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Beverly Ann Hanes Swale

November 21, 1990

Fluorescence Detection of DNA-Ethidium Complexes in the Diffusion Layer of a Modified Electrode

A Thesis

presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Beverly Ann Hanes Swaile

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DEDICATION

The following thesis is dedicated to all those who supported this endeavor emotionally, financially and otherwise. Thanks go to my mother and father, Barbara and Harold Hanes and brother, Billy, and to my in-laws Pat and Bill Swaile who lovingly nudged me forward throughout my graduate experience. I thank my friends: Gina Stanley, The Sepaniak group (Jim, Rod, Craig, et al.), Patti Kreke, Nivens, Costas, and Stephen Schudel. Special thanks also go to my research advisor, Dr. James Q. Chambers who financially supported and patiently guided my research efforts. To my toughest critic and dearest friend, my husband, David F. Swaile, I offer my deepest thanks. Finally, this thesis was made possible in part by Killer the cat who helped both with typing and organization - more often than not!!!

ABSTRACT

An extremely rapid and highly sensitive technique for the quantitative determination of bulk (total) deoxyribonucleic acid (DNA) samples at the picogram level is described. The method is based on the release of ethidium cation (2,7-Diamino-10-ethyl-9-phenyl-phenanthridinium), Et^+, into a DNA solution via oxidation of a tetracyanoquinodimethane ($TCNQ^{-}$) acceptor anion at a carbon paste/Et^+TCNQ^- composite electrode surface. Detection of the DNA was performed fluorometrically by exciting the DNA-ethidium complex in the diffusion layer using the 514.5 nm line of an argon ion laser aligned parallel to the electrode surface. Detection occurred in less than 20 seconds after application of +0.45 V potential. Sample preparation was quickly and easily done by dissolving calf thymus DNA in HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffer solution, pH 7.0 without the need for sample denaturization by heat.

Experimental parameters which have been optimized include (i) electrode composition, (ii) applied electrode potential, (iii) distance of the excitation source from the electrode surface, (iv) electrolysis time, and (v) laser power. Although various ratios of EtTCNQ to carbon proved acceptable for data collection, a 1:20 ratio enabled complex measurement over 6 orders of magnitude of DNA concentration. It was determined that the optimum applied potential for ethidium release was +0.45 V. Varying the distance of the excitation source from the electrode surface provided a means of monitoring the diffusion front as it traversed the containment cell. A

very small distance of the source from the electrode resulted in short analysis times and reasonable reproducibility. Because the surface of a carbon paste electrode had to be renewed after the chemical concentration was exhausted, a method was developed so that the electrode could be pulsed several times before the ethidium supply was depleted. Various electrolysis times were compared for peak reproducibility and sufficient signal to noise ratio. A 5 seconds pulse time met the criteria. Laser power was examined in attempts to further improve signal to noise ratios, but high excitation power resulted in rapid and uneven diffusion from heating and increased background signal.

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I. INTRODUCTION

(A.) The History of DNA

Elucidation of the DNA structure occurred less than 40 years ago. Since then, research on DNA has produced an entirely new way of thinking in the scientific, industrial, and medical communities. Thousands of papers have been published on various aspects of DNA, its functions, capabilities, properties, etc. Although Watson, Crick, and Rosalind Franklin stand out in most minds as the great DNA investigators, there are many others who played key roles in shaping our understanding of this amazing molecule. A chronology of events and those persons responsible for them is listed below in Table 1. These are but a small portion of the milestones that shaped DNA science. As one can see this endeavor was a cumulative effort spanning the first half of the century (1, 2, 3, 4).

In April 1953, J. D. Watson and F. H. C. Crick published their paper entitled *Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid* in NATURE which suggested what has since been determined to be the correct structural model for DNA. This structure contains two right-handed antiparallel polydeoxyribonucleotide strands wound around one another in a helical fashion. The strands are held together by hydrogen bonding which occurs through the purine and pyrimidine base linkages, namely Guanine, Cytosine, Thymine and Adenine (1, 5). The helix

Table 1. Chronological events leading to the determination of the correct structure for DNA

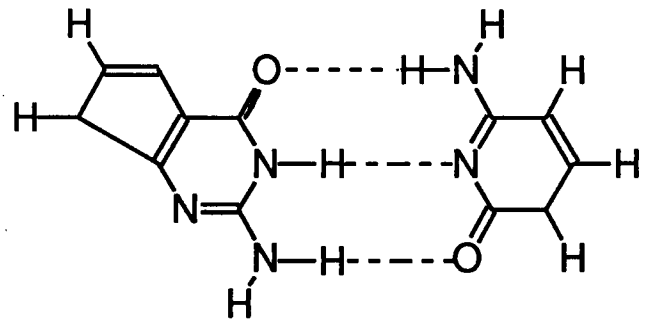
DATE	EVENT
1938 --	DNA was shown to be an extremely large molecule by Casperson and others.
1945-1952--	The nature of the internucleotide bond was specified by Todd and coworkers.
1948--	Hydrogen bonding between the bases was proposed by Gulland and coworkers to explain the titration curves of DNA.
1948 On --	The quantitative relationships of the purine and pyrimidine bases in DNA were determined by Chargaff and Wyatt.
1949--	Furberg postulated a single-stranded helix for the structure of DNA and theorized that in a nucleoside the sugars and base pairs had to be perpendicular.
1951 On --	The α-helical structure for the polypeptides was established by Pauling and Corey using X-ray diffraction and molecular modeling.
1951-1953--	An X-ray diffraction study of hydrated DNA performed by Franklin and Gosling showed that phosphate-sugar backbone was external and the bases internal, and indicated that the molecule was double- or triple-stranded and had a dyad axis of symmetry.
1952--	The genetic significance of DNA was confirmed by Hershey and Chase.
1953--	The correct structure of DNA was presented in the paper *Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid* published in Nature by Watson and Crick. (1, 2, 3, 4)

has a diameter of 20 A with a repeat distance of 34 A occurring every ten nucleotides. The phosphate moieties are located on the outside of the structure and the base pairs faced the inside of the helix. The structure of the base pairs held together by hydrogen bonds are shown in Figure 1 (2, 3). The only constraint on the nucleotide sequence was that the sequence of the second strand must be complementary to the first (4). A schematic drawing of DNA strands showing part of the chain sequence may be found in Figure 2 (1, 2, 3, 4, 6).

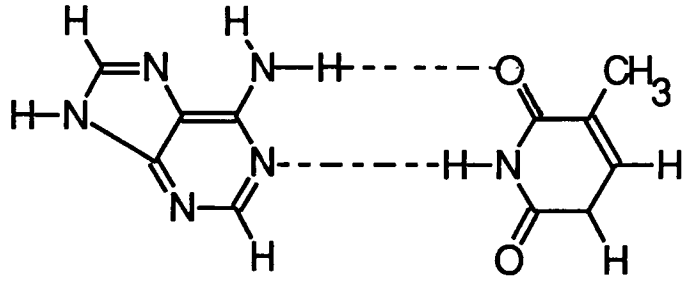
DNA functions as a template for replication and transcription of genetic information in cells. It can also provide a one dimensional surface that can take part in molecular reactions and direct diffusion of large molecules (7, 8, 9, 10). It is also instrumental in protein synthesis within the cell. All cells contain at least one DNA molecule that has a molecular weight around $5x10^6$. In the average mammalian cell the total DNA weight is approximately $7x10^{12}$ daltons (6).

(B.) Intercalating Dyes

Many drugs that have planar, aromatic structures can bind to DNA by insertion between base pairs. This process is often referred to as intercalation (11, 12, 13, 14). Treatment with these drugs can inhibit polymerase reactions by DNA and cause metabolic changes and even death in the cells and organisms. There are a number a



Guanine Cytosine



Adenine Thymine

Figure 1. Schematic drawing of Adenine, Thymine, Cytosine, and Guanine bound through hygrogen bonds

Schematic Drawings of DNA

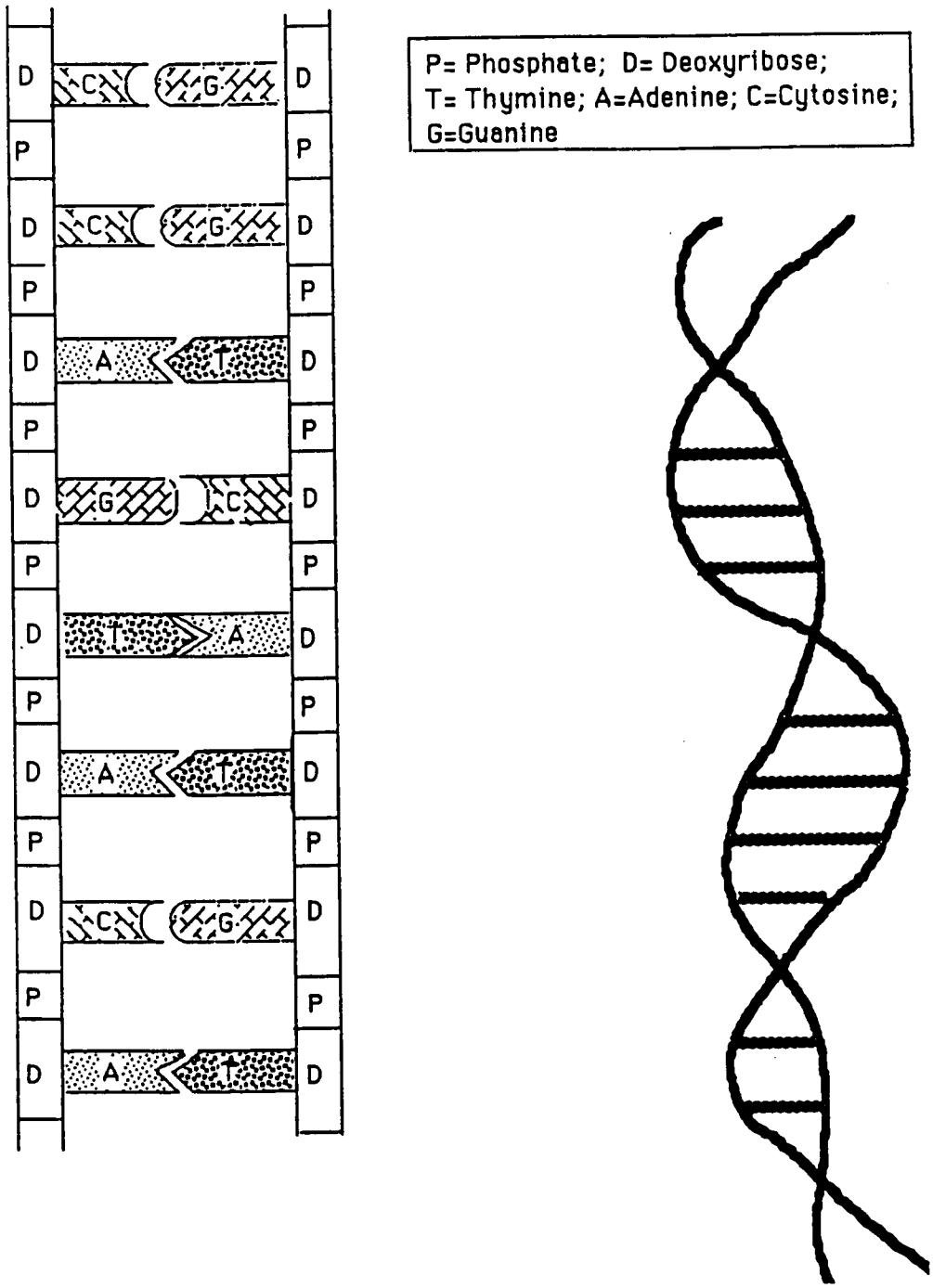


Figure 2. Representation of the structure of DNA showing both the base pairing and helical quality

intercalating drugs that have been studied by various investigators such as dimidium, 9-aminoacridine, quinacrine, proflavin, adriamycin, actinomycin D, ethidium bromide (2,7-Diamino-10-ethyl-9-phenyl-phenanthridinium bromide), DAPI (4'6'-diamidino-2-phenylindole), and Hoechst 33258 (2-[2-(4-hydroxyphenyl)-6-benzimidazolyl-6-(1-methyl-4-piperazyl)-benzimiazol·3HCl)] (14, 15, 16, 17). The use of many of these compounds has been established for chemotherapy (14). Ethidium and Hoechst 33258 have been used for various DNA studies because of their fluorescent properties upon intercalation (15, 18).

The structure of the ethidium molecule features a planar tricyclic phenanthridium ring and a secondary phenyl group perpendicular to the primary ring system (14, 19). The primary mode of binding tc right-handed double stranded polynucleotides is by intercalation between adjacent base pairs. This process has been characterized by a large binding constant, 2.1 x 10^6 M^{-1}, rapid kinetics (milliseconds), and has been shown to have the following sequence preference in both ribo- and deoxyribooligonucleotides: pyrimidine-(3'-5')-purine> purine-(3'-5')-purine> purine-(3'5')-pyrimidine (19) . Ethidium bromide (EtBr) has also been widely used as a histological stain for nucleic-acid-containing structures in cells (20, 21, 22). As a drug, ethidium bromide exhibits a number of detrimental biological effects such as the inhibition of DNA replication, transcription, recombination, self-splicing, and ribosome function. The compound also shows potent antiviral activity related to its ability to inhibit retroviral reverse transcription (8, 14, 19).

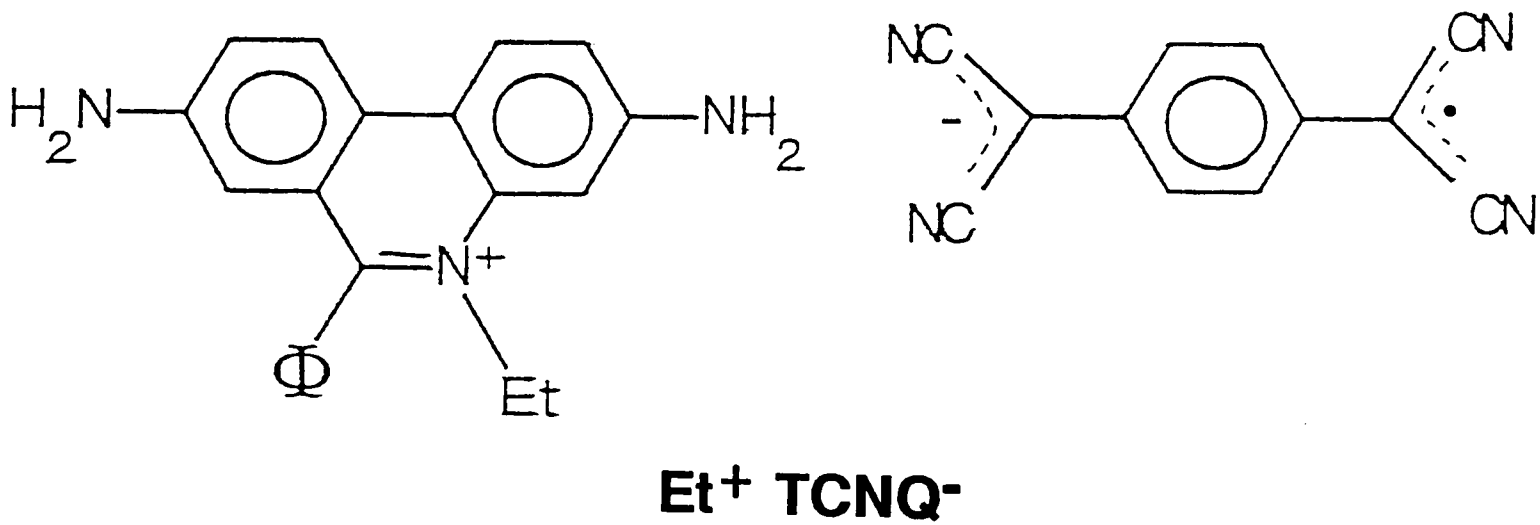
The fluorescence of ethidium is enhanced 50-fold upon intercalation into double-stranded nucleic acids (20, 21, 23, 24, 25, 26, 27). It has been proposed that the rate of intersystem crossing of ethidium is modified upon binding with DNA and that the fluorescence properties of ethidium are dominated by intersystem crossing to a triplet level nearly degenerate with the lowest singlet (29). This phenomenon is the result of the removal of quenching by the polar solvent when the dye is absorbed in the hydophobic region of the double helix structure (20, 21).

Ethidium binds both to DNA and RNA (19, 28). Other compounds such as Hoechst 33258 exhibit similar characteristics (15). Circular dichroism of the DNA-ethidium complex has been studied in an effort to determine the mechanisms of binding and fluorescence enhancement (29). Fluorescence ~~can be~~ a powerful tool for the detection of DNA because of the strong signal given off by the DNA/ethidium complex. DNA which absorbs little above 300 nm is basically nonfluorescent when excited in the visible range of the spectrum (30). The complex, however, can be excited near 500 nm (8, 31) and has a broad emission band around 600 nm. The polynucleotide(ethidium)$_n$ intercalation complex fluoresces at approximately 600 nm after excitation at 514.5 nm (21, 32, 33). Ethidium has been shown to provide excellent analytical sensitivity for the detection of DNA without a great deal of sample manipulation and was therefore chosen for the method presented below.

(C.) Carbon Paste Electrodes

Carbon pastes have been used as working electrodes in a variety of formulations since originally introduced by Adams in 1960 (34). These electrodes, consisting of graphite particles bound together by an inert and usually organic liquid such as mineral oil, display low background currents under voltammetric conditions. A variety of compounds or materials can be included in the formulation of the carbon paste to alter the properties of the working electrode. In these applications, the graphite particles, which are usually necessary to make electrical contact, function as an array of ultramicroelectrodes.

The main disadvantage of this type of electrode is its non-reversible surface. Because the chemical concentrations at the surface become exhausted, the surface must be renewed periodically. In the experiments that follow this problem was alleviated to a degree, but the surface had to be renewed between experimental series. Incorporation of ethidium-tetracyanoquinonedimethane (EtTCNQ) into the carbon paste permitted the amount of ethidium released to be controlled electrochemically. Application of an appropriate potential induced the release of Et^+ by oxidizing $TCNQ^-$ to the neutral compound. The $TCNQ^-$ counterion was chosen because of the ease with which it can be oxidized and the characteristically low solubility in water of the neutral TCNQ molecule (35, 36). The Et^+TCNQ^- structure is shown in Figure 3.



Et^+ $TCNQ^-$

Figure 3. Structures of ethidium cation and $TCNQ^-$ anion

(D.) Fluorometric Detection

Fluorescence, defined as a radiative transition between electronic states of the same multiplicity, usually involves transitions from the electronically excited state S_1 to various vibrational levels in S_0 (S_1---> S_0 + hν). Typically fluorescence requires 10^{-9}-10^{-7} seconds to occur (37) and usually exhibits only one fluorescence band (38). Because of factors such as solvent reorientation, the fluorescence radiation given off is lower in energy and appears at longer wavelengths than that of the excitation. Besides this characteristic, the fluorescence spectrum often is an approximate mirror image of the absorption profile (37, 38).

Fluorescence is traditionally measured against a black or near zero background resulting in high sensitivity and has thus assumed a major role in the determination of trace contaminants. A quantitative relationship between fluorescence intensity and concentration is derived from Beer's law:

$$P_F = \Phi_F K P_s \varepsilon bc \qquad (1)$$

where P_F is the florescence power, Φ_F is the fluorescence efficiency, K is a parameter representing the amount of light collected and detected, P_s is the excitation power, ε is the molar absorptivity of the analyte, b path length, and c is concentration (37, 38, 39, 40).

A laser was chosen as the excitation source for its power and stability in the wavelength range of interest. It also has a collimated beam. Under most conditions, the photoluminescence signal is directly proportional to the incident radiant power; this is sometimes called the fluorescence advantage. In the experiment below, an

attempt to take advantage of this characteristic was made. Furthermore, the collimated beam enabled excitation in a small volume and allowed easy imaging on the small slits of the monchromator.

(E.) Importance of DNA Measurement

As aforementioned, DNA molecules carry the information necessary for the biosynthesis of proteins. Because all life forms contain DNA, the ability to detect it is directly related to the detection of living organisms on the microscopic level. The detection of microorganisms in produce and canned goods could signal spoilage or contamination. There is also need for DNA detection for medical purposes. An extremely DNA-specific technique that could be used as a blood stream probe for detecting particular viruses strains or mutated DNA strains found in cancer cells would be very much welcomed.

Many areas of the biomedical industry and nutrition-based corporations have a great need for detecting bioorganisms and specific quantities of DNA. The Food and Drug Administration and biopharmaceutical industry have adopted strict guidelines regarding product purity. Current regulations require that DNA contamination be limited to 10 pg/dose in mammalian cell culture products (41, 42). For many commercially available techniques the problem is not only one of sensitivity, but of complexity as well.

At present there are several ways of measuring DNA, some of which employ nucleic acid stains, gel electrophoresis (30, 43), radioisotopes (44, 45, 46), etc. A list of the most important characteristics that a method should have for DNA measurement are as follow: high sensitivity, rapid analysis time, fast and easy sample preparation, low toxicity, and inexpensive instrumentation and apparatus. A number of methods have been developed for the detection of DNA based on radiochemistry, spectrophotometry, and fluorimetry. While many of these have proven quite useful for DNA detection, none meet all the criteria set above.

Radioactive ^{32}P incorporated into the DNA structure coupled with a dot hybridization technique has been successful in determining amounts of nucleic acids in a sample mixture (45). Although this technique is extremely specific for particular chain sequences, it is not very reproducible and a detection limit has not been ascertained. The presence of radiolabled isotopes also makes this procedure both fairly complicated and hazardous.

Laboratory manuals suggest that for quantification of DNA or RNA, absorption readings should be taken at wavelengths of 260 nm. The 260 nm signal is used to calculate the concentration of nucleic acid in the sample. An absorbance of 1 corresponds to approximately 50 μg/mL of double-stranded DNA (42). Because absorption is proportional to concentration from the Beer's Law equation, $A = \varepsilon bc$, where A is absorbance, ε is molar absorptivity, b is path length, and c is concentration, the quantity of DNA in the sample can be easily estimated. While as little as 1-5 ng of DNA can be detected by this

method in the absence of contaminants that competitively absorb at that wavelength, this is rarely the case (47). In biological samples such as blood and urine, a number of compounds other that DNA absorb at 260 nm. Proteins, for example absorb strongly in this wavelength range (48), rendering this an unsatisfactory technique for real sample DNA detection.

Labarca and Paigen described a procedure that could be used for DNA detection in crude tissue samples. Here the deoxyribonucleic protein structure of the chromatin must first be dissociated. This technique employs the fluorochrome Hoeschst 33258 as a DNA stain. The fluorescence of the resulting complex was then measured. While this method reports a detection of 10 ng DNA as the limit, a significant amount of sample preparation and manipulation is required (15). Kung et al. reported a method of DNA detection that is sensitive to 2 pg with a 10% variation in the 10 to 200 pg range. This sandwich assay technique using two high-affinity DNA-binding proteins requires a 1 hour sample incubation for DNA complexation with biotinstreptavidin and anti-DNA-urease. This complex must then be attached to a nitrocellulose membrane by filtration. An elaborate electrochemical cell employing a light-addressable potentiometric sensor (LAPS) measured pH changes in this system. It was reported that the total assay time was about 2 hours (42). While this method is extremely sensitive, it is labor intensive and time consuming.

A number of methods using ethidium bromide as an intercalating dye have been proposed. Lange and Chambers took

advantage of the large fluorescence enhancement of the DNA-Ethidium complex to develop an electrode developed specifically for quantitative DNA measurement. They designed an electrochemical cell employing carbon paste as the working electrode in which EtTCNQ was incorporated into the paste. By applying a potential that oxidized the $TCNQ^-$ anion to TCNQ the ethidium cation was released from the paste mixture into a DNA solution placed in the cell. Laser-based excitation followed by fluorescence detection using a photodiode array system enabled detection of calf thymus DNA to ca. 3.8 ppm (mg/L) in the presence of a buffer (32, 33). While the limit of detection for this method was not particularly low, the electrode developed was quite innovative and served as a model for further study.

(F.) Research Purpose

The goal of the following research was to improve upon the methods developed by Lange and Chambers. As mentioned above, an electrode was designed which allowed quantitative detection of bulk DNA samples based on the fluorescence enhancement of the DNA-ethidium complex. Some specific alterations were made to the system discussed above which include a change in type of detection system and fabrication of a new cell.

It was hoped that from method improvement a lower limit of detection would be reached. For this purpose, many of the experimental parameters were optimized. The electrode composition

was studied, for example, in order to ascertain a suitable amount of dye to use with lower concentrations of DNA. Other parameters improved in this experiment will be discussed below.

II. EXPERIMENTAL

(A.) Instrumentation

During preliminary experiments, fluorescence signals were detected using a Spectrovision Model FD-300 fluorimeter. The first experiments employed a triangularly shaped electrode housing in hopes of collecting more of the fluorescence signal by directing it at a 90 degree angle. A Coherent Innova Model I-100-15 argon ion laser was then used to excite the fluorophore using the 514.5 nm line. A Jarrell Ash monochromator Model 82-410 was used with a Hamamatsu photomultiplier tube Model R212 housed in a Photon Technologies Inc., Model 01-512 housing. The intensity profiles for each sample were collected by chart recorder. Although this system improved the limit of detection, it had a number of inherent flaws which were eliminated by development of a new cell design and use of more sophisticated spectroscopic apparatus graciously made available by Dr. David C. White (Institute of Applied Microbiology).

A schematic drawing of the final experimental apparatus is shown in Figure 4. An argon ion laser (Spectra-Physics Model 2016-11, Mountain View, CA) was used as an excitation source (λ = 514.5 nm, 10-12 mW). The fluorescence signal was collected and collimated by a 3.6 cm diameter quartz lens with a focal length of 18 cm and focused onto the monochromator entrance slit by a 4.0 cm diameter quartz lens with focal length of 12.5 cm. An aperture (Newport) with a 2.4 cm inner diameter was positioned between the

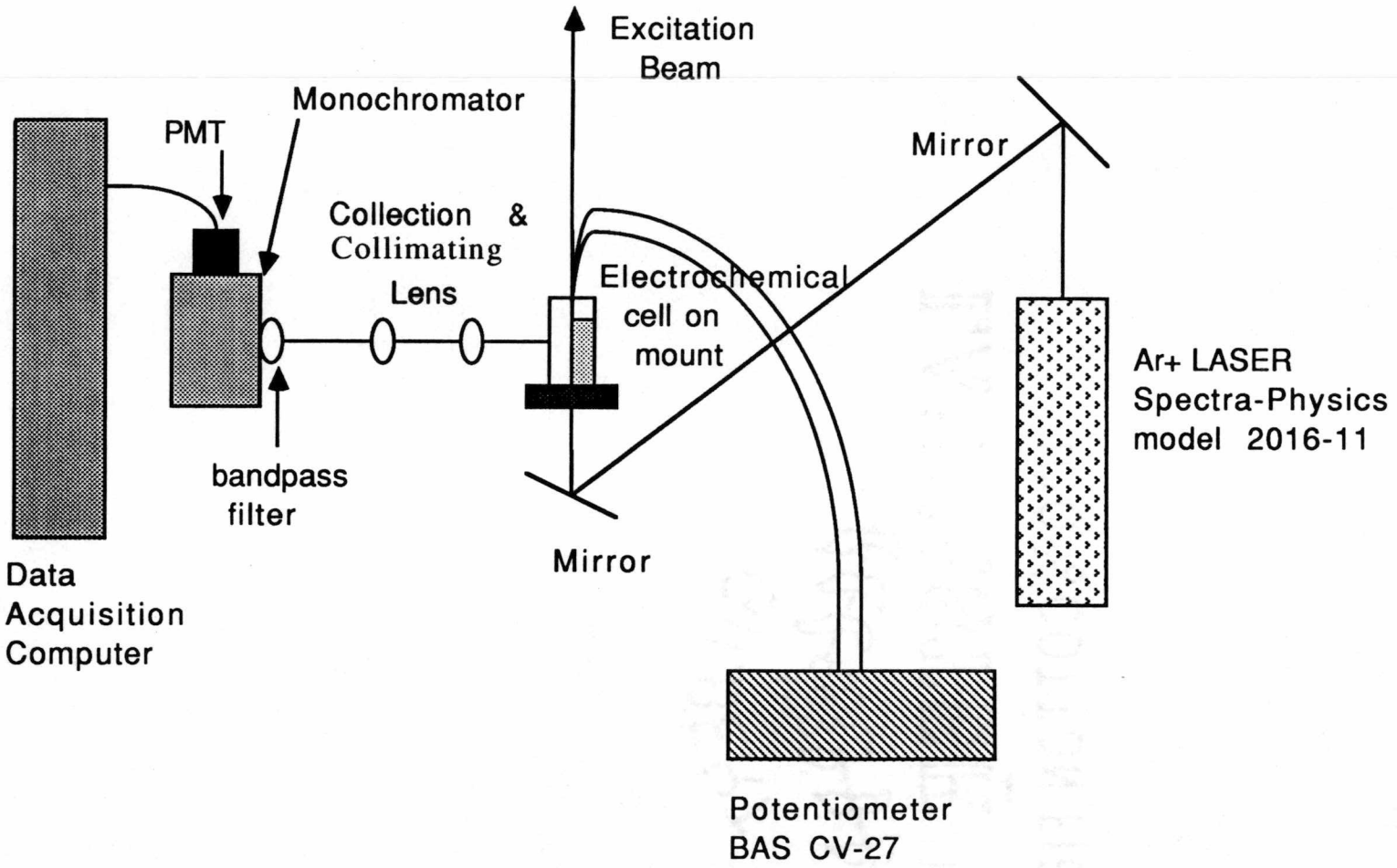


Figure 4. Block diagram of the experimental apparatus

two lenses. A high pass filter (Omega Optical Co.) with a 533 nm cutoff was placed after the second lens and in front of the entrance to the monochrometer. A monochromator (Instruments S. A. Scarsdale, NY Model HR640) with a grating of 1800 grooves/mm, resolution of 0.159 nm, and entrance and exit slits set to 100 microns isolated the 599 nm emission. Detection was performed by a photomultiplier tube (pmt) (Burle Model C31034-28) used in photon counting mode at 1800 V. To reduce the dark current noise, the tube was cooled to -25 C via a Galium-Arsenide cooling system (Peltier cooler) inside the pmt housing (Products for Research, INC Danvers, MA Model TE-104RS Chamber). An ACER 900 computer equiped with the Enhanced Prism software package version 1.3 (Instruments S.A./Jobin Yvon) received and manipulated the data which was then printed by a Hewlett Packard Colorpro plotter.

All weights were obtained using an American Scientific Products (SP) electronic balance Model 120. Dispo glass pipets (SP) were used for all solution transfers. A BAS CV-27 potentiostat was used to control the electrode potential. A simple pulse generator constructed from a monostable one shot circuit was used to trigger the potentiostat. The charge was monitored using a chart recorder.

(B.) Electrode Construction

The spectroelectrochemical cell, Figure 5, consisted of a Hellma 101-OS glass cuvette which held the electrode housing constructed of Delrin, a synthetic polymer obtained from Commercial Plastics,

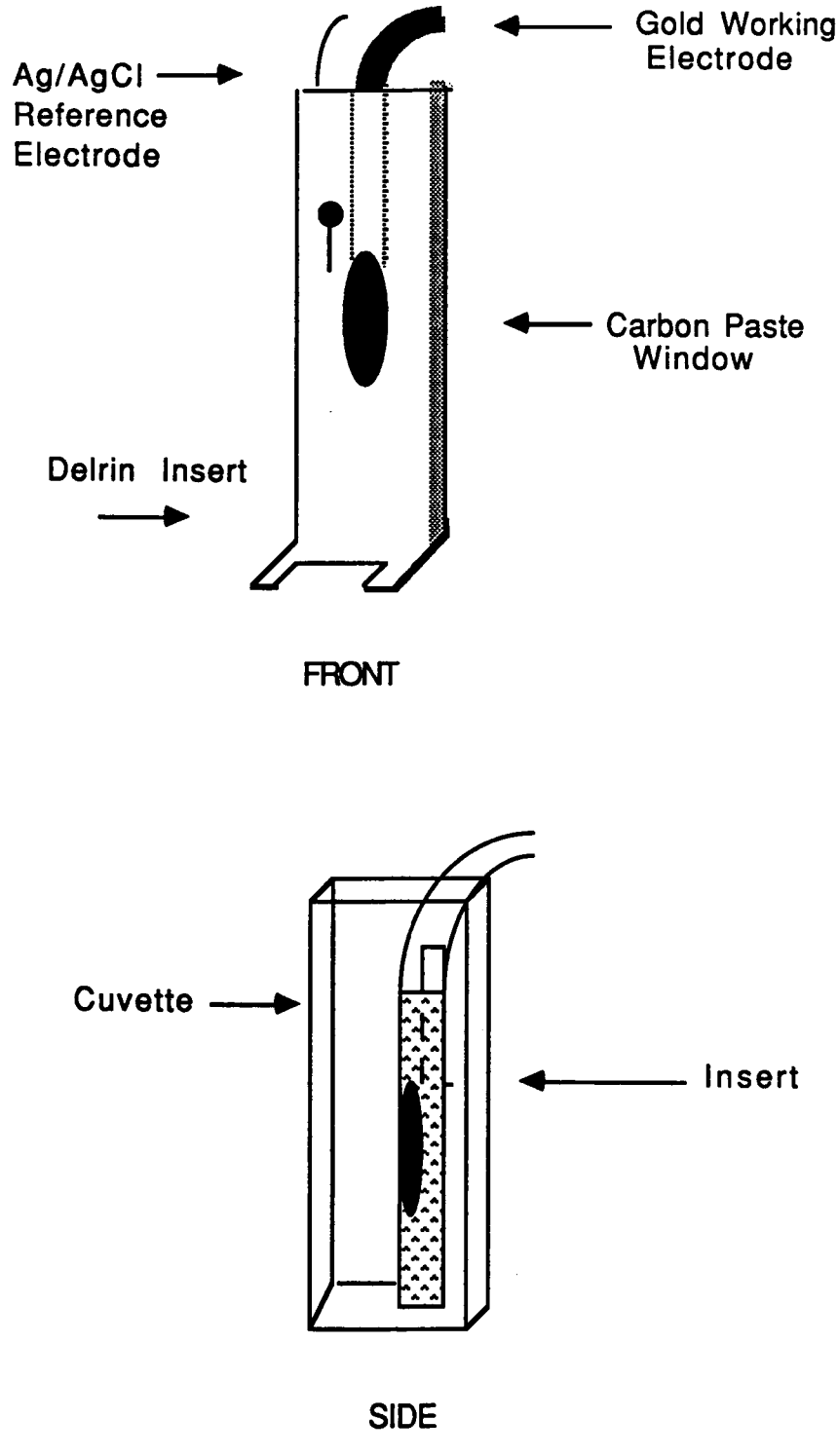


Figure 5. Diagram of the cell design and electrode orientation

Knoxville, TN. The 0.5 cm^2 working electrode area was oval in shape and located in the front face of the insert. A small hole was located 0.25 cm to the left of the oval window which housed the reference electrode. Gold wire (Aldrich 0.5 mm) was used for the auxiliary electrode and 0.5 mm Ag/AgCl wire for the reference electrode. Electrical connection to the graphite/Et^+TCNQ^- paste working electrode was made by gold foil onto which the paste was packed. The electrode insert was positioned in a clean (acid-washed) glass 1.0 cm^2 standard cuvette such that the back was flush with one of the walls. The gold auxiliary electrode was positioned in a channel between the glass face and the Delrin insert, and approximately 1/8 inch separated the Ag/AgCl reference electrode from the working electrode.

(C.) Chemicals

Carbon paste was prepared by thoroughly mixing 0.8 g of mineral oil (Squibb) with a 1.0 g mixture of various ratios of graphite (Scientific Products) to ethidium tetracyanoquinodimethane (EtTCNQ). The EtTCNQ was synthesized according to the procedure outlined by Lange and Chambers (32, 33) via the reaction of ethidium bromide (Aldrich) and LiTCNQ (13). The paste in the electrode was always released into either a solution of HEPES buffer or into DNA dissolved in the buffer.

Solutions of 10mM HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffered solution brought to concentrated

HCl were prepared using either double distilled water purified by a Millipore system or HPLC grade water (Baxter). DNA solutions were prepared from calf thymus DNA (Sigma, St. Louis, MI) dissolved in HEPES buffer solution. DNA did not readily dissolve into solution, therefore sonication for approximately 30 minutes or manual mixing followed by overnight refrigeration were the techniques commonly employed.

(D.) Overview of Experimental Method and Brief Theory

Electrode composition, excitation distance from electrode surface, potential application time, and laser power were the experimental parameters optimized for improved analytical sensitivity. The cell design (above) was modified from Lange and Chambers to enhance collection of the emitted fluorescence signal. The electrode was placed in a clean cuvette and approximately 2 mL of solution was used to fill the cuvette to a point above the working electrode surface. A potential of +0.45 V vs. Ag/AgCl was applied across the electrode to release the ethidium cation into the solution. As the Et^+ was released it combined with the DNA to form the fluorescent complex. A schematic of the experimental process occurring inside the cuvette is shown in Figure 6. The electrochemical release, mentioned briefly above, occurs when a potential pulse is applied to the carbon paste/Et^+TCNQ^-//Gold working electrode. A cyclic voltammogram (CV) of the complex is shown in Figure 7. The CV's displayed a broad irreversible oxidation

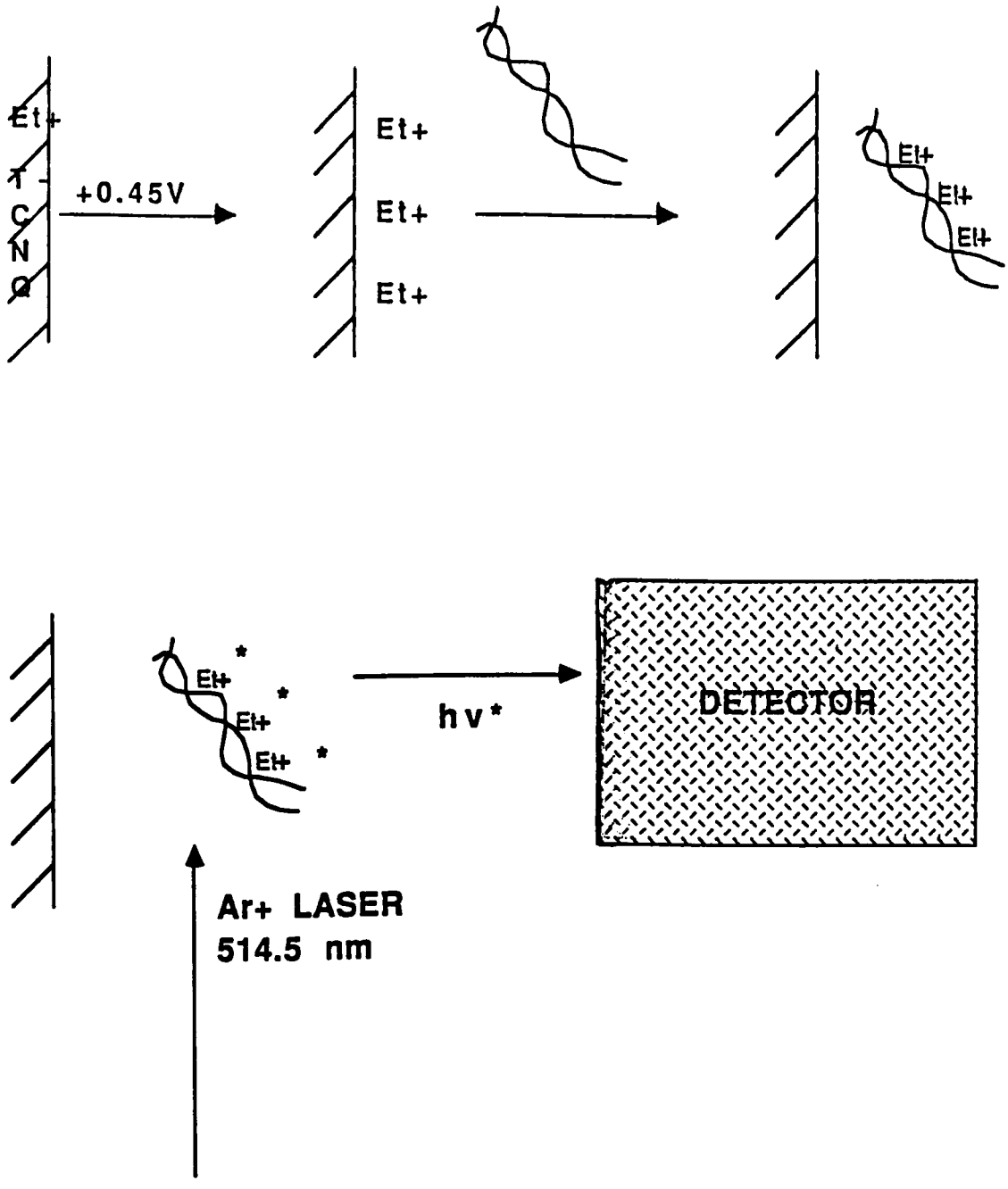


Figure 6. Schematic of the release and intercalation of the ethidium cation in the presence of DNA

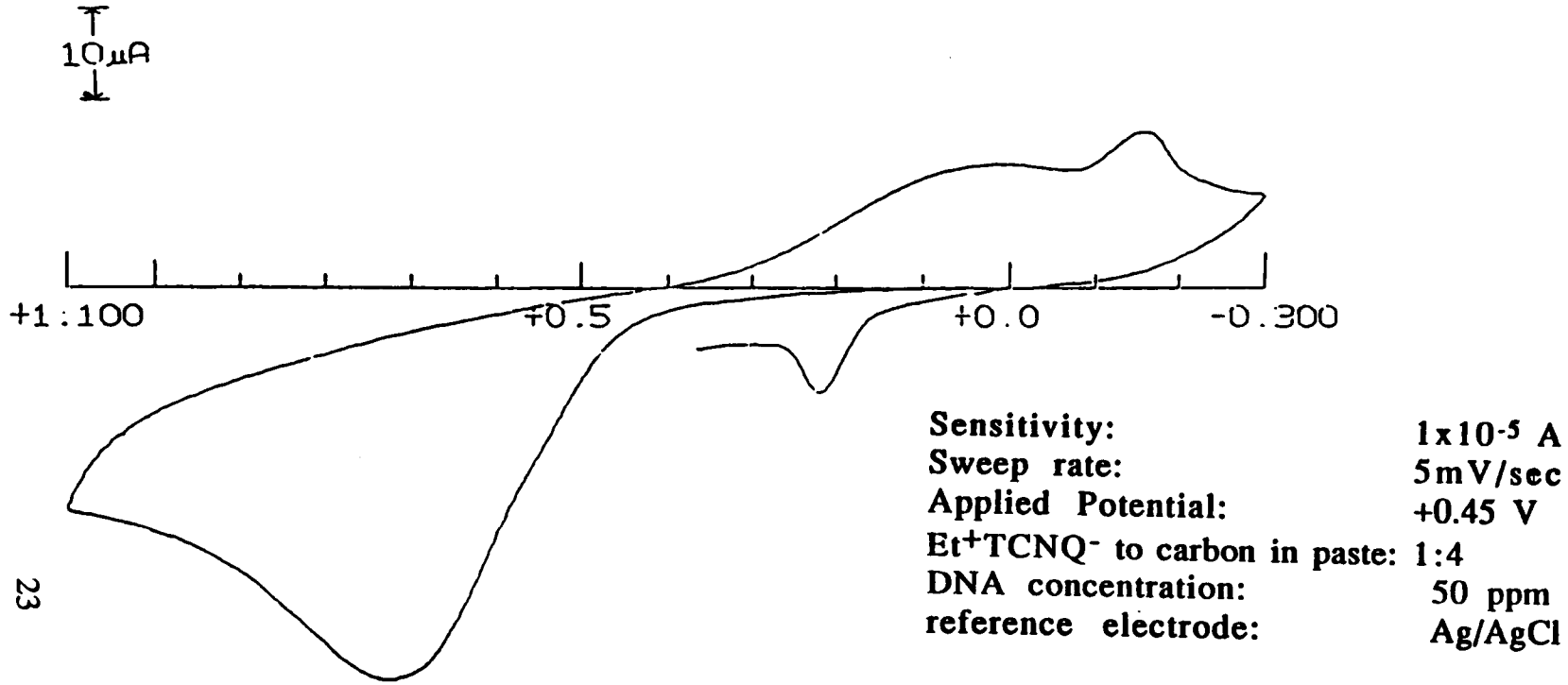


Figure 7. Cyclic voltammogram of the DNA-Ethidium complex

wave at ca. E_{peak}= 0.7 V vs. SCE at slow sweep rates. The peak widths were typically greater than 100 mV. On the reverse scan poorly developed waves appeared which were presumably due to reduction of TCNQ formed during the oxidation process. Lange found that this behavior was markedly dependent on the nature of the supporting electrolyte. In the present study electrorelease of the ethidium cation was effected by applying potential to the carbon paste electrode on the foot of the wave, E = 0.5 V vs. SCE where the electrode process was activation controlled. This caused minimal perturbation of the electrode solution interface such that the electrode could be used for repeated experiments. After potential application, the ethidium cation diffuses into the solution where it intercalates into the DNA molecules:

$$Et^{+}TCNQ^{-}_{(s)} \xrightarrow[0.45\ V]{} Et^{+}_{(aq)} + TCNQ + e^{-} \qquad (2)$$

$$\text{Polynucleotide} + nEt^{+} \longrightarrow \text{Polynucleotide}(Et^{+})_{n} \qquad (3)$$

In Figure 8 only the wavelength region around the peak maximum is shown which reveals the fine structure of the emission spectrum containing four major peaks. For this spectrum a solution of 5 x 10^{-5} M Et^{+} in 25 ppm DNA prepared by dissolving ethidium bromide directly into stock DNA/HEPES buffer solution was used. This scan was taken from 585 nm to 620 nm. The maximum intensity occurred at 599 nm, therefore the monochromator was set at this wavelength for all the experiments that follow.

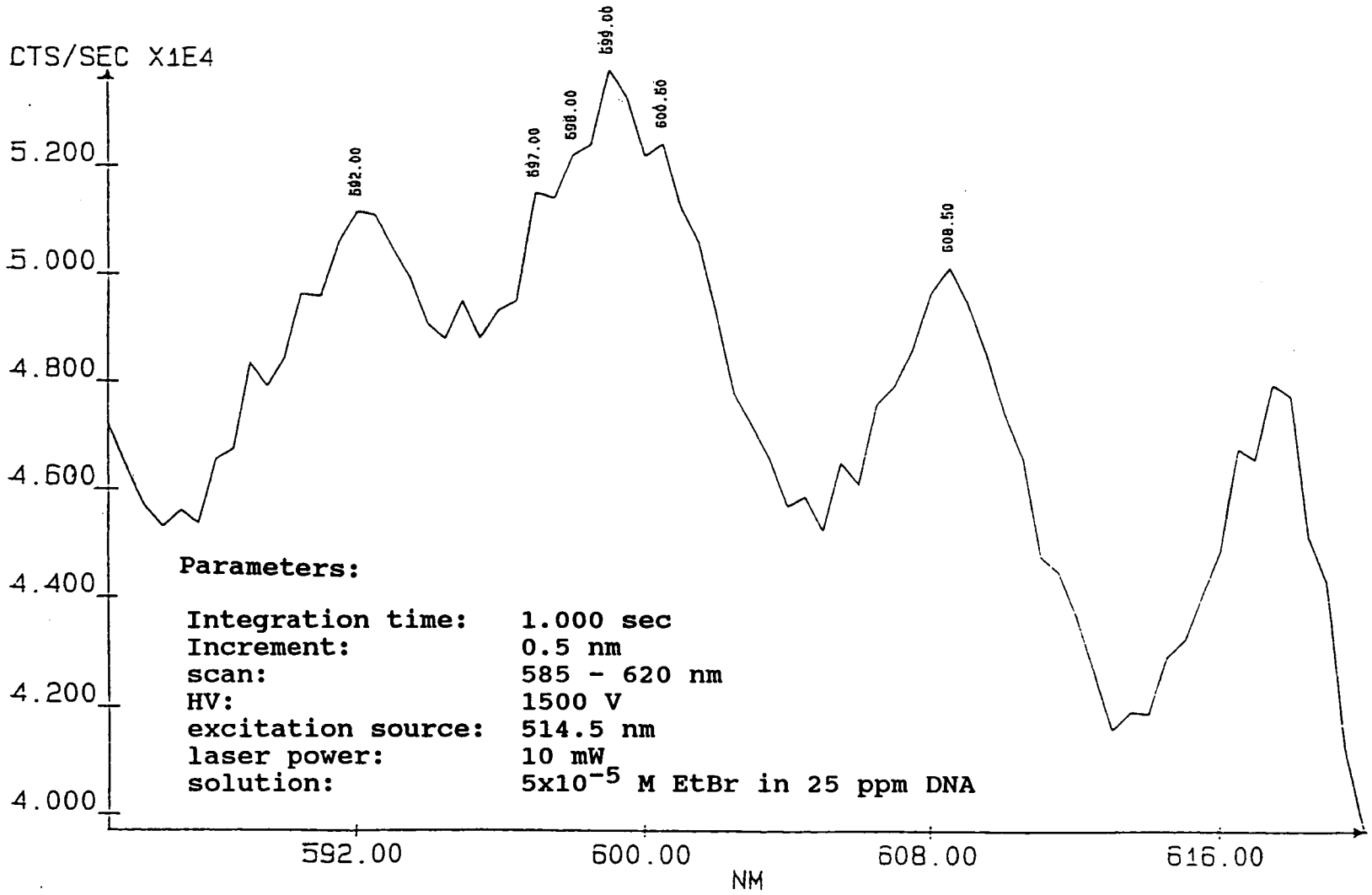


Figure 8. Fluorescence emission spectrum of DNA-Ethidium complex.

Figure 9 shows the typical fluorescence intensity response after +0.45 V is applied to the graphite/Et^+TCNQ^- electrode with a ratio of 1:20 EtTCNQ to carbon in the paste in the presence of 0.1 ppb DNA. The laser beam was aligned parallel and positioned very close to the electrode surface. There was an approximately 5-10 second delay for the complex to reach the excitation beam after release of the cation. The small increase in intensity from a few hundred to about 2000 counts per second is caused by background noise associated with solution impurities when the shutter is initially opened. This region is called called t_0. The sudden rise (t_{onset}) marks the consequential release and DNA intercalation of the ethidium cation upon application of the 0.45 V pulse. The maximum peak value corresponding to time (t_{max}) preceded a drop in intensity as the ethidium concentration wave passed through the excitation beam. The onset of the intense fluorescence signal was in accord with Fick's law of diffusion and the equation for the diffusion layer thickness:

$$t = \delta^2 / \pi D, \qquad (4)$$

where t is the transient time, δ is the distance from the electrode surface to the excitation beam, and D is the diffusion coefficient for the released ethidium cation.

Several experiments were performed with the excitation laser beam position at relatively large distances from the electrode surface. In each case, considerably longer times were required for the orange fluorescence to develop. For $D = 2 \times 10^{-5}$ cm^2/sec and d = 0.2 cm, a transient time of 600 sec was calculated. This is in qualitative agreement with our observation of 580 sec (data not

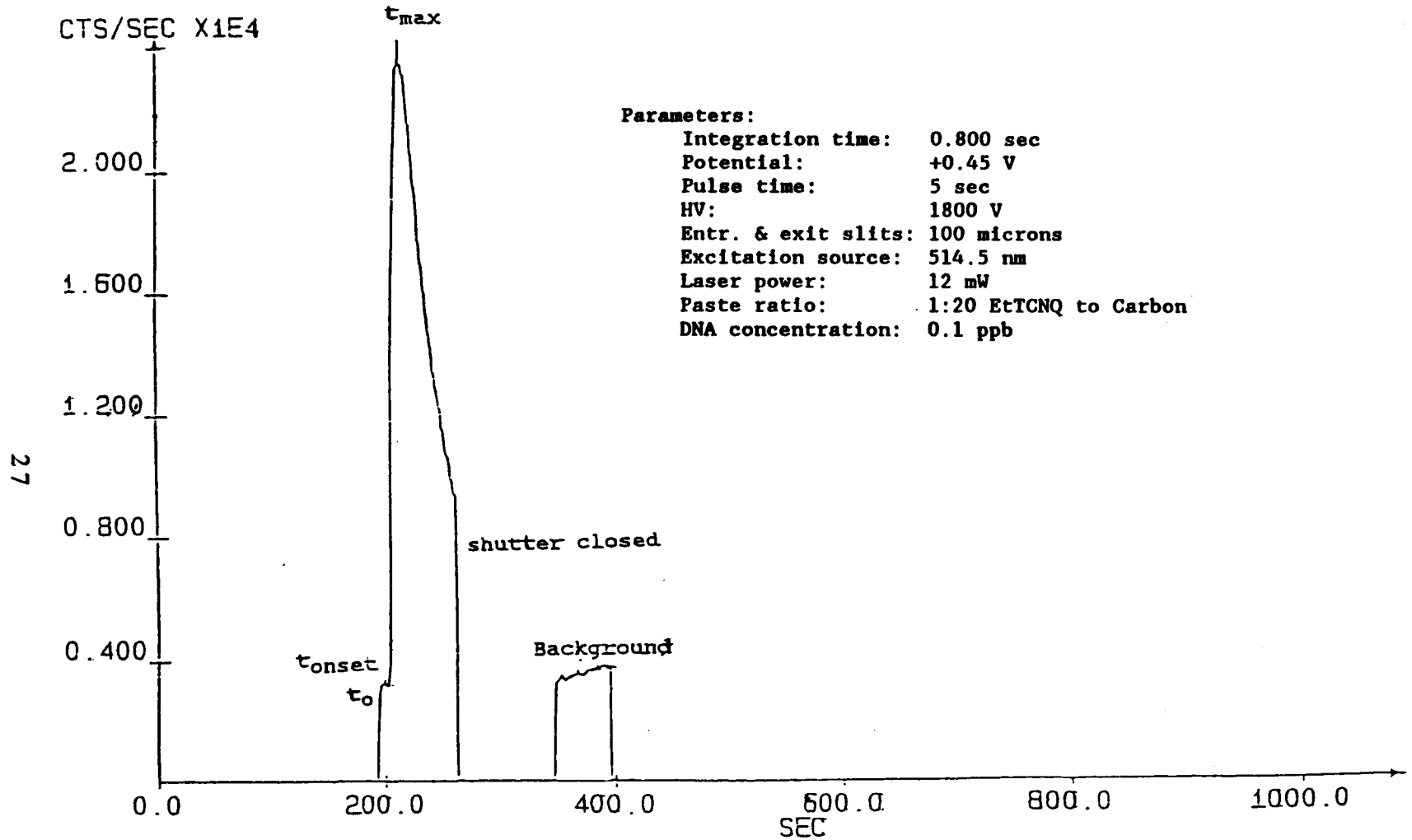


Figure 9. Typical fluorescence response.

shown). Unless otherwise noted, experiments were performed using the methods and parameters specified below. Electrodes were prepared by removing previously used paste and packing fresh paste onto the gold foil. The surfaces were smoothed manually using a scraping motion with a spatula. Each data point presented in the results that follow represents the average of the maximum intensities (at t_{max}) of three separate potential applications. These data were collected using an excitation power between 10 and 12 mW in accordance with the thesis of Lange (32, 33). The beam was aligned parallel to and positioned as close as possible to the electrode surface. A series of 3 potential applications in the form of 5 second pulses was made to a given electrode surface. Fresh solutions of either HEPES buffer or some concentration of DNA in HEPES buffer were delivered to the cell after each pulse application.

For all experiments that follow, a blank was run for each measurement taken. Blank intensities were measured to correct for the fluorescence caused by the uncomplexed ethidium cation whose broad fluorescence band overlaps the complex emission at 600 nm. Potential was applied to the electrode in the same fashion as in each experiment, except the ethidium cation was released into HEPES buffer containing no DNA.

For the pulse width study and those following, a correction factor for the data was made using the "cut and weigh" method on the intensity data. The chart paper on which the current responses were recorded was xeroxed and enlarged. The individual responses were then cut out with scissors and weighed on an electronic balance.

Surgical gloves were worn during the cutting and weighing exercises to reduce erroneous weight changes from fingerprint residue. Correction factors made by dividing the weight of an individual response by that of a 1 cm^2 piece of the xeroxed paper were multiplied by the maximum intensity of each peak. Figure 10 shows a typical current response for a given pulse. These corrections lead to a 10-15% RSD in the linear dynamic range.

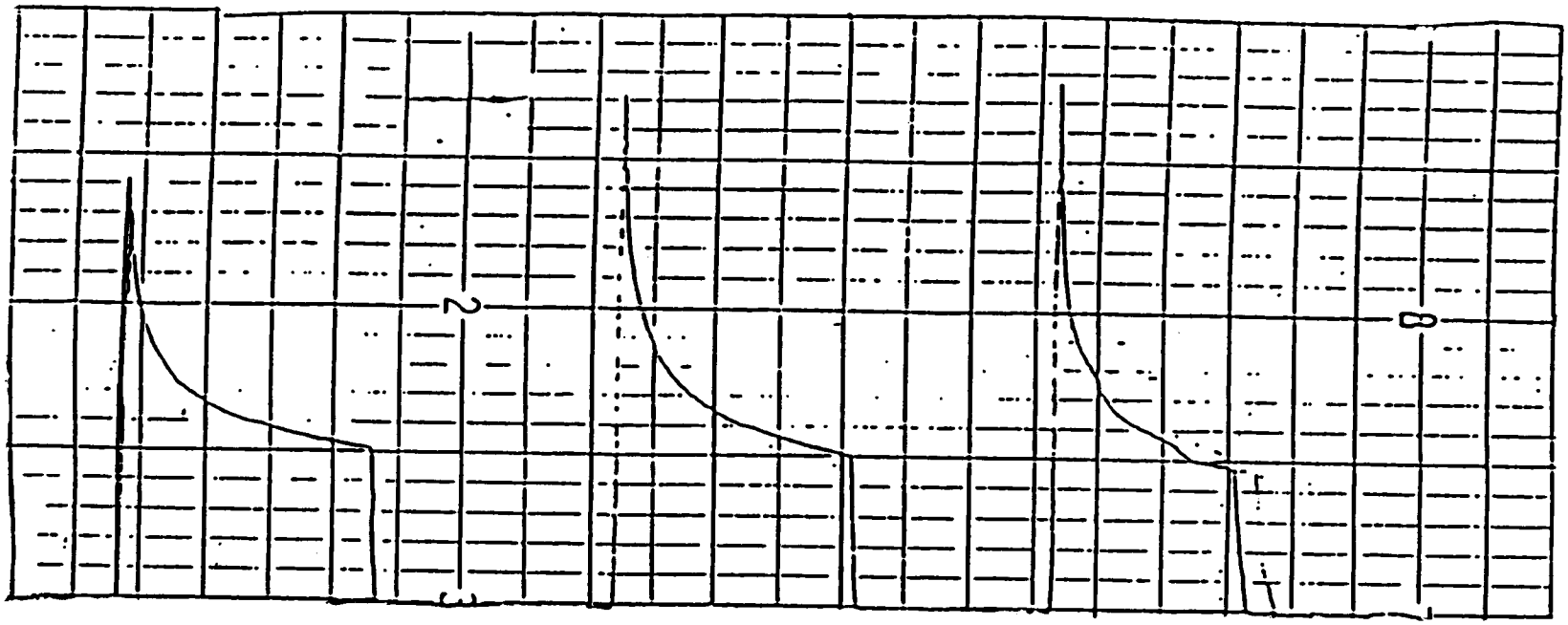


Figure 10. Chronoamperometric response for pulse generator. 5 second pulses, +0,45 V applied, 1 cm/sec chart speed, gain of 1 volt full scale, 1:20 EtTCNQ to carbon in paste released into 10 ppb DNA solution

III. PARAMETER OPTIMIZATION

(A.) Preliminary Experiments

1. Applied Voltage Study

In order to release an optimum amount of ethidium cation a study was performed to determine the best potential. Various potentials from 0.0 to +1.0 V were applied to a fresh electrode surface composed of a 1:9 ethidium electrode composition and a fresh solution of 25 ppm DNA. The intensity was measured using a fluorimeter. A triangularly shaped electrode housing was constructed to fit into a cuvette corner. This design was suitable for use in a fluorimeter. The results corresponded well with that of Lange and Chambers (32, 33) (data not shown). The optimum potential was between 0.4 and 0.5 V and was taken to be +0.45V. This potential was used throughout the remainder of the experiments to release the ethidium cation.

2. Laser Alignment

Various positions were tried in attempts to attain reproducible results in intensity measurements using the final experimental apparatus. To minimize distortion effects from the meniscus and to maximize the amount of fluorescence entering the monochromator

the beam was directed by two mirrors up through the bottom of the cuvette and parallel to the electrode surface. Originally the beam was brought down into the cuvette from the top using mirrors. It was found that this was not acceptable because the meniscus of the solution diffracted the light and changed the path direction. The second option shown in Figure 11 was then explored with success.

The optimum distance of the laser beam from the electrode surface was then determined. If the beam was aligned far from the surface (ie. 0.25 cm. away), intensity measurements took a very long time due to slow diffusion across the cell. Furthermore, convective effects were evident which resulted in marked fluctuations of the signal. Partial light blockage by the Delrin housing occurred when the beam was positioned too near the surface. The optimum distance was as close to the electrode surface as possible, but avoiding blockage by the Delrin housing. Although this position was achieved with success, an exact distance from the electrode surface was not measured. Because the alignment was very close to the electrode surface, it was hoped that small variations would not dramatically effect reproducibility. For a given set of measurements the electrode/cell/laser alignment was not altered. Thus, reproducibility depended on the operator's ability to reposition the Delrin housing in the cell after a new electrode surface was prepared. These distance variations however, were perhaps the greatest single source of error in this experimental technique.

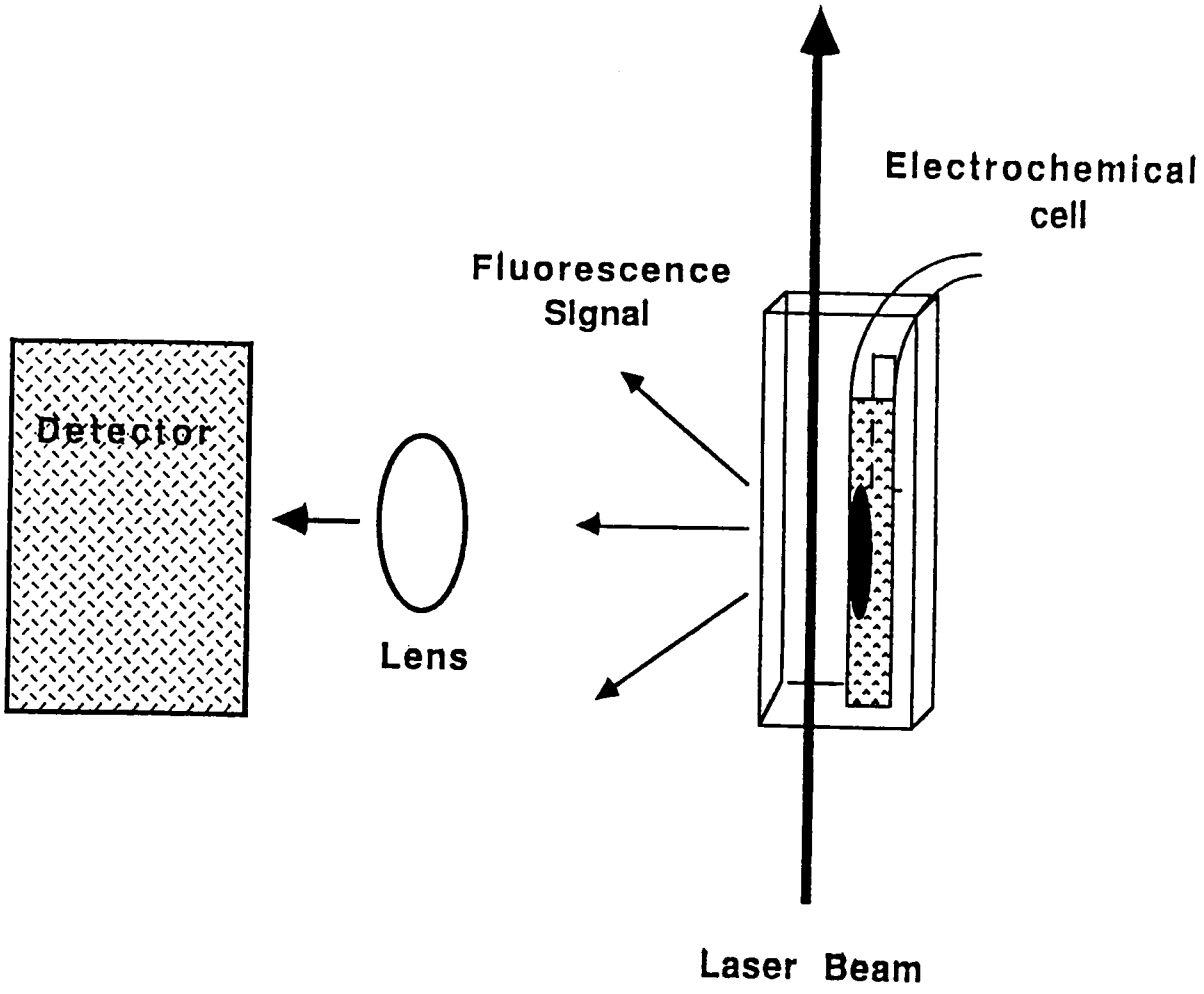


Figure 11. Laser alignment in the spectroelectrochemical cell

(B.) Electrode Composition Study

The composition of the carbon paste was varied to determine the optimum ratio for development of the fluorescence signal. Initially, ratios from 1:1 to 1:9 Et^+TCNQ^- to graphite were used in a 25 ppm DNA solution (data not shown). A fresh electrode surface was prepared before collection of each data point. While most of these ratios were suitable for 25 ppm DNA, at lower DNA concentrations the existence of excess ethidium resulted in a large fluorescent background. Furthermore, when releasing the 1:1 and 1:2 ratios into the DNA solution the excitation beam was absorbed to such an extent that the light could not travel through the solution.

A second study was performed to find a more suitable ethidium ratio for lower DNA concentration measurements. Carbon paste was prepared with ratios from 1:10 to 1:50 Et^+TCNQ^- to graphite and the intensities measured using $1x10^{-4}$ ppm DNA. The data are shown in Figure 12. The lower ethidium ratios performed much better for DNA detection. Because there was no flooding of the solution by the red cloud of ethidium all DNA concentrations were detectable. The 1:20 (Et^+TCNQ^-) to graphite mixture was chosen for further studies because it was sufficiently concentrated to give values over a wide range of DNA concentrations without flooding the solution with excess ethidium cation.

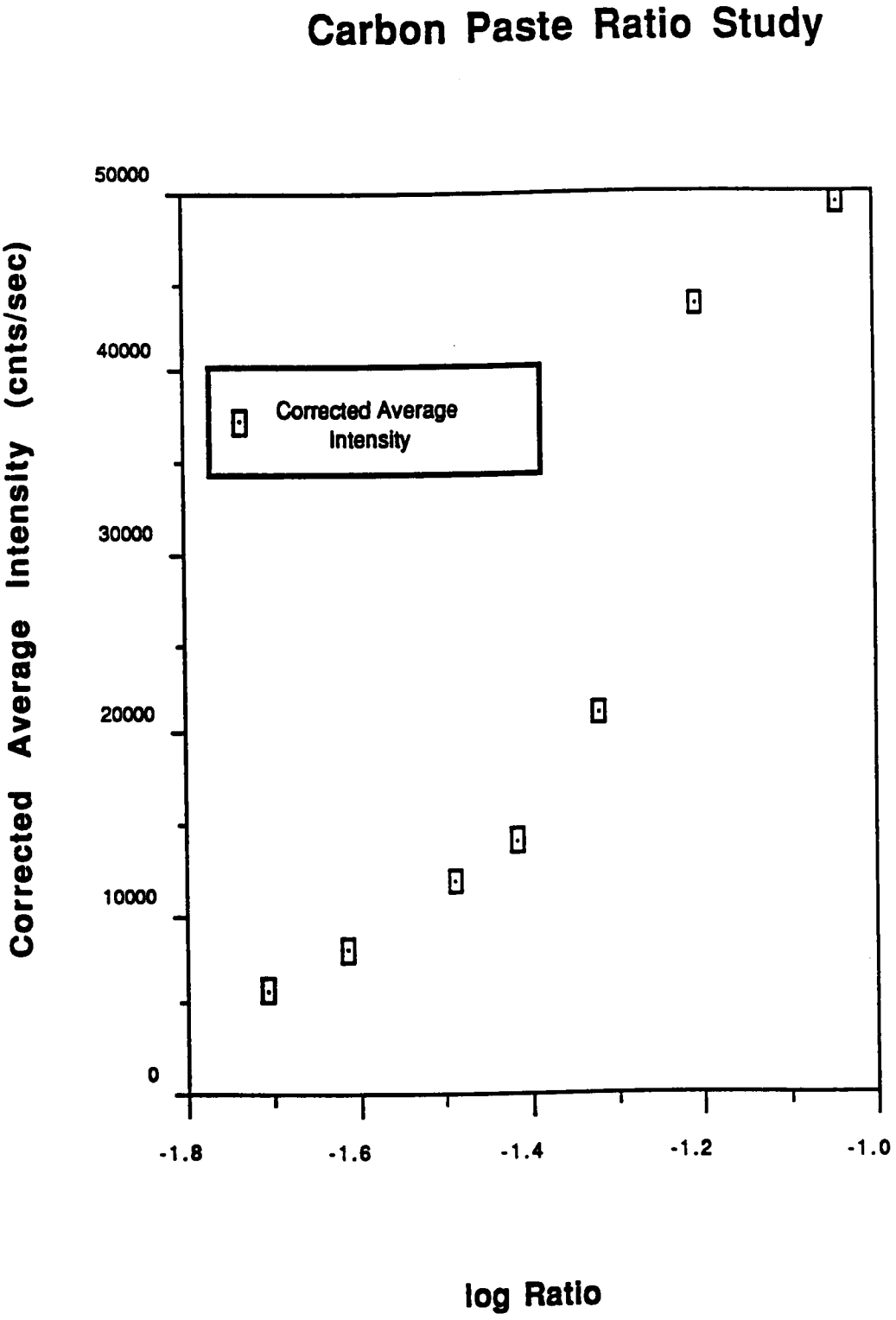


Figure 12. Graph of average peak intensity versus ratio of $Et^{+}TCNQ^{-}$ to carbon in paste

(C.) Electrolysis Time Measurements

Since the Et^+TCNQ^-/graphite electrode did not perform in a reversible or repeatable fashion, the electrode surface was renewed throughout a series of measurements. In order to shorten experimental time and to find a width that was both reproducible and released an adequate amount of ethidium, a pulse study was performed in which the width of the 0.45 V pulse was varied from 1 to 6.8 seconds using a 1:20 ratio of Et^+TCNQ^- to graphite in the paste in a 1 ppm DNA standard solution.

A simple one-shot circuit pulse generator was fabricated to allow application of a particular potential for various time periods. The pulse generator not only facilitated determination of the optimum pulse time, but also improved reproducibility of the applied pulse. Previously, potentials were applied to the potentiostat manually. The generator improved precision in that potentials could now be applied by the push of a button.

A series of +0.45 V pulses was applied to the electrode until the peak maxima decreased by more than 20%. The cell surface was renewed after each set of pulses. Peak intensities are shown in Figure 13 as a function of the pulse width and number of pulses per electrode. Although the peak heights for the shorter pulse widths did not decay as markedly as those for the longer widths, the signal to noise ratio was lower because each single peak height was lower. The peak heights are related to the amount of ethidium released. It was found that three pulses of 5 seconds each per electrode surface

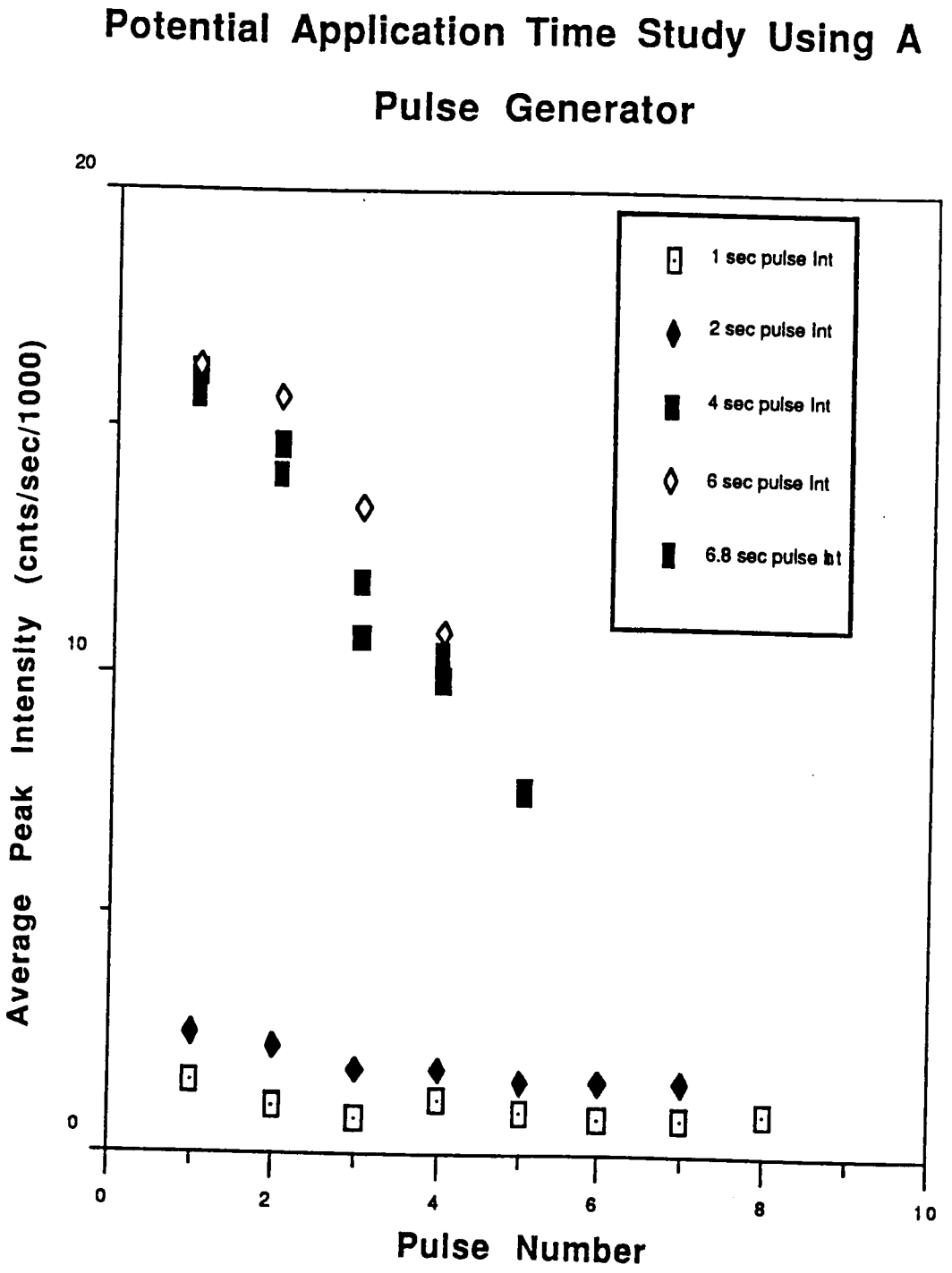


Figure 13. Graph of peak intensity versus pulse number. Parameters include: +o.45 V applied potential, 1:20 EtTCNQ to Carbon in 0.1 ppb DNA solution

produced both fairly reproducible results with a good signal to noise ratio.

Although the pulse generator was of significant use in collecting data, it had one major problem; it did not apply an exactly reproducible pulse width. It became apparent when averaging sets of data points that some of the peak maxima were significantly lower that others. To ascertain the problem, a chart recorder was attached to the potentiostat and the current (I_{out}) versus time (t) was recorded. Henceforth, the current vs. time profiles will be called charge (Q) responses since $I_{out} \times t = Q$. Occasionally the pulse generator would not apply a full 5 seconds of potential. When this occurred, less ethidium was released, and the peaks were not as large. The cut and weigh correction method, mentioned in the experimental section, was applied to these data.

Although this method was not a completely precise correction for the data, it improved the error in this experiment to an extent. The general conclusion drawn from this study was, although the pulse generator was a useful device for potential application, a more sophisticated instrument should be developed to send a consistent electronic signal to the potentiostat. With a new pulse generator, the error for this experimental data should be reduced.

(D.) Laser Power Study

In an attempt to exploit the fluorescence advantage, the complex was excited using a higher laser power. Increased signal to

noise ratio, and therefore greater sensitivity, was expected as a result of this laser power study. The effect of 12, 20, and 50 mW power levels on 1:20 ratios of $Et^{+}TCNQ^{-}$ to graphite paste in 1 x 10^{-4} ppm DNA solution, near the limit of detection for this system, and on a blank were tested. The pulse generator used in the experiment above was employed giving three 5-second +0.45 V pulses per electrode surface.

Figure 14 shows a graph of fluorescence signal versus laser power for both the complexed and uncomplexed ethidium cation. Both theblank and the complex showed greater intensities in a linear fashion with power. It was found that the error increased as well. Furthermore, rapid heating and thermal perturbation which cause changes in the diffusion front and distortions in the intensity profiles could be associated with an increase in laser power. Greater sensitivity was therefore not achieved at the higher power levels in this experiment.

Higher power did not result in increased sensitivity in part because of the lack of differences between the complexed and uncomplexed species. This could be associated with a large increase in background fluorescence caused by uncomplexed ethidium cation. Ethidium was released in excess to force the intercalation reaction to the right. Although most of the DNA sites were probably filled by the cation, there was such an excess of ethidium that differences in fluorescence between the DNA and non-DNA solutions were minimal. Releasing lower concentrations of ethidium into the DNA solution would probably not change the fluorescence behavior significantly

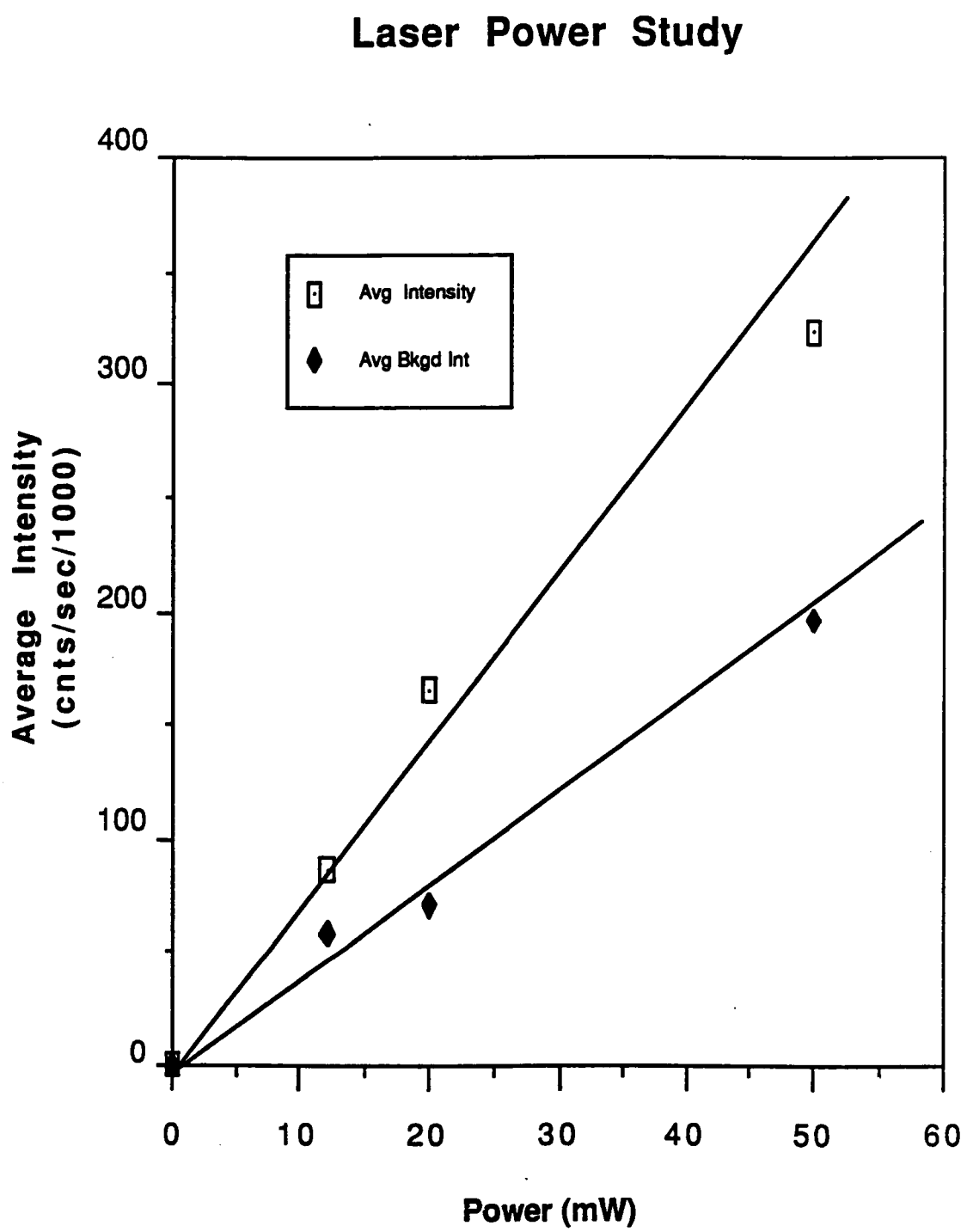


Figure 14. Graph of laser power vs. fluorescent intensity

whereas the signal for the uncomplexed ethidium would most likely decrease dramatically. Thus, better sensitivity at higher laser power should be achieved by reducing the $Et^{+}TCNQ^{-}$ concentration in the paste.

IV. DNA CONCENTRATION MEASUREMENTS

(A.) Calibration Study

Previously Lange and Chambers reported a limit of detection (LOD) of ca. 3.8 ppm DNA using a similar method (32, 33). A principal objective of the concentration study was to determine whether the sensitivity of this method could be improved by using the optical and spectral components described above. To that end, the response as a function of DNA concentration was determined for many experimental conditions.

Using the optimized parameters discussed above, the fluorescence intensities of a series of DNA solutions were measured. The first experiment measured concentrations from 10 to 0.05 ppm DNA in HEPES buffer (blanks were also measured for each experiment). The second study involved concentrations from 0.05 to 1×10^{-4} ppm. The third measured DNA concentrations from 10^{-4} to 10^{-7} ppm. Figure 15 is a graph showing the three studies grouped together. A correction factor was used to remove discrepancies caused by variation of the alignment of the electrode housing and fluctuating laser powers on the separate days. The correction involved multiplying the intensities of the first experiment by the ratio of the last intensity measured in the first set of data by the first intensity of the second. This type of calculation was applied to the second data set of numbers. While this graph spans a wide range of DNA concentrations with numerous data points enabling

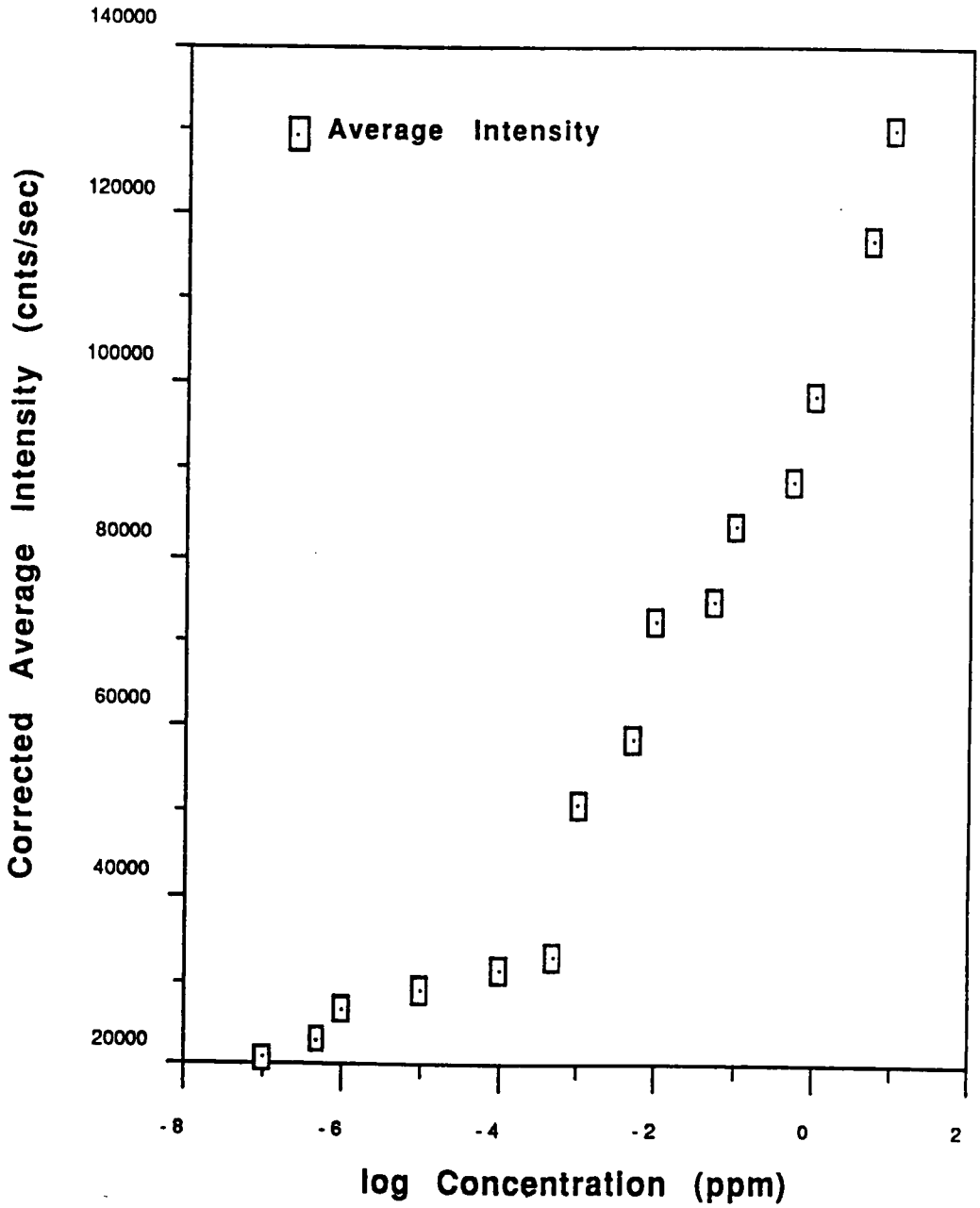


Figure 15. Graph of DNA concentration curve composed of corrected data from three separate studies

determination of the approximate limit of detection, it gives only fair precision due to rough data correction.

A second and more precise experiment was performed by measuring DNA concentration over a wide range with all parameters the same. Figure 16 shows a graph of the data obtained. Once the limit was reached, a second study spanning the entire range of concentrations explored previously was made and appears below. The reproducibility of data collection by this system is shown in Table 2. Intensities are shown for the points appearing in Figure 16 corrected only for background noise. The relative standard deviation lies between 10% and 15% for these data.

The two graphs shown below indicate that below approximately 0.1 ppb DNA the response becomes insensitive to the presence of DNA in solution. Therefore bulk concentrations of DNA in solution at the 100 picogram level were detectable. This corresponds to approximately 2×10^{-6} Abs_{260} units per mL and represents an improvement of more than four orders of magnitude over the results of Lange. Thus the sensitivity of the method is considerably better than traditional methods for the determination of DNA concentration and it is comparable with more elaborate methods based on sandwich assay and enzyme amplification techniques. Furthermore, this sensitivity level is achieved for analysis times less than one minute, a value compatible with a flow cell operation.

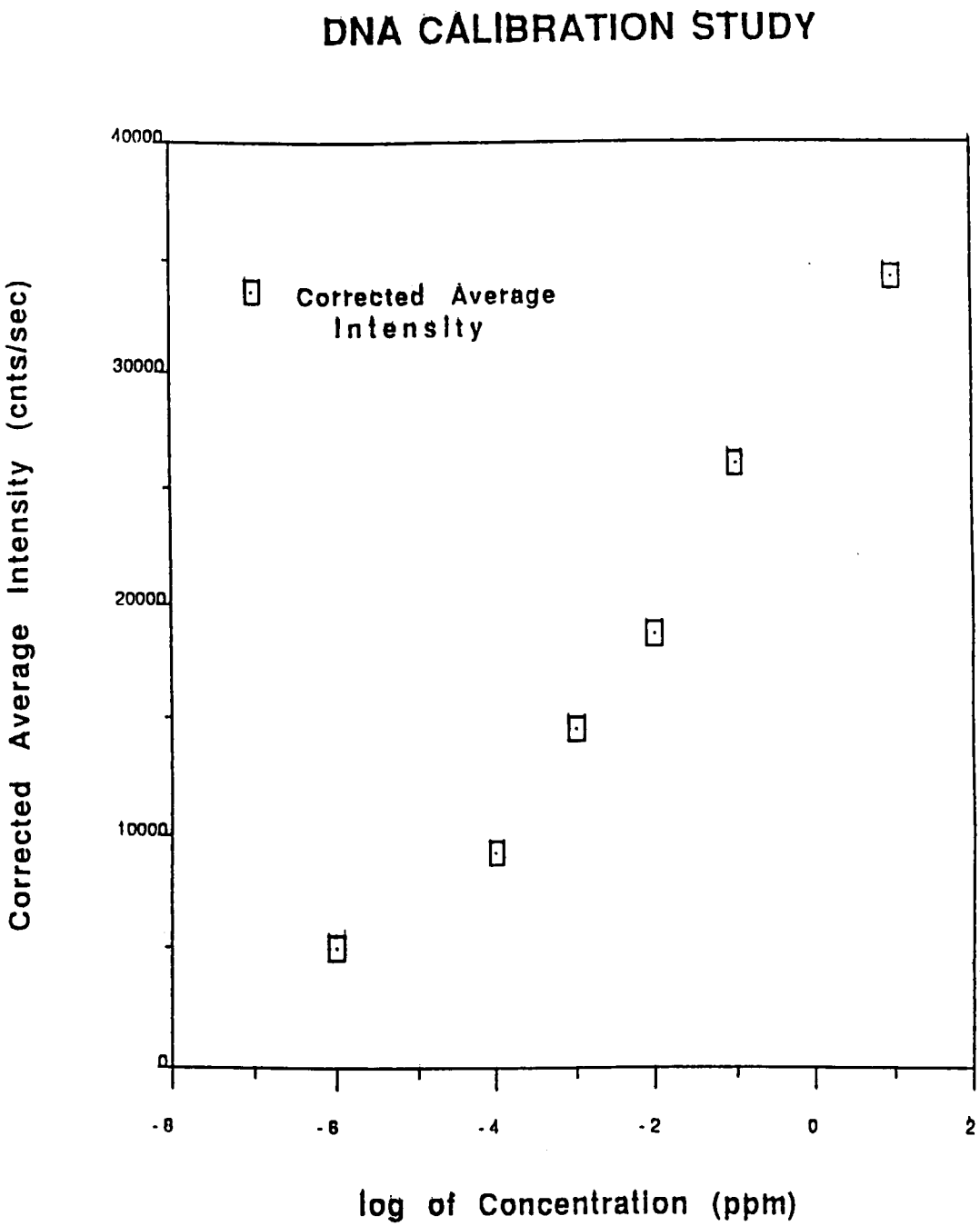


Figure 16. DNA concentration curve

Table 2. Reproducibilities of the data. *The intensity is the number obtained after subtrating the background noise from the peak maximum. These numbers have not been corrected for charge discrepancies using the cut and weigh method described in the text.

DNA Conc (ppm)	Intensity* (cnt/sec)	Average Intensity	Standard Deviation	Relative Std. Dev.(%)
$1x10^{-6}$	16446 15761 13205	15137	1708	11
$1x10^{-4}$	20870 19000 15891	18587	2515	14
$1x10^{-3}$	21843 25448 21438	22910	2208	10
$1x10^{-2}$	29833 28524 25396	27918	2280	8
$1x10^{-1}$	41588 34759 28200	34849	6694	19
10	42869 38921 3218	37991	5403	14

(B.) Real Sample Study

The electrochemical cell was tested on an actual, non-purified sample to determine feasibility for real sample DNA determinations. Tropicana(R) orange juice in a glass bottle was purchased from a grocery store. A sample from the juice was placed into a plastic container with a lid and allowed to spoil outside in the summer sun for two days. Another sample was placed in an identical plastic container with a lid and kept in a refrigerator for the same 48 hour period. Exactly one mL of each sample was then placed in a 25 mL volumetric flask and diluted to the mark with HEPES buffer prepared with HPLC grade water. The DNA detection experiment was then performed according to the same procedures used for the calf thymus DNA samples outlined above.

Figure 17 shows the results of the orange juice bacteria DNA samples. The first peak represents the refrigerated sample. The second peak is the spoiled sample. As expected, the peak for the spoiled DNA exhibits significantly more counts per second than the refrigerated one. It is hypothesized that this phenomenon corresponds to increased amount of bacteria and thus DNA in the spoiled sample. Although this experiment was not repeated, it implies that the electrochemical cell could perform well outside the laboratory on real life samples.

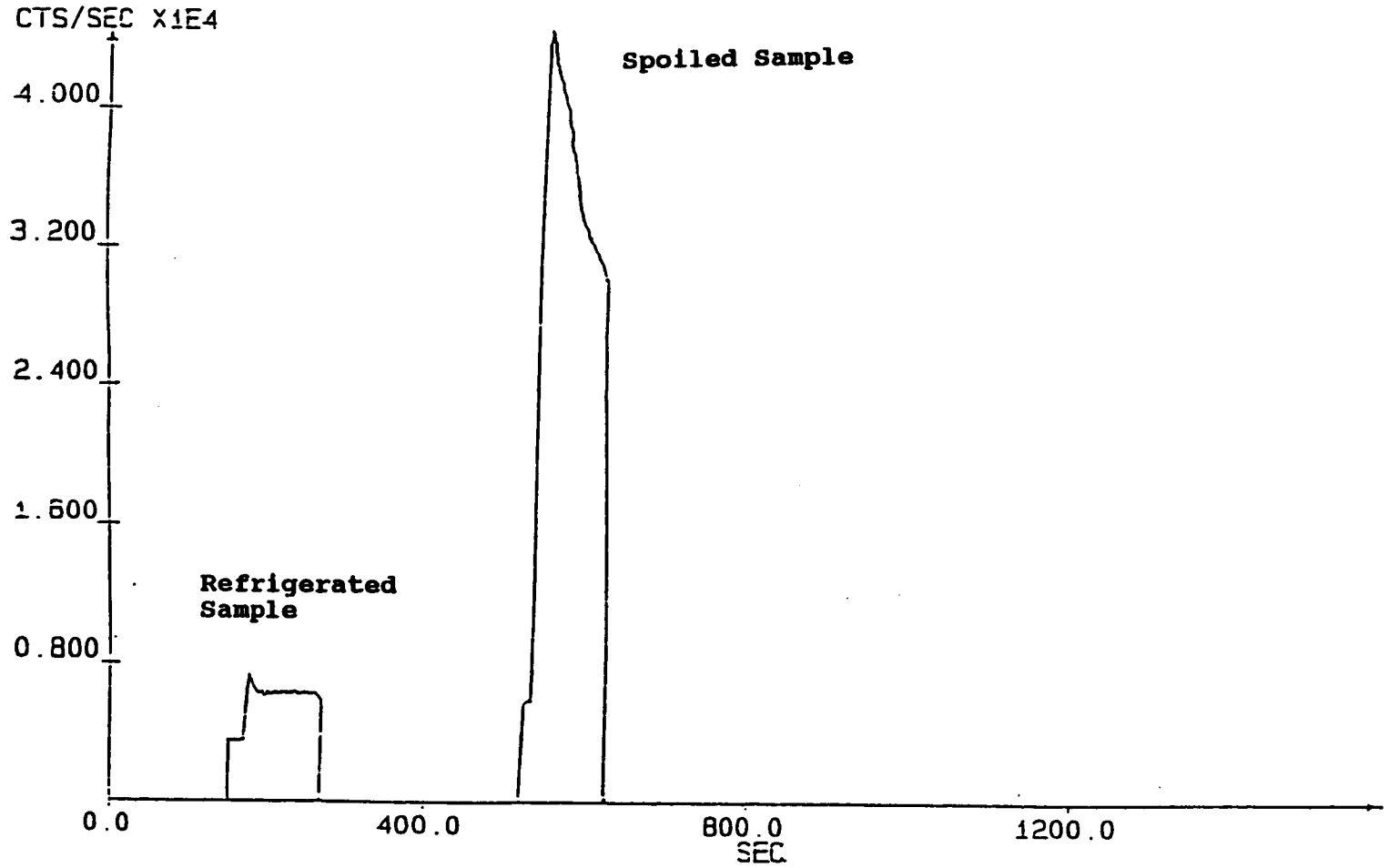


Figure 17. Real sample intensity profile using both spoiled and unspoiled orange juice samples

(C.) Conclusion

A rapid and very sensitive method for measuring bulk samples of double-stranded DNA has been presented using a specially designed spectroelectrochemical cell. A limit of detection at the 100 pg level within a 10-15% relative standard deviation of the log of concentration was achieved. The major sources of error were varied excitation beam distance from the electrode surface and inconsistent potential application pulse widths. At low concentrations of DNA ethidium may have overwhelmed the system causing large background fluorescence interference due to excess uncomplexed ethidium cation. While many of these experiments were fairly crude, the technique offers considerable promise for spatially detecting species released or formed in the diffusion layer. Furthermore, this method could be adapted to a flow cell design or coupled with fiber optic sensors or flow cell arrangements.

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VITA

Beverly Ann Hanes Swaile was the first born to Harold And Barbara Hanes on April 26, 1966 in Winston-Salem, North Carolina. Upon graduating from West Forsyth Senior High School in the spring of 1984, she entered Wake Forest University in fall of that same year. She graduated in May 1988 with a Bachelor of Science degree in Chemistry. While at Wake Forest she met David Frederick Swaile of Lakewood, New York whom she married on July 16, 1988.

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 and partially financed her efforts with a split research/teaching appointment. She successfully completed the requirements for a Master of Science degree in Chemistry in the Fall of 1990 and graduated in December.