OTE cell was used in the spectroelectrochemical measurements. A gold OTE was prepared by electron beam coating using the IAM sputtering electron beam evaporator Cooke vaccuum product Model SPS-.5K at the UT lazer laboratory on the Pellissippi Parkway. A one nanometer layer of chrome was initially applied to each quartz OTE; a five nanometer layer of gold was then coated on each electrode.

Nafion, a perfluorocation exchange polymer, used in an attempt to decrease the biological risks of ethidium bromide, was initially applied to a platinum sealed-in-glass electrode by using the technique described by Martin (34). The film was observed to be much more adhering to a Teflon jacketed platinum electrode than to the glass electrode even after applying Martin's application method. Nafion, was applied using an eppendorf pipet; a drop was smeared on the Teflon and dried under vaccuum for approximately one hour. After the Nafion film was applied, the electrode was soaked in a 0.5mM solution of ethidium bromide for approximately 20 minutes. The electrode was then washed thoroughly with doubly distilled water and allowed to dry.

C. CHEMICALS

Several chemicals were used in experimentation. These included potassium monophosphate buffer (Mallinckrodt), ethidium bromide (Aldrich), Triphosphopyridine nucleotide sodium salt,NAD+, (Aldrich), methyl viologen (Aldrich),and Nafion (Aldrich).

Potassium phosphate buffer, KH_2PO_4, was prepared with a pH range of 1-12 using an Orion pH meter model SA520. The pH was adjusted by adding dropwise .1M HCl to decrease or .1M NaOH to increase the pH with consistent stirring.

A stock solution of 25mM ethidium bromide was prepared in 0.1M potassium phosphate and diluted to lesser concentrations. NAD+ and MV^{2+} solutions were prepared in the same manner as ethidium.

One of the more expensive chemicals used was Nafion. Nafion, a perfluorinated ion exchange powder 5 wt% solution in mixture of lower aliphatic alcohols and 10% water, was used in the form received.

III. RESULTS AND DISCUSSION

A. Nafion Film Study

The perfluorinated polymer, Nafion, was applied as a thin film on a platinum disk electrode and used to study the oxidative process of ethidium bromide. Several preliminary experiments including film application and electrode composition were attempted. Three methods of film application (oven heat, blow drying, and vaccuum) were attempted. Oven heat and blow drying left the films nonuniform and cracking. Vaccuum drying was the only successful method. Two electrode compositions were tested, glassy carbon and platinum. Platinum was the electrode of choice because the surface of the glassy carbon electrode was too difficult to reproduce and the cyclic voltammograms were not consistent. After completion of preliminary experiments, cyclic voltammetry and spectroelectrochemistry were performed at the Nafion coated platinum electrodes.

1. Cyclic Voltammetry

In a beaker-like electrochemical cell comprised of a SCE (saturated calomel electrode) reference and a platinum mesh counter, a .203 cm^2, Teflon-jacketed, Nafion-coated, ethidium-soaked, platinum working electrode was subjected to

the electrochemical technique of cyclic voltammetry, in a twenty-five milliliter portion of supporting electrolyte, 0.1M potassium phosphate pH 7 buffer. The cell was purged with argon for approximately twenty minutes before each cyclic voltammogram. Several trials were attempted under the previous conditions; a representative voltammogram is seen in Figure 13. The diffusion of ethidium from the Nafion film could not be seen visually; the observed peak is possibly the oxidation of the platinum electrode, since a blank produces a peak at approximately 0.80V. Previous experiments reported an observed peak at +0.75V for the oxidation of ethidium ion.(35)

Facile diffusion of ethidium and the electroactivity of ethidium entrapped the Nafion film, unlike other organic compounds of similar characteristics, did not appear to occur under these conditions. Multiple regions within the Nafion film have been reported with the use of ferrocene\ferricenium couple (6). A hydrophobic inner layer and a hydrophilic outer layer appear to exist; the outer layer consists of an interfacial and an ion cluster region. Once the ferrocene was incorporated into the hydrophobic region, a small percentage (10%) was shown to be electroactive; Apparently, the ferrocene molecules in this region were trapped in the Nafion and electroinactive. Ethidium could possibly be trapped in the hydrophobic region making it electrochemically inactive.

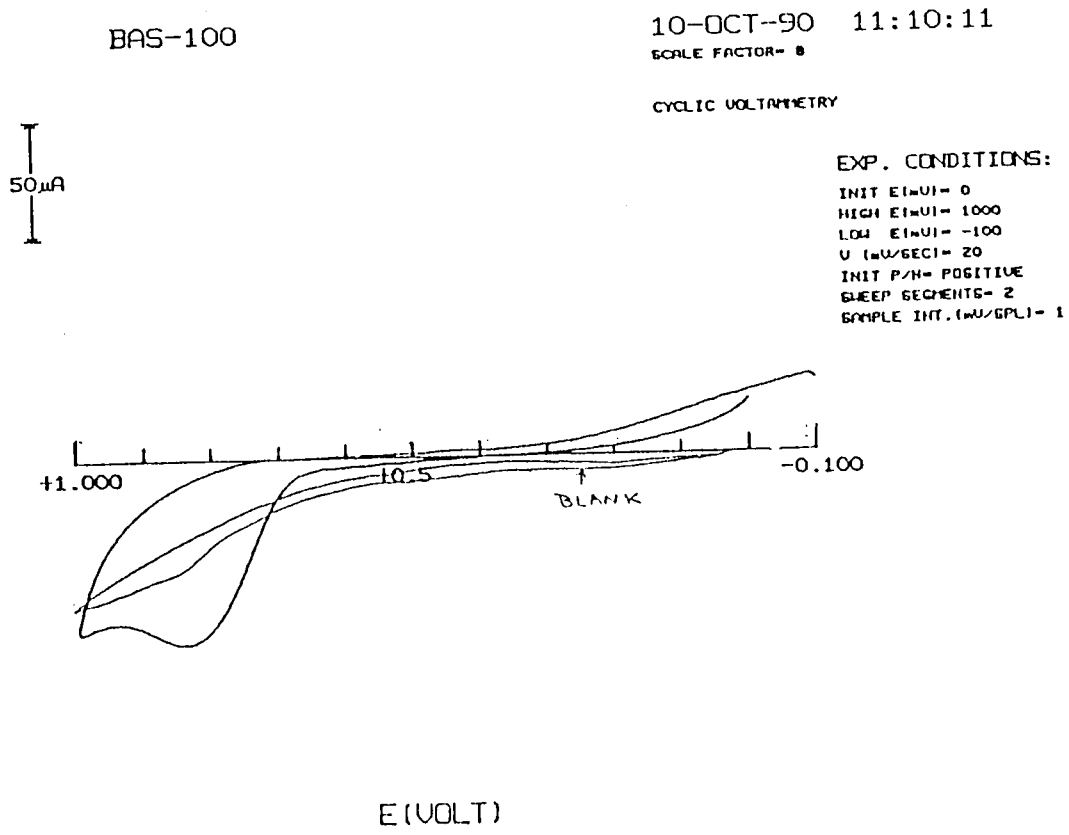


Figure 13. Ethidium soaked nafion film cyclic voltammogram on a platinum working electrode.

2. Spectroelectrochemistry

The electroinactivity of ethidium bromide in Nafion was confirmed by studying the spectroelectrochemistry of the system. A gold OTE, Ag/AgCl wire reference, and a platinum wire counter were incorporated in a spectroelectrochemical cell filled with supporting electrolyte, 0.1M potassium phosphate buffer, to measure and record a blank spectrum which was used as a reference. The supporting electrolyte was then removed and replaced with 0.5mM ethidium bromide for approximately half an hour. A pink coloration was observed after soaking the Nafion film in ethidium bromide. The ethidium visually appeared to be incorporated in the Nafion film. The cell was then washed several times with supporting electrolyte, filled with fresh supporting electrolyte, and a scan was taken to determine the wavelength at which ethidium absorbs (Figure 14). Tenth of a volt potential step increases (0.0V to 1.0V) were applied at time intervals of thirty minutes, and a wavelength scan was taken after each pulse as seen in Figure 15. In the absorbance spectra in Figure 15, (a) represents 0.0V and (b) represents 1.0V. At a wavelength of 400 nm you can observe a slight absorbance change; but at 500 nm, the wavelength at which the ethidium ion absorbs, there is no apparent electrochemical activity occurring. As the potential was increased from 0.0V to 1.0V, essentially no difference was

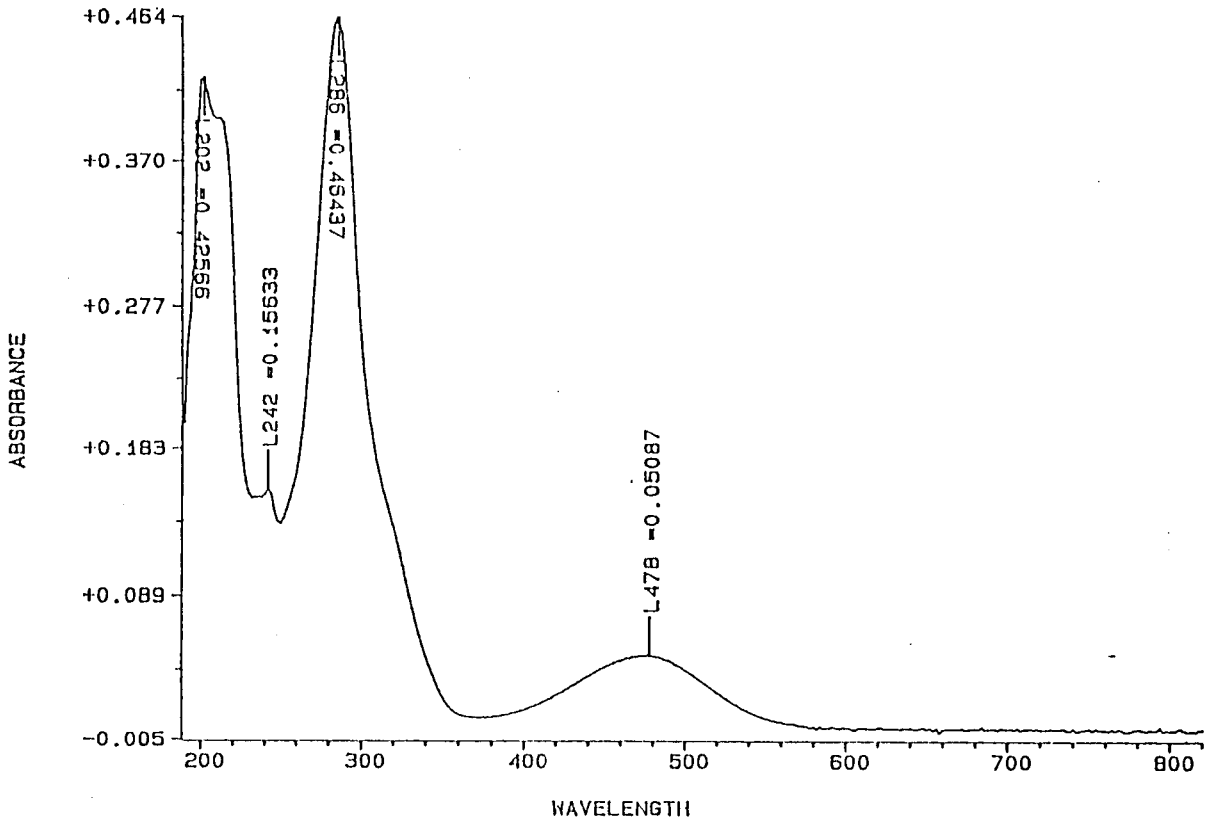


Figure 14. Absorbance Spectra of a .5 mM EtBr solution.

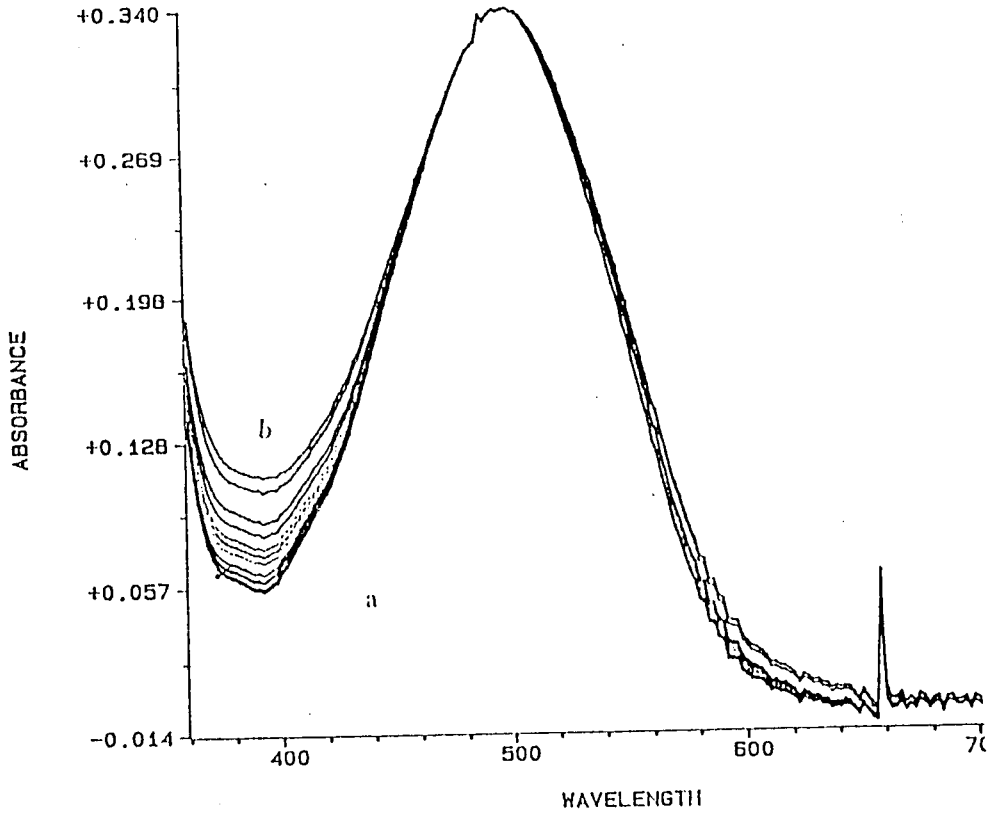


Figure 15. The ethidium absorbance wave being pulsed from .1V to 1V. This is a typical Spectroelectrochemical response for ethidium bromide.

apparent in the ethidium absorbance measurements suggesting electrochemical inactivity when dealing with ethidium loaded nafion films.

B. pH Study

A pH study of range 1-12 was conducted using polarographic electrochemical techniques. Solutions of ethidium bromide, ranging from pH 1-12, were prepared in 0.1M potassium phosphate buffer and studied to determine the effects of pH on the redox system. The known one-electron reduction of NAD+ (7) was used in comparison to the reduction of ethidium ion.

The electrochemical cell consisted of a SCE reference, a platinum mesh counter, and a DME working electrode. A rise in DPP peak potentials of the 0.5mM ethidium bromide solutions was observed. At a pH 1, the DPP peak potential was measured at -0.95V; and at a pH 4, the peak potential was measured at -1.26V. The slope was determined to be equal to rise/run = 1168mv-1010mv/pH3.0-pH1.5 = 105.33 mv/1.5pH = 70.2 mv/1.0 pH. The determined slope of 70.2 suggests a one- or a two-electron exchange for ethidium bromide. Between pH 4.0 and 8.0, the DPP peak potential levels to a consistent value of -1.26V vs. SCE (Figure 16). Because of the change in slope at a pH 4.0, the pKa of ethidium is suggested to be approximately 4.0. Through comparison to analine,

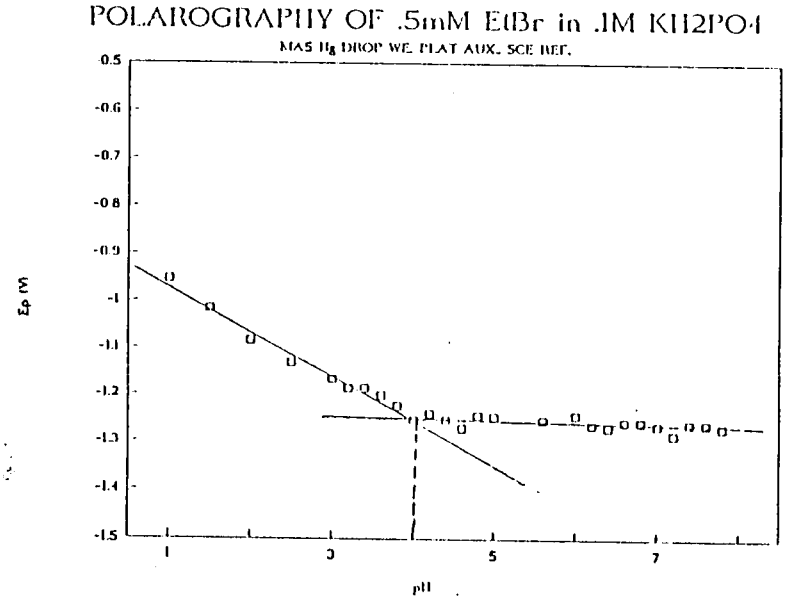


Figure 16. Response of potential to change in pH of 0.5mM ethidium bromide.

a compound with similar amino group substitution whose pKa value is 4.6 (36), the value approximated for ethidium seems reasonable. Above a pH 8.0, the peak potentials were sparatic and not documented.

At a pH 7, the DPP current peak of the initial one-electron reduction of NAD+, peak potential = -1.15V. was found to be approximately half the DPP current peak of the ethidium ion reduction potential at -1.26V, under the same conditions, Figure 17. This suggests a two-electron redox system for ethidium bromide, Figure 18.

A documented diffusion coefficient for NAD+ was found to be 4.2 x 10^{-6} cm^2/s (31). The diffusion coefficient from literature for Methyl viologen (dichloride hydrate) used in these results was 8.6 X 10^{-6} cm^2/s (37) using a pH 7. Using chronoamperometric methods, the diffusion coefficient for ethidium bromide was calculated with background correction to be 7.5 x 10^{-6} cm^2/s. Quiet time applied was 1 minute. The diffusion coefficient was calculated using Equation 9,

$$51.31(cm/As)\ i = D^{1/2}/\ t^{1/2} \qquad \text{Equation 9.}$$

from the Cottrell equation (where n is approximated at 2, F=96484.56 As/mol,C=.0005 mol/1000cm^3, A=.0385 cm^2 are comprised in the constant) . The slope of the line resulting from the current,i(uA), as a function of the time, $t^{-1/2}$(1/s), gives the diffusion coefficient, D (cm^2/s), Figure 19. A

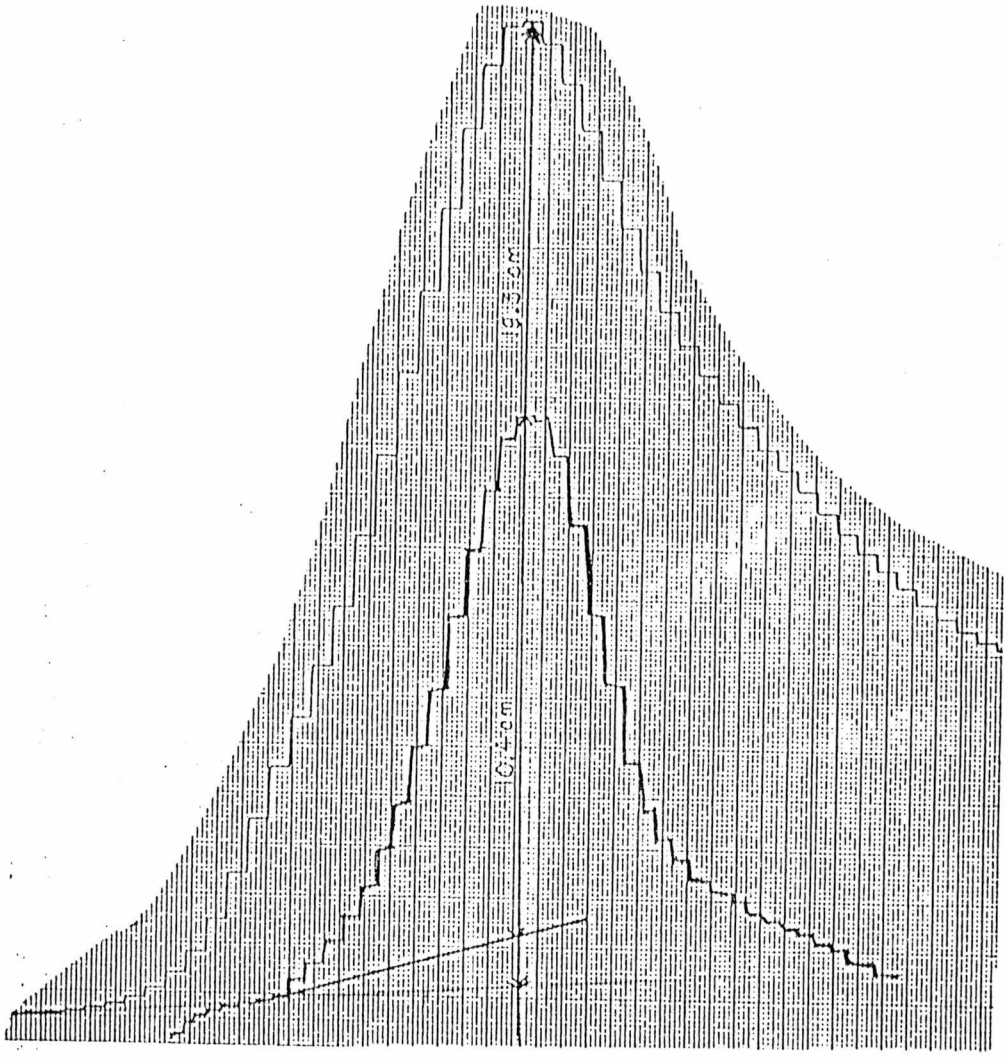


Figure 17. NAD+ (DPP) transposed on to an ethidium bromide (DPP) under the same conditions to show the current peaks ratio 1:2, NAD:EtBr. E peak of ethidium bromide is -1.26V and E peak of NAD+ is -1.10V. (y=.5uA/cm;x=25mv/cm)

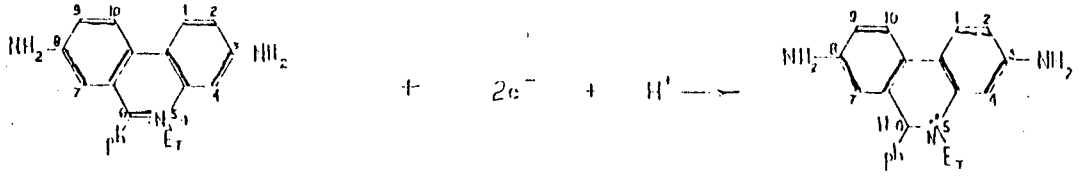
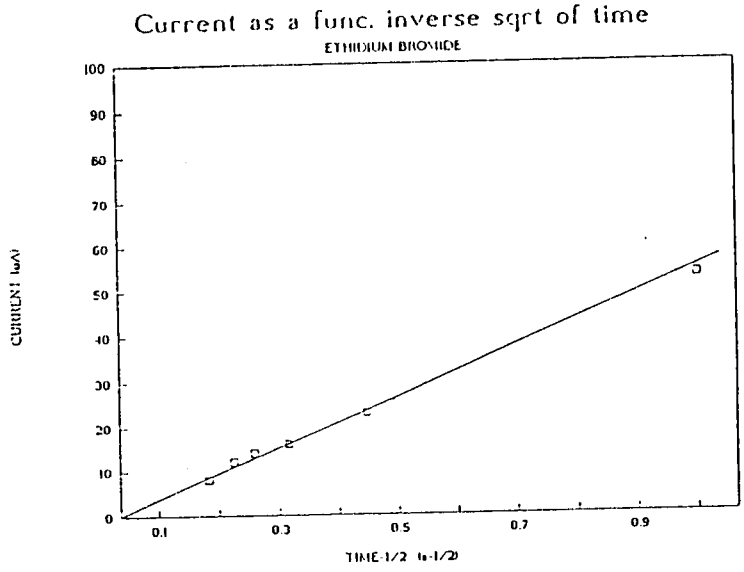
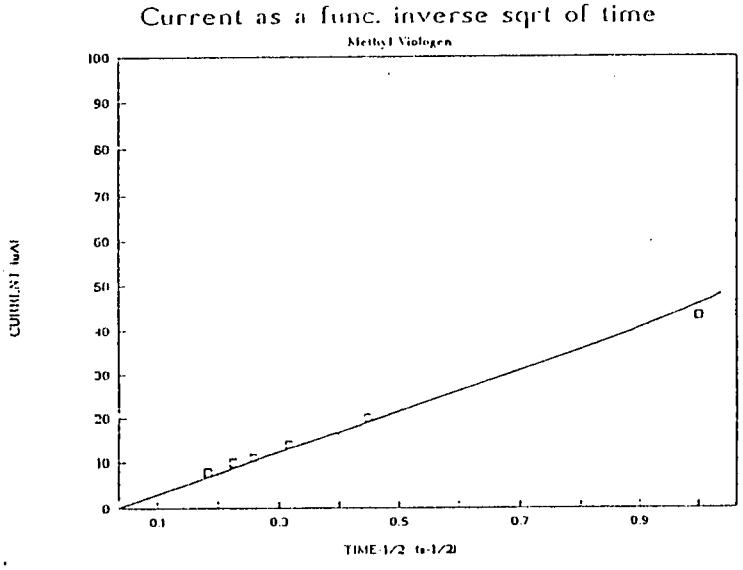


Figure 18. A suggested redox scheme for ethidium bromide.



(a)



(b)

Figure 19. Current as a function of the inverse square root of time for (a) ethidium bromide and (b) MV^{2+}.

the electrochemical technique of cyclic voltammetry, in a twenty-five milliliter portion of supporting electrolyte, 0.1M potassium phosphate pH 7 buffer. The cell was purged with argon for approximately twenty minutes before each cyclic voltammogram. Several trials were attempted under the previous conditions; a representative voltammogram is seen in Figure 13. The diffusion of ethidium from the Nafion film could not be seen visually; the observed peak is possibly the oxidation of the platinum electrode, since a blank produces a peak at approximately 0.80V. Previous experiments reported an observed peak at +0.75V for the oxidation of ethidium ion.(35)

Facile diffusion of ethidium and the electroactivity of ethidium entrapped the Nafion film, unlike other organic compounds of similar characteristics, did not appear to occur under these conditions. Multiple regions within the Nafion film have been reported with the use of ferrocene\ferricenium couple (6). A hydrophobic inner layer and a hydrophilic outer layer appear to exist; the outer layer consists of an interfacial and an ion cluster region. Once the ferrocene was incorporated into the hydrophobic region, a small percentage (10%) was shown to be electroactive; Apparently, the ferrocene molecules in this region were trapped in the Nafion and electroinactive. Ethidium could possibly be trapped in the hydrophobic region making it electrochemically inactive.

HDME working electrode vs. SCE and a pH 7 0.5 mM ethidium bromide solution was used. The diffusion coefficient of methyl viologen under the same conditions was determined to be 5.1 x 10^{-6} cm/s (Figure 19); this diffusion coefficient is very similar to the value found in literature. The calculated diffusion coefficent for ethidium bromide (7.5 x $10^{-6}cm^2/s$) is similar to the calculated value of MV^{2+} (5.1 x $10^{-6}cm^2/s$) under the same conditions. Since MV^{2+} is a well documented two-electron exchange, this agrees with the suggestion that ethidium under goes a two electron exchange. MV^{2+} and ethidium bromide are comparable because of their similarity in size.

C. Concentration Study

Cyclic voltammograms of ethidium bromide were obtained over a range from 0.05mM to 25mM and compared to voltammograms of NAD+ over a similar range. A stock solution of 25 mM ethidium bromide and NAD+ were prepared and diluted. Cyclic voltammetry was the electrochemical technique of choice utilizing a HMDE vs. SCE at pH 7 with potential limits -0.5V to -1.5V.

The cathodic peak potential is observed at -1.04V vs. SCE with a current of 10 uA at a scan rate of 100 mv/s; a drastic increase in scan rate, 51200 mv/s, produces a negative shift in the cathodic peak potential to -1.25V with a current of 300 uA. This is expected for an irreversible electrochemical

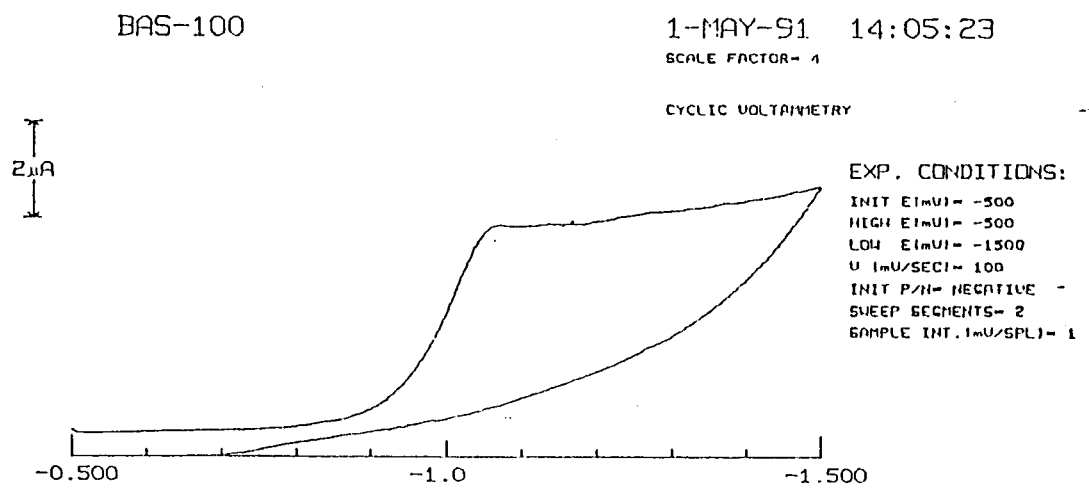


Figure 20. Cyclic Voltammetry after cycling 25 times, showing the irreversibility of the system.

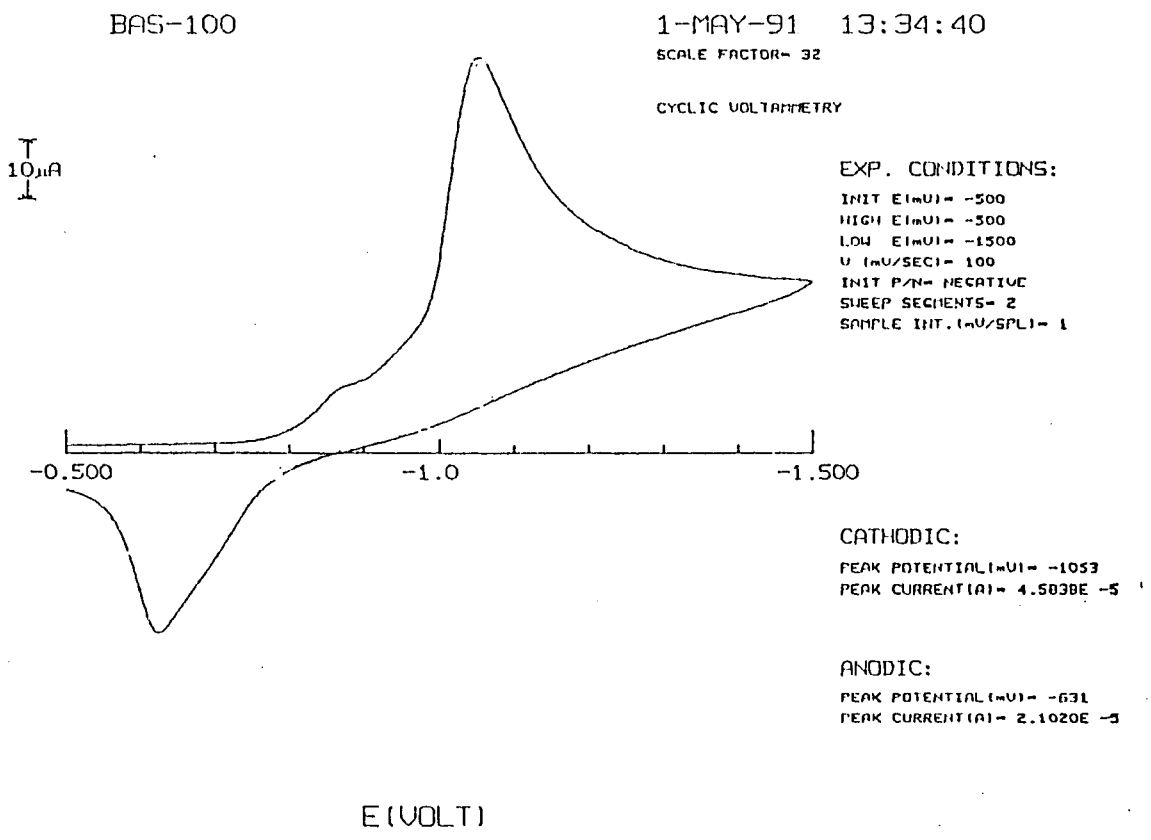


Figure 21. A Cyclic Voltammogram at a high concentration (25mM) shows surface wave on the return cycle.

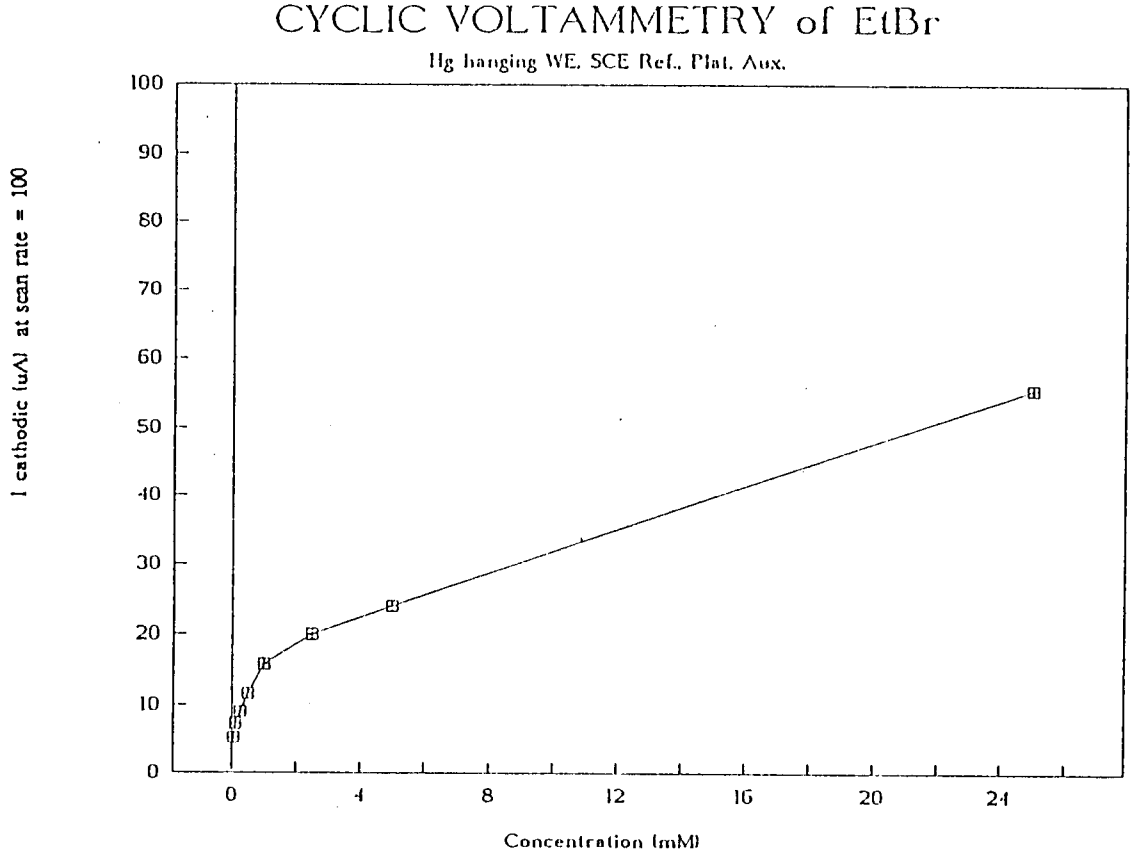


Figure 22. The cathodic current of ethidium bromide as a function of concentration.

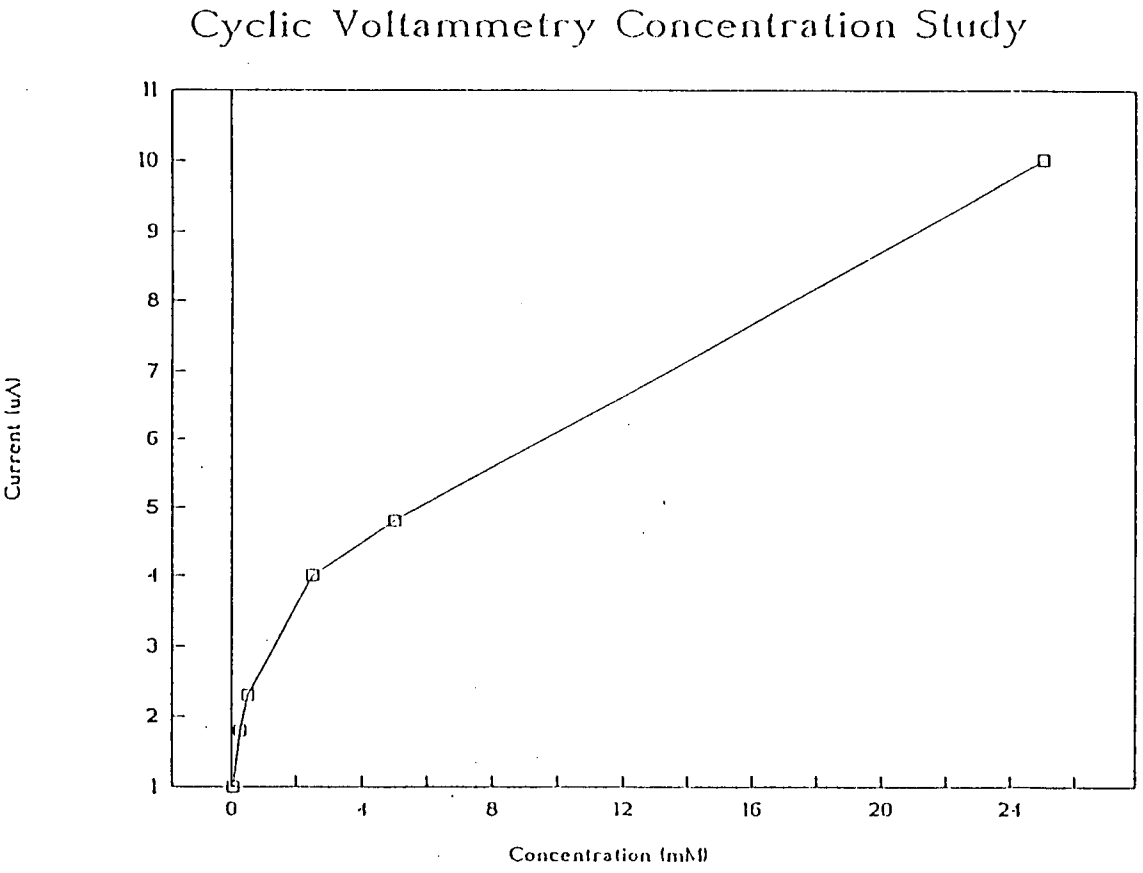


Figure 23. The cathodic current of NADH as a function of concentration.

Table I. The results of concentration study of ethidium bromide, at a sweep rate of 100mv/s.

Conc. (mMol/L)	Ip average (uA)	Ep average (Volts)	Current Function
25	61.7	-1.05	.0081
5	40	-1.01	.0024
2.5	30.5	-1.02	.0033
.5	14.4	-1.08	.091
.25	13.2	-1.08	.144
.05	5.5	-1.08	.35

Table II. The results of Concentration Study of NAD,100mv/s.

Conc. (mMol/L)	Ip average (uA)	Ep (Volts)	Current Function
25	10	-1.14	.0003
5	5	-1.14	.0006
2.5	4.0	-1.125	.001
.5	2.3	-1.17	.003
.25	1.8	-1.19	.005
0.05	.10	-1.16	.0117

IV. CONCLUSION

Ethidium bromide, an intercalating dye, is a compound of biological interest. Fluorescence and antiviral properties are two of the more interesting qualities of ethidium bromide (38,39).

Nafion, a perfluorinated polymer, was applied as a thin film on a platinum sealed-in-Teflon electrode. Ethidium apparently was incorporated in the Nafion films because of the pink coloration after soaking the film in ethidium bromide solution. However, once incorporated, electrochemical inactivity was observed and proven by spectroelectrochemistry.

The electrochemical characterization of ethidium was then studied with respect to pH and concentration. The pH study observed from a plot of potential as a function of pH that a slope of 70.2 mV from pH 1 to 4 levels to a slope of zero from a pH 4 to 8. The point at which the slope changes, pH 4, is suggested to be the value of the pKa of ethidium. The potential at which the slope of is zero is -1.26V SCE.

At pH 7.0, DPP peak of ethidium is twice the one-electron reduction NAD+. This suggests a two-electron redox system. A diffusion coefficient (7.5×10^{-6}) for ethidium bromide is calculated, assuming n=2, by measuring current as a function of time through electrolysis. This factor is comparable to the diffusion coefficient of NAD+ (4.2×10^{-6} cm^2/s), MV^{2+} (8.6×10^{-6} cm^2/s) found in literature, and a value

of 5.1 x 10^{-6} cm^2/s determined for MV^{2+} under the same conditions as the diffusion coefficient for ethidium bromide. Since MV^{2+} AND NAD+ are documented two-electron exchanges, the determined diffusion coefficient for ethidium bromide suggests that ethidium bromide undergoes a two-electron exchange.

A concentration study was conducted from 25mM to 0.05 mM ethidium bromide and compared to NAD+, with the same concentration range. At a scan rate of 100 mv/s a potential peak of -1.04V and a current peak potential of 10 uA was observed. At a scan rate 51200 mV/s a potential peak of -1.25V and a current peak of 300uA was observed. An irreversible system is suggested.

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VITA

Gina Ann Stanley Becker was born on September 28, 1966 in Bristol, Tennessee, to Elby and Betty Stanley. After graduating from Sullivan East High School in the Spring of 1984, she entered East Tennessee State University in the Fall of 1984. She graduated in May of 1984 with honors and received the Bachelor of Art degree in Chemistry. She moved to Knoxville, Tennessee to begin graduate school at the University of Tennessee in August 1988. Dr. James. Q. Chambers was chosen as her research advisor; he partially financed her efforts with a split research/teaching appointment. The National Science Foundation is gratefully thanked for support of her graduate education.

She was married to William Edward Becker on June 1, 1991. She intends to complete her Master of Science degree in Chemistry in the Fall of 1991 and graduate in December.