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We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

# APPLICATIONS OF SURFACE-ENHANCED RAMAN SPECTROSCOPY FOR THE STUDY OF INDICATORS OF EXPOSURE TO SELECTED GENOTOXINS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Anne Marie Helmenstine

December, 1992

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## DEDICATION

This dissertation is dedicated to my husband Todd.

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## ABSTRACT

Surface-enhanced Raman scattering spectroscopy (SERS) was used to detect and analyze certain genotoxic chemicals, their metabolites, and selected deoxyribonucleic acid/ribonucleic acid (DNA/RNA) adducts that may be produced from exposure to specific toxins. Substances examined included nucleic acid (purinc/pyrimidine) etheno adducts of adenine and cytosine and carcinogens benzo[a]pyrene and aflatoxins. SERS was shown to be an effective tool for sensitive, selective detection and analysis of these genotoxins and related substances. Detection was accomplished even at such low concentrations as might be present in biological systems, and in minute volumes of approximately one microliter per sample. Most substances were found to possess unique spectral signatures and could be distiguished from other chemicals in this study. The sample molecules were also analyzed in body fluids (e.g. plasma, serum, and urine) and adducted to DNA.

In this study SERS was demonstrated to open a new dimension in chemical analysis not previously available by utilizing other spectroscopies or methods of chemical detection. Special attention was given to the various factors affecting the quality and reproducibility of a SERS spectrum, emphasizing the importance of optimization of Raman cross-scattering enhancement. Methods for adapting SERS to deal with specific research problems and for expanding the capabilities of SERS to address the health effects from human exposure to toxic pollutants were addressed in the concluding remarks section of this manuscript.

# PREFACE

There were several possible approaches to these research problems. In keeping with the author's diverse background, the experimental problems were dealt with in an interdisciplinary fashion. This study was not intended as a comprehensive probe into any single topic or chemical system. Many additional experiments could have been performed to support or supplement the data presented here. A compromise had to be made, however, between completeness and conciseness. Ample spectra were presented to clarify the statements made in the text, but space restrictions prevented full exploration of all aspects of the research problems addressed here. The main purpose of this dissertation was to establish the different technologies which may be developed using SERS to detect and analyze biologically important substances. Despite the length of this work, the basic findings are tantalizingly brief. Many more genotoxins remain to be examined with SERS; those examined here may be analyzed more thoroughly.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Adenine |
| Ab | Antibody |
| Ag | Antigen |
| b | Broad |
| B[a]P | Benzo[a]pyrene |
| ben. | Bending |
| BG | Substrate and Optics Background |
| BGY | Bright Greenish-Yellow Fluorescence |
| BPDE | r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene or Benzo[a]pyrene Diolepoxide |
| BPT | r-7,t-8,9,c-10-Tetrahydoxy-7,8,9,10-tetrahydrobenzo[a]pyrene or Benzo[a]pyrene Tetraol |
| C | Carbon |
| CAA | Chloroacetaldehyde |
| CEO | Chloroethylene Oxide |
| cm | Centimeter |
| $cm^{-1}$ | Per Centimeter |
| CZE | Capillary Zone Electrophoresis |
| dADENO | 2'-Deoxyadenosine |
| DBAE | 1,2-Dibenz[a,h]anthracene-5,6-dihydroepoxide |
| dCYTIDINE | 2'-Deoxycytidine |
| DNA | Deoxyribonucleic Acid |
| EADENINE | $1,N^6$-Ethenoadenine |
| EADENO | $1,N^6$-Ethenoadenosine |

| | |
|---|---|
| ECYTIDINE | 3,$N^4$-Ethenocytidine |
| ECYTOSINE | 3,$N^4$-Ethenocytosine |
| EdADENO | 1,$N^6$-Etheno-2'-Deoxyadenosine |
| EDTA | Ethylenediamine Tetra-Acetic Acid |
| ELISA | Enzyme-Linked (or Labeled) Immunosorbent Assay |
| EtOH | Ethanol |
| f m | Femtomole |
| $H_2O$ | Water |
| HPLC | High-Performance Liquid Chromatography |
| I | $C_6N_{10}C_{11}C_{12}N_1$ Ring |
| I R | Infrared Spectroscopy |
| IUPAC | International Union of Pure and Applied Chemistry |
| k V | Kilovolt |
| m | Meter |
| m (Tables) | Medium |
| m m | Millimeter |
| M | Molarity |
| m g | Milligram |
| m W | MilliWatt |
| N | Nitrogen |
| NA | Numerical Aperature |
| NCI | National Cancer Institute |
| NIH | National Institutes of Health |

| | |
|---|---|
| n m | Nanometer |
| O | Oxygen |
| ORNL | Oak Ridge National Laboratory |
| PABA | 4-Para-Aminobenzoic Acid |
| PAC | Polynuclear Aromatic Compound (also PNA) |
| pH | Pouvior Hydrogene (Hydrogen Power) or |
| | Negative Logorithm of Effective $H^+$-Ion Concentration |
| PMT | Photomultiplier Tube |
| PNA | Polynuclear Aromatic Compound |
| p p b | Parts Per Billion |
| PBS | Phosphate Buffered Saline |
| R | Ribose |
| RNA | Ribonucleic Acid |
| r p m | Revolutions Per Minute |
| S | Strong |
| s | Seconds |
| SEM | Scanning Electron Microscope |
| SERS | Surface-Enhanced Raman Scattering Spectroscopy |
| s h | Shoulder |
| str. | Stretching |
| Thz | Terahertz |
| TPA | Terephthalic Acid |
| v | Very |
| vib. | Vibration |

| | |
|---|---|
| v/v | Volume/Volume |
| w | Weak |
| w/v | Weight/Volume |
| w/w | Weight/Weight |
| °C | Temperature in Degrees Celsius |
| µg | Microgram |
| µL | Microliter |
| µm | Micrometer |

# PART I: GENERAL INTRODUCTION AND OVERVIEW

# CHAPTER 1

## INTRODUCTION AND OBJECTIVES

One of the major difficulties with detecting and assessing the damage caused by exposure to toxins is the extremely dilute concentrations at which these substances are found in biological systems. Many pollutants have chemical groups of toxicological importance that can be detected and characterized by Surface-Enhanced Raman Scattering (SERS) spectroscopy. In response to the need to develop sensitive and selective techniques to study toxicity related to low-level exposure to hazardous chemicals, SERS and laser-based fiberoptic systems were integrated to analyze bioindicators of exposure to genotoxic chemicals. SERS detection of sample molecules was found to be possible in minute sample volumes and at very low concentration levels. The results of these experiments are of great analytical interest, since these chemicals are difficult to detect or characterize by other techniques such as luminescence spectroscopy, because of low luminescence quantum yields for these substances. Specific aims and accomplishments of this research are listed in Table I-1.

This dissertation was divided into five sections. The first part introduces the theories of Raman scattering and SERS and the rationale for selection of fiberoptics-based instrumentation. General methodologies for the SERS experiments presented later are also outlined in this section. The second part reviews the experimental parameters affecting SERS spectra and illustrates the importance of optimization of the experimental setup. The third section focuses on SERS experiments designed for the initial detection and analysis of selected genotoxic chemicals. The application of SERS to the study of

**TABLE I-1**
**Research Objectives and Accomplishments**

| Objective | Accomplishment |
|---|---|
| Investigate SERS spectral properties of biologically important molecules. | SERS was used to detect several appropriate samples including carcinogen aflatoxin $B_1$ and etheno adducts of adenine and cytosine. |
| Develop a SERS substrate non-toxic to biological systems, yet useful for detection and characterization of trace analyte quantities. | An agarose-covered, silvered alumina-roughened substrate was developed that actually enhanced SERS spectral signatures, while shielding against silver toxicity. |
| Utilize the SERS technique to analyze a chemical system that may not be detected in the same manner by another spectroscopic method. | SERS was used to detect and analyze purines/pyrimidines and etheno adducts. Fluorescence could not be used to detect both modified and unmodified mononucleosides and required a much larger sample volume. |
| Assess the potential ability of a SERS fiberoptic probe to detect and differentiate relevant substances *in vivo*. | Determined and described several obstacles in application of SERS biosensor to *in vivo* system for low-level chemical exposure risk assessment. |
| Refine the SERS technique so that the limit-of-detection of a substance approaches that of other sensitive spectroscopic techniques. | Nucleic acid adduct 3,$N^4$-etheno-cytosine was detected by SERS at femtomole levels, making SERS sensitivity comparable to that of fluorescent techniques. SERS samples were routinely only 1.0-μL in volume. |
| Apply SERS to study a substance in a minimally purified sample from a chemically-exposed organism. | Analyzed human BPDE/DNA adducts and aflatoxin-exposed human urine extracts. |

genotoxins in biological matrices is the general subject of the fourth section. In this part, substances are examined in body fluids, after reaction with DNA, and on specially coated SERS substrates. Adaptation of the SERS fiberoptic setup for possible *in vivo* remote sensing is also discussed. The final section presents future directions for SERS research and summarizes the accomplishments and significance of this research.

# CHAPTER 2

# SURFACE-ENHANCED RAMAN SCATTERING SPECTROSCOPY

## *Interactions Between Light and Matter*

Before discussing SERS, it may be helpful to describe the basic theory and define some of the terms pertaining to spectroscopy. It will be expedient to begin by describing the basic quantum-mechanical aspects of interactions between light and matter. The interested reader is directed to the reference portion of this section for technical details of spectroscopy and optics.

When a photon of frequency $\nu_0$ collides with an atom, the photon's energy may be absorbed by the atom. Energy transmission to a bound electron in the atom may result in excitation of the atom. The absorption probability is greatest when the frequency of the incidient photon is equal to an excitation energy of the atom. In solids, liquids, and dense gases, absorption occurs over a wide range of frequencies and the energy is usually dissipated through intermolecular collisions as heat. The excited atoms in a rarified gas, in contrast, can make a downward energy transition by emitting a photon in a random direction with the same frequency $\nu_0$ as the incident photon. This process is known as *resonance radiation* and was first described by R. W. Wood in 1904 (1).

At frequencies other than resonance, electrons vibrating with respect to the nucleus may be regarded as oscillating electric dipoles that will re-radiate electromagnetic energy at the same frequency as that of the incident photon. This nonresonant emission propagates according to a dipole radiation

pattern where the scattered flux density is directly proportional to the fourth power of the driving frequency. The removal of energy from an incident wave and the subsequent emission of some portion of that energy is termed *scattering*. The majority of scattering frequencies are coincident with the excitation energies of the atoms. Scattering also occurs, with less likelihood, at frequencies other than those corresponding to stable energy levels of the atom. In these situations, a photon will be re-radiated without any appreciable time delay and generally with the same energy as that of the incident photon. This process is termed *elastic* or *coherent scattering* because there exists a phase relationship between the incident and scattered electromagnetic fields. Since Lord Rayleigh was credited with determining the dependence of scattered flux density on frequency, this scattering of light by particles small in comparison to the incident wavelength is called *Rayleigh scattering*.

It is possible that upon emission of a photon, an excited atom will not return to its initial state. In this case, when the atom only drops down to an intermediate state, the emitted photon is of lower energy than the incident primary photon. In honor of George Stokes, who studied this behavior extensively before the advent of quantum theory, this phenomenon is referred to as a *Stokes transition*. When this process happens rapidly (order of $10^{-7}$ s) it is called *fluorescence*. If the process is delayed (seconds, minutes, hours) it is known as *phosphorescence*.

### *A Brief Introduction to Raman Spectroscopy*

Raman scattering is essentially comprised of the spontaneous Raman effect and the stimulated Raman effect. The theory of conventional Raman

spectroscopy is directly relevant to any discussions about SERS because the basic results of the interactions between light and matter are the same, although SERS theory is complicated by the presence of a scattering enhancement factor to be discussed later.

Spontaneous Raman

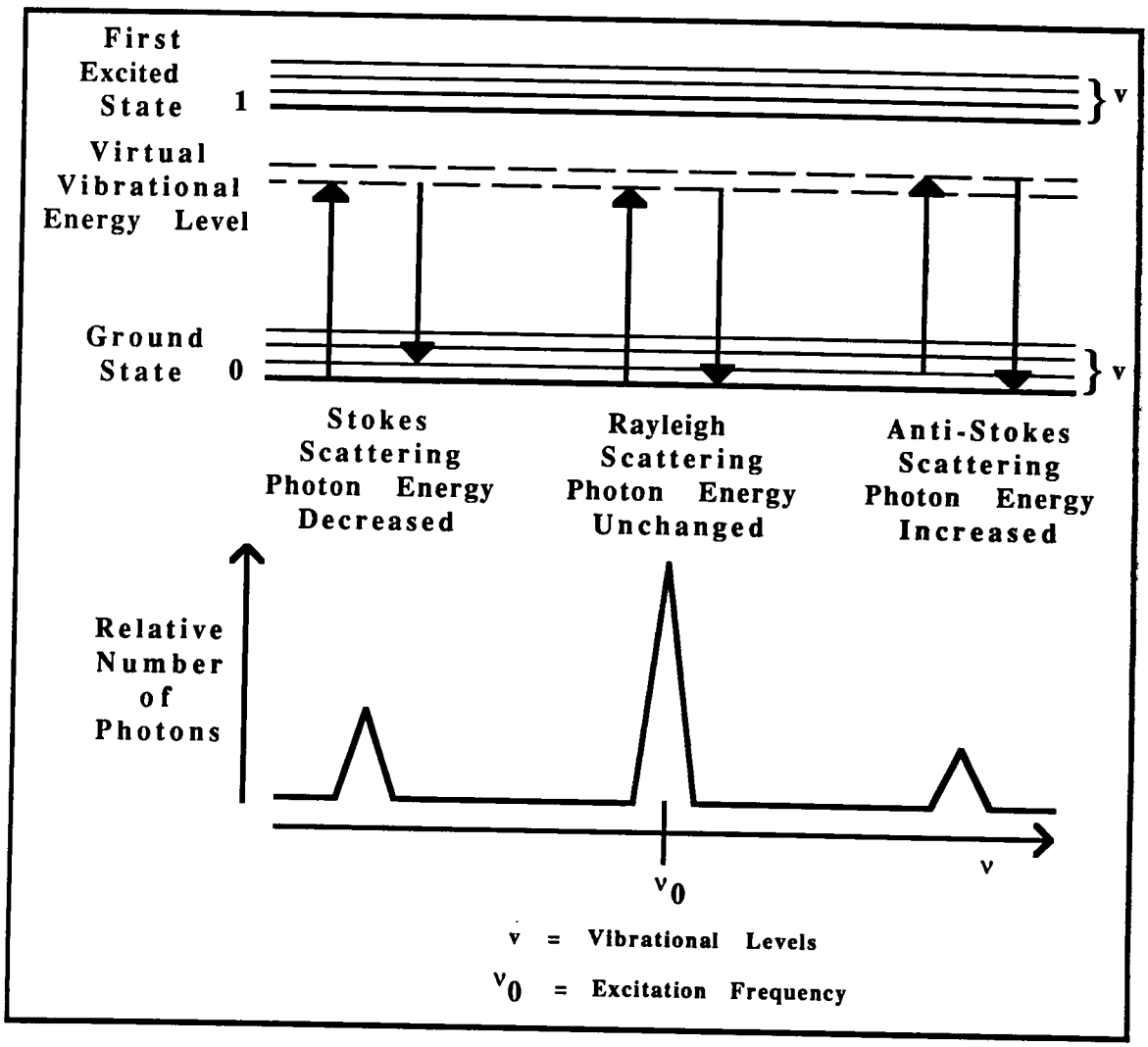
When quasimonochromatic light is scattered by matter, the greater portion of it will thereafter consist of light of unchanged frequency. There will exist, however, very weak components of the light having either higher or lower frequencies (side bands) than that of the incident light. The difference between the frequencies of the side bands and that of the incident light is a characteristic of the scattering material. The existence of these side bands was predicted by Adolf Smekal in 1923 and was experimentally observed by Sir Chandrasekhara Vankata Raman in 1928 (1) and is called the *spontaneous Raman effect.* Spontaneous Raman shifts were initially difficult to detect because the effect required strong light sources (usually mercury discharges) and large sample sizes. When strongly ultraviolet light sources such as mercury discharges were used for excitation, experimentation would be further complicated by decomposition of the sample molecules. Raman spectroscopy came into its own with the invention of the laser and the subsequent ability to deliver quasimonochromatic light at less energetic and more readily controlled wavelengths.

Several types of scattering contribute to a molecular spectrum. A molecule that absorbs radiant energy in the far-infrared and microwave regions of the electromagnetic spectrum can convert it into rotational kinetic energy. A molecule can absorb infrared photons and transform that energy

into vibrational energy. A molecule can absorb energy in the visible and ultraviolet regions of the spectrum through the mechanisms of electronic transitions similar to those of an atom. Rotational, vibrational, and electronic transitions all affect the profiles of molecular spectra.

If a molecule is in some vibrational state (not necessarily an excited state), an incident photon of frequency $\nu_0$ may be absorbed. The system will be raised to an intermediate or virtual state where it immediately makes a Stokes transition emitting a (scattered) photon of lesser energy than that of the primary photon. The leftover energy is conserved by excitation of the molecule to a higher vibrational level. Electronic or rotational excitation may result as well. Alternatively, if the molecule is initially in an excited state, upon absorbing and emitting a photon the molecule may drop to an even lower energy state, thereby making an *anti-Stokes transition.* In an anti-Stokes transition some of the vibrational energy of the molecule is converted into radiant energy.

Thus, the total radiation from the molecule consists of the excitation frequency, the sum of the excitation and vibrational frequencies (anti-Stokes radiation), and the difference of the excitation and vibrational frequency (Stokes radiation). These components of Raman scattering are depicted in Figure I-1. With either Stokes or anti-Stokes transitions, the difference between the initial and scattered photon frequencies corresponds to specific energy-level differences for the molecule being studied, and so may provide insights into the sample's molecular structure. Note that the proportion of Rayleigh scattered light (photon frequency unchanged) is much greater than the Stokes or anti-Stokes proportion, and that more scattered photons are likely




<FIGURE I-1> Components of Raman Scattering

to result with decreased photon energy (Stokes scattering) than with increased photon energy (anti-Stokes scattering).

Stimulated Raman.

In principle, each spontaneous scattering mechansim (e.g. Rayleigh) has its stimulated counterpart. The *stimulated Raman scattering effect* was discovered in 1962 by Eric J. Woodbury and Won K. Ng while they were working with a million-watt pulsed ruby laser. The optical setup incorporated a nitrobenzene shutter for controlling light input to the sample cell (1). Woodbury and Ng noticed that approximately ten percent of the incident energy at 694.3-nm was shifted in wavelength to what appeared to be a coherent beam at 766.0-nm. It was subsequently determined that the frequency shift of about 40-Thz was characteristic of one of the vibrational modes of the nitrobenzene molecule. Stimulated Raman scattering can occur in solids, liquids, or dense gases under the influence of focused high energy laser pulses. When two photon beams are simultaneously incident on a molecule -- one beam with the frequency of the laser and the other with a scattered frequency -- the laser beam can lose a photon while the scattered beam gains a photon. The remaining energy is then transmitted to the sample molecules. The chain reaction by which a large percentage of the incident beam may be converted into stimulated Raman light can occur only above a certain high-threshold flux density of a laser excitation beam.

For comprehensive descriptions of Raman theory, the reader is directed to Colthup *et al.* (2), Parker (3), or Baranska *et al.* (4). Excellent additional material on the theory of optics may be found in the text by Hecht and Zajac (1).

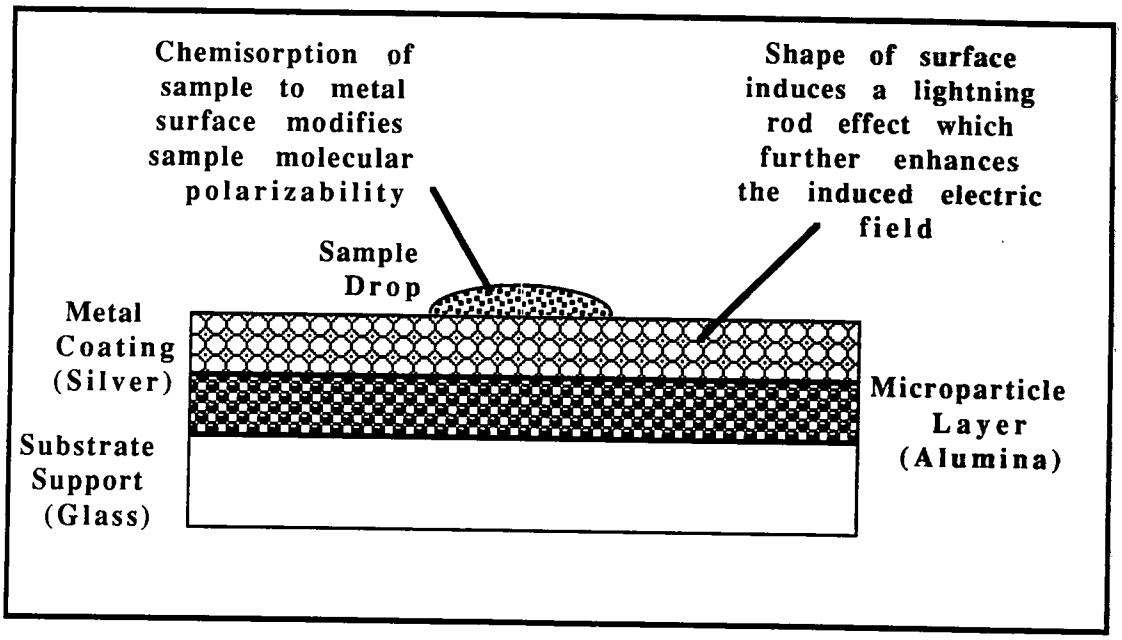
## *SERS Versus Conventional Raman Spectroscopy*

Raman spectroscopy has been used as an important analytical tool in studies requiring chemical group specificity (5,6). The small scattering cross-section inherent in the technique has, however, generally limited its use to situations where powerful and expensive lasers can be used for sample excitation. A pure, crystalline specimen may generate suitable Raman scattering intensity for chemical detection, but molecules in a biological matrix may be too dilute for detectable spectral intensity. The enhancement of molecular cross-scattering intensity in SERS makes the study of biologically-important molecules feasible.

*Surface-enhanced* Raman spectroscopy has been an active research area since its discovery in 1974 by Fleischmann, Hendra, and McQuillan on the molecule pyridine (7) and independent confirmation in 1977 by Jeanmaire and Van Duyne (8) and Albrecht and Creighton (9). In SERS, the spectral signal from a substance is enhanced approximately a million to ten billion times over the molecular scattering efficiency of conventional Raman spectroscopy (7-9). This enhancement is the result of an interaction between the analyte and a roughened, electron-conducting surface. This special electron-conducting surface is generally referred to as a SERS *substrate*. An example of a SERS substrate is depicted in Figure I-2.

## *Proposed Basic Mechanisms of SERS*

There are several theoretical models for Raman enhancement from the substrate (10,11), but the mechanisms fall into essentially two groups:




<FIGURE I-2> An Example of a SERS Substrate

electrical effects and chemical effects. While there is no general agreement among theorists on the exact enhancement process, it likely results from a combination of effects within these two mechanism types.

In one aspect of the electrical enhancement mechanism, the electron-conductive surface of the SERS substrate has an electromagnetic resonance called the *surface plasmon*. When the excitation radiation (laser light in these studies) impinges on the substrate surface, the plasmons oscillate and produce an induced electrical field that affects the polarizability of the sample molecules. Small particles such as the agents used to roughen a SERS substrate also possess electromagnetic resonances similar to the surface plasmon and similarly affect molecular polarizability. The roughening agent creates substrate-specific protrusions that may increase the local electromagnetic field of the substrate surface by acting as "lightning rods" that concentrate the electromagnetic energy.

The chemical enhancement factors also affect molecular polarizability and hence the Raman scattering cross-section. Modification of the molecular polarizability is thought to result from an interaction between the analyte molecules and the electron-conducting (usually metallic) substrate surface. This adsorption process, called *chemisorption*, consists of the formation of a chemical complex between the sample molecules and metal atoms on the substrate surface. Chemisorption implies the presence of active sites on the substrate that promote particular orientations of the sample molecule, thus affecting the polarizabilty of the sample. Several reviews of proposed SERS theories are available (11-16).

*SERS and the Optical Setup*

SERS was selected to perform this study because of its several advantages over other spectroscopic methods. SERS yields excellent detection sensitivity and chemical group specificity with direct laser excitation. In these experiments, however, fiberoptics were integrated with SERS to provide additional sample detection advantages including site specificity, the option for remote sensing of samples, and minimization of sample exposure to radiation. Fiberoptic probes have been developed for spectrometry in the ultraviolet to visible light range, but these techniques can provide little *structural* information about a specimen and are not highly selective. Infrared absorption spectrometry can provide chemical structural information, but it is not amenable to interfacing with fiberoptic probes because fibers composed of glass and plastic are typically poor transmitters of infrared radiation. SERS spectra are usually taken within the visible light spectrum. Since visible light is efficiently transmitted by optical fibers, SERS may be used in conjunction with fibers to provide selective vibrational information about a sample. Thus, one of the major advantages of SERS over other visible spectroscopies such as fluorescence and absorption is the large quantity of structural information which can be obtained from a SERS spectrum. Another advantage is that the presence of water in a sample does not interfere greatly with the quality of a SERS or conventional Raman spectrum. This phenomenon is due to the relatively weak inelastic photon scattering by water outside of the OH-stretch and HOH-bending bands of the molecule (17,18). SERS analysis of biological systems is preferable to Raman or

resonance Raman analysis because fluorescent impurities and low sample concentrations may prevent molecule detection by the latter techniques.

# CHAPTER 3

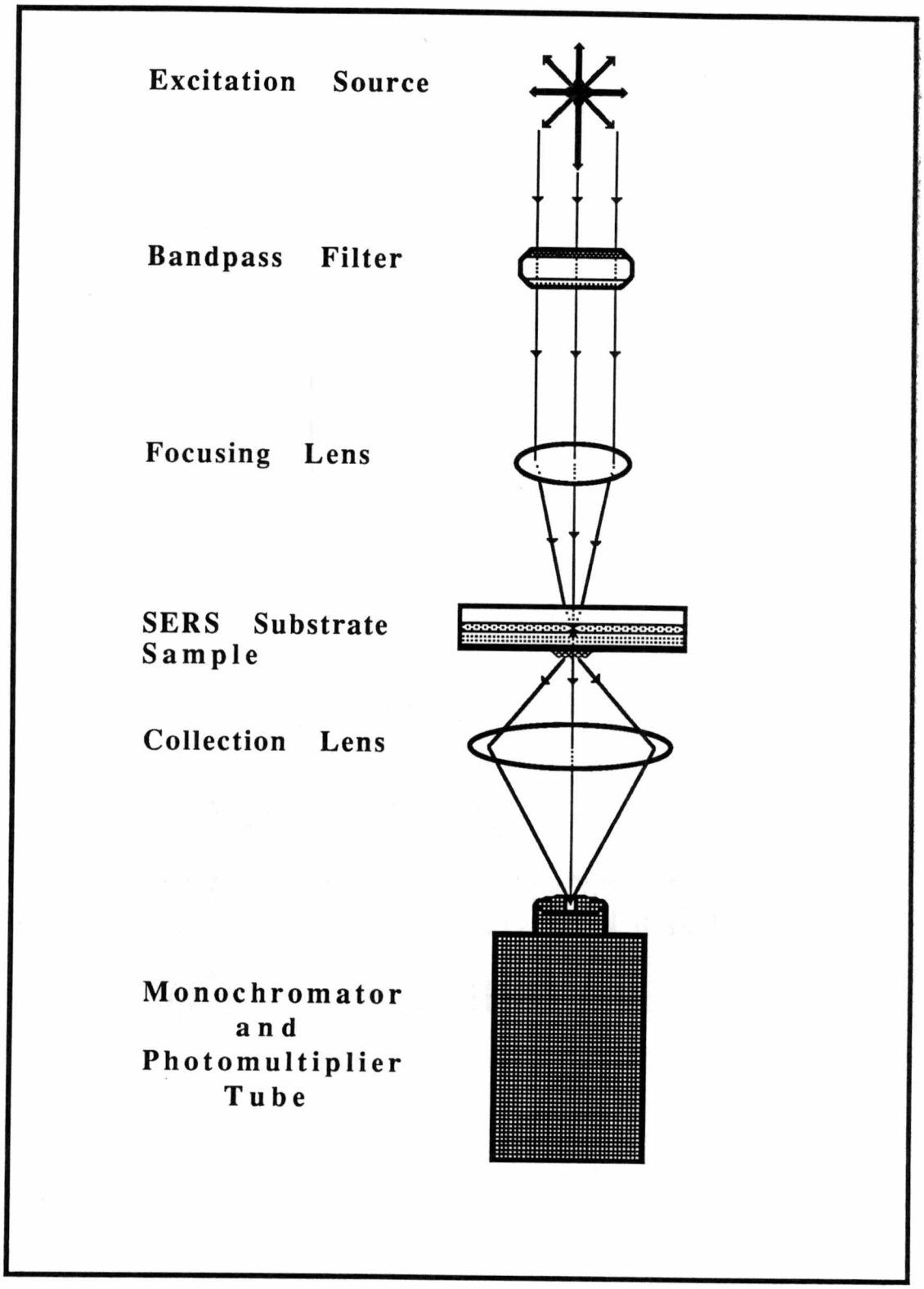
# METHODOLOGY

Specific variations in instrumentation, chemicals, and procedures will be noted when appropriate in later sections. Methodology in this section applies to all data except where stated otherwise.

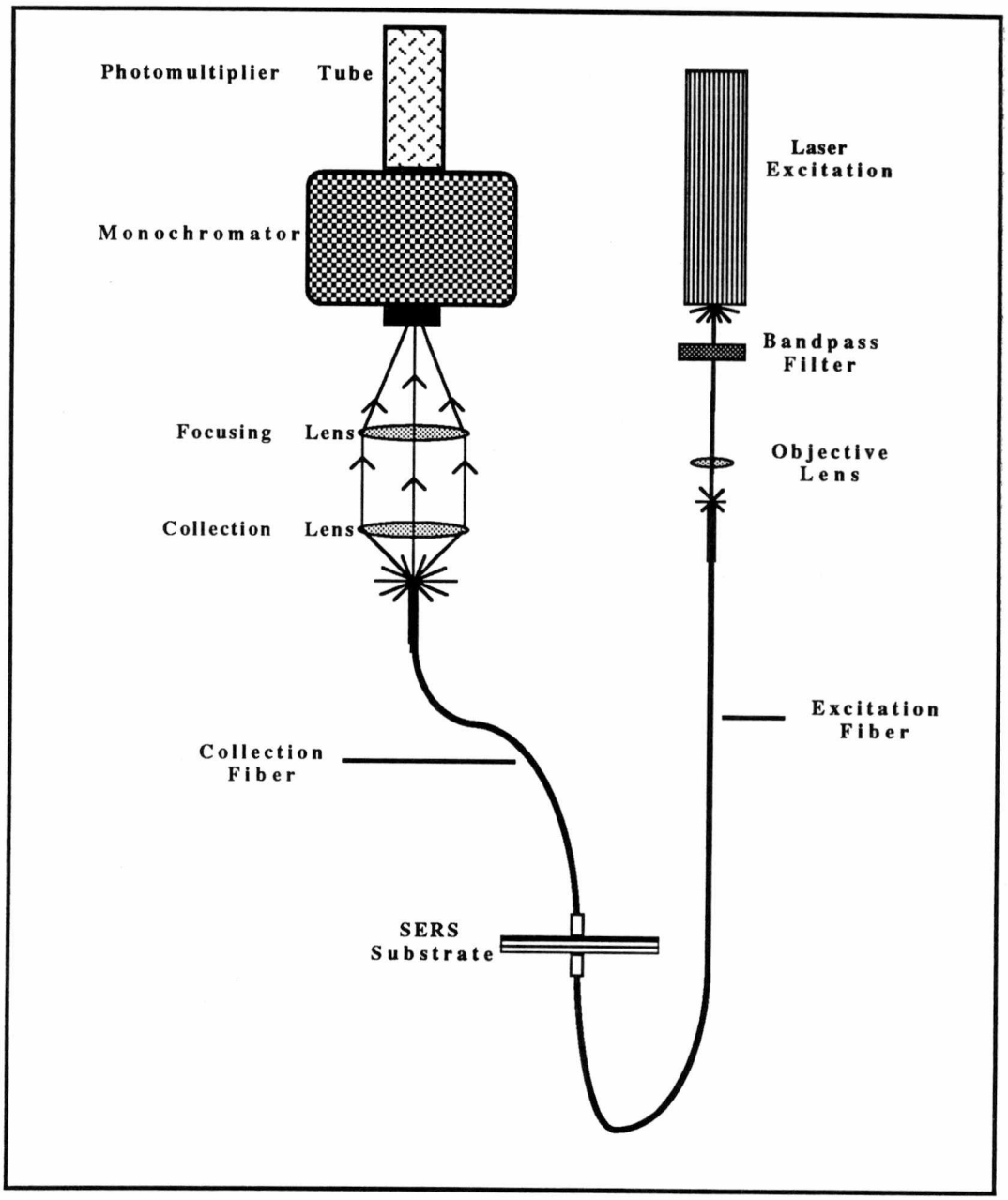
## *Instrumentation*

There has been considerable interest in the use of fiberoptic probes for remote spectroscopic sensing (19-23). Optical fibers have been applied to Raman spectroscopy for approximately a decade (24-27), but their uses for SERS had been relatively unexplored when this research was initially undertaken in 1989 (28-30). A rudimentary optical setup for SERS is shown in Figure I-3. Figure I-4 illustrates a modified setup where optical fibers carry the excitation and collected light. Unless otherwise noted (e.g. "direct setup" denotes no optical fibers used in the instrumentation), optical setups identical or similar to the one diagrammed in Figure I-4 were used to conduct the experiments described in later sections.

Substrates were coated with microparticles (e.g. polystyrene beads, alumina) using a photoresist vacuum spin-coating device (Model Electrocraft 101, Headway Research, Inc., Garland, TX). All spectra were obtained with a SPEX (Metuchen, NJ) Model 1403 double-grating spectrometer equipped with a thermoelectrically-cooled gallium arsenide photomultiplier tube (Model C31034, RCA Corporation, Lancaster, PA) operated in single-photon counting mode. The monochromator bandpass was 2 $cm^{-1}$ for all of these experiments.




<FIGURE I-3> A Rudimentary Experimental Setup for SERS




<FIGURE I-4> A SERS Fiberoptic Setup

Either a krypton-ion (647.1-nm) or an argon-ion (514.5-nm or 488.0-nm) laser was used for sample excitation, both water-cooled Model Innova 70 lasers (Coherent Laser Products Division, Palo Alto, CA). For some spectra, a Coherent (Palo Alto, CA) Model 599-01 broadband dyehead was used in conjunction with the argon-ion laser. Excitation light was passed through narrow bandpass laser filters (Oriel Corporation, Stratford, CT) for the appropriate wavelength. When further beam attenuation was desired, light was passed through a neutral density filter. In the experiments using fiberoptics, all fibers were obtained from General Fiber Optics, Inc. (Cedar Grove, NJ). All fibers were composed of silica with soft plastic cladding. Optical fiber diameter was generally 600-μm, with a numerical aperature of 0.26.

### *Chemicals*

The commercial source of agglomerate-free aluminas was the Baikowski International Corporation (Charlotte, NC). All studies included in this manuscript using alumina-type substrates utilized 0.1-μm diameter particles in thoroughly sonicated aqueous suspensions. Either 4.0% or 5.0% (w/w) alumina suspensions were used.

Latex polybead microspheres (2.5% solids in an aqueous suspension) were obtained from Polysciences, Inc. (Warrington, PA). In these studies, sphere size was generally 0.22-μm in diameter. This bead size had previously been determined to yield the best results for SERS. (Best bead size was determined by other scientists at ORNL using the spin-coating device and thermal evaporator available for this research. Note that the use of the substrate roughening agents used for this research may not yield comparable

results if these experiments were reproduced using different equipment. Scientists at other facilities may find a different alumina diameter or latex bead size works best with their equipment.)

*Procedures*

Silvered, microparticle-coated substrates were prepared as follows: Approximately 100-μL of a microparticle suspension was spin-coated (usually 2000-rpm, 30-s) onto a precleaned 20x10x1-mm piece of microscope slide glass (VWR Scientific, Inc., San Francisco, CA). Next, silver was thermally evaporated onto the roughened plates under a pressure of approximately $2x10^{-6}$-Torr (Model CVE 301, Cooke Vacuum Products, Norwalk, CT). Unless otherwise noted, the evaporation rate was 0.15-nm/s, to a final silver film thickness of 100.0-nm. Silver island substrates were prepared by thermally evaporating 10.0-nm of silver onto cleaned microscope slide glass. SERS substrates were reused by soaking used plates in dilute (unspecified concentration) nitric acid (J. T. Baker, Inc., Phillipsburg, NJ) to remove the silver, then washing the glass in water to remove any microparticles. Glass was stored in absolute ethanol until use. Prior to coating, the glass plates were dried under a high-velocity stream of dessicated air.

For all fiberoptic studies, spectra were taken through the back (glass) side of a SERS substrate. When used, the collection fiber was positioned as close to the sample/silver surface as possible without disrupting the silver film. Using a stepping motor to position the fiber over the sample, this distance was reproducibly about one micrometer. Negligible variation between spectra or signal loss resulted from this distance.

Spectra taken with a "direct beam" setup were illuminated on the silvered side of the substrate. Scattered light was collected on the same side of the substrate with a large lens placed at a forty-five degree angle with respect to the excitation beam. The primary beam of the laser was directed away from the collection lens to increase the signal-to-noise ratio of the spectra.

Samples were usually prepared for SERS by depositing 1.0-μL to 3.0-μL of an analyte solution onto the silvered side of a substrate. Unless otherwise noted (e.g. "wet sample" denotes a sample spot that remained liquid throughout scan duration), the solvent was allowed to evaporate at room temperature before exposure to the laser beam. SERS could also be performed with solid, liquid, or gaseous samples. The experimental setup and the production of the silvered microparticle substrates have been described in detail previously (31-33).

# SECTION I

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## REFERENCES


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# PART II: EXAMINATION OF EXPERIMENTAL PARAMETERS

# CHAPTER 1

## INTRODUCTION AND RATIONALE

The results of some studies of experimental parameters were included in this dissertation to clarify nuances of the SERS spectra presented in later sections and to emphasize the flexibility of the SERS technique through variation of those parameters. Unlike many laboratory techniques, SERS does not rely on a standardized set of experimental conditions, even when identical substances are being examined. The many possible approaches to an experimental problem may yield similar but rarely identical results -- one laboratory may be incapable of detecting a chemical or of duplicating a spectrum readily analyzed by another laboratory. Because of the permutations of experimental parameters, some of which are unalterable environmental conditions, even in a given laboratory there may be a degree of difficulty reproducing experimental results. Variations between substrates (slight differences in cleanliness and quality of glass supports, quantity and quality of roughening agent, thickness and rate of application of metal, method of substrate storage) and between background conditions (optical alignment, ambient light, and vibration of optics) account for the majority of the differences between spectra.

In this study, the most *important* factors found to affect spectral reproducibility were the optical alignment, type of setup, variations between individual silvered microparticle-coated substrates, and the inconsistencies across the surface of any one substrate. These and other parameters experimentally determined to affect SERS are listed in Table II-1. Some of these factors will be discussed in detail. To minimize inconsistencies between

**TABLE II-1**
**Some Experimental Parameters That May Affect a SERS Spectrum**

| Parameter | Degree of Importance (Scale of 1-3) | Level of Control (Scale of 1-3) |
|---|---|---|
| **Atmospheric Dust**<br>May contaminate sample; affects efficiency of optics | 2 | 3 |
| **Atmospheric Pressure and Optics**<br>May affect accuracy of monochromator scanning of sample | 3 | 2 |
| **Excitation Power Intensity**<br>Too high intensity may photodecompose or photodegrade sample; too low power may render the sample indetectable because of resultant poor signal-to-noise ratio | 1 | 1 |
| **Excitation Wavelength**<br>Ideally corresponds to sample color; photon energy affects stability of sample in laser beam | 2 | 1 |
| **Humidity**<br>Alters substrate effectiveness and performance of instrumentation | 2 | 2 |
| **Method of Photon Amplification**<br>May alter SERS sensitivity and length of scan | 2 | 1 |
| **Method of Sample Application**<br>May be gas, liquid, solid, dried liquid spot -- affects relative intensities of spectral bands | 1 | 1 |
| **Optical System Temperature**<br>Affects instrumentation performance | 2 | 3 |
| **Portion of Substrate Scanned**<br>Substrate surface may not be homogeneous; affects reproducibility of spectrum | 2 | 1 |
| **Sample Concentration**<br>Monolayer of sample molecules believed to produce optimal SERS spectrum -- too low sample concentration may not be detected | 2 | 1 |
| **Sample Molecular Orientation**<br>Depends largely on substrate and sample phase of matter | 2 | 2 |
| **Sample Purity**<br>Trace impurities may appear on spectrum; may alter sample solubility; may chemically interact with sample | 1 | 2 |

TABLE II-1 (cont.)
**Some Experimental Parameters That May Affect a SERS Spectrum**

| Parameter | Degree of Importance (Scale of 1-3) | Level of Control (Scale of 1-3) |
|---|---|---|
| **Sample Temperature** | 2 | 1 |
| Affects sample phase of matter, sample stability, and rate of solvent evaporation | | |
| **Scan Duration** | 2 | 1 |
| Related to sample stability and solvent evaporation | | |
| **Solvent Purity** | 1 | 1 |
| Trace impurities detectable by SERS | | |
| **Solvent Type** | 1 | 1 |
| Interacts with substrate and sample; determines sample distribution on substrate; rate of evaporation alters sample concentration | | |
| **Stability of Optics** | 2 | 3 |
| Affects quality of spectrum; may cause unwanted mixing of liquids | | |
| **Substrate Type** | 1 | 1 |
| Largely responsible for SERS enhancement over Raman; selection of substrate greatly affects spectrum quality | | |

Scale:

Degree of Importance
1 = Very Important
2 = Important Under Some Circumstances
3 = Usually Insignificant

Level of Control
Easily Controlled
Controlled with Difficulty
Beyond Normal Control

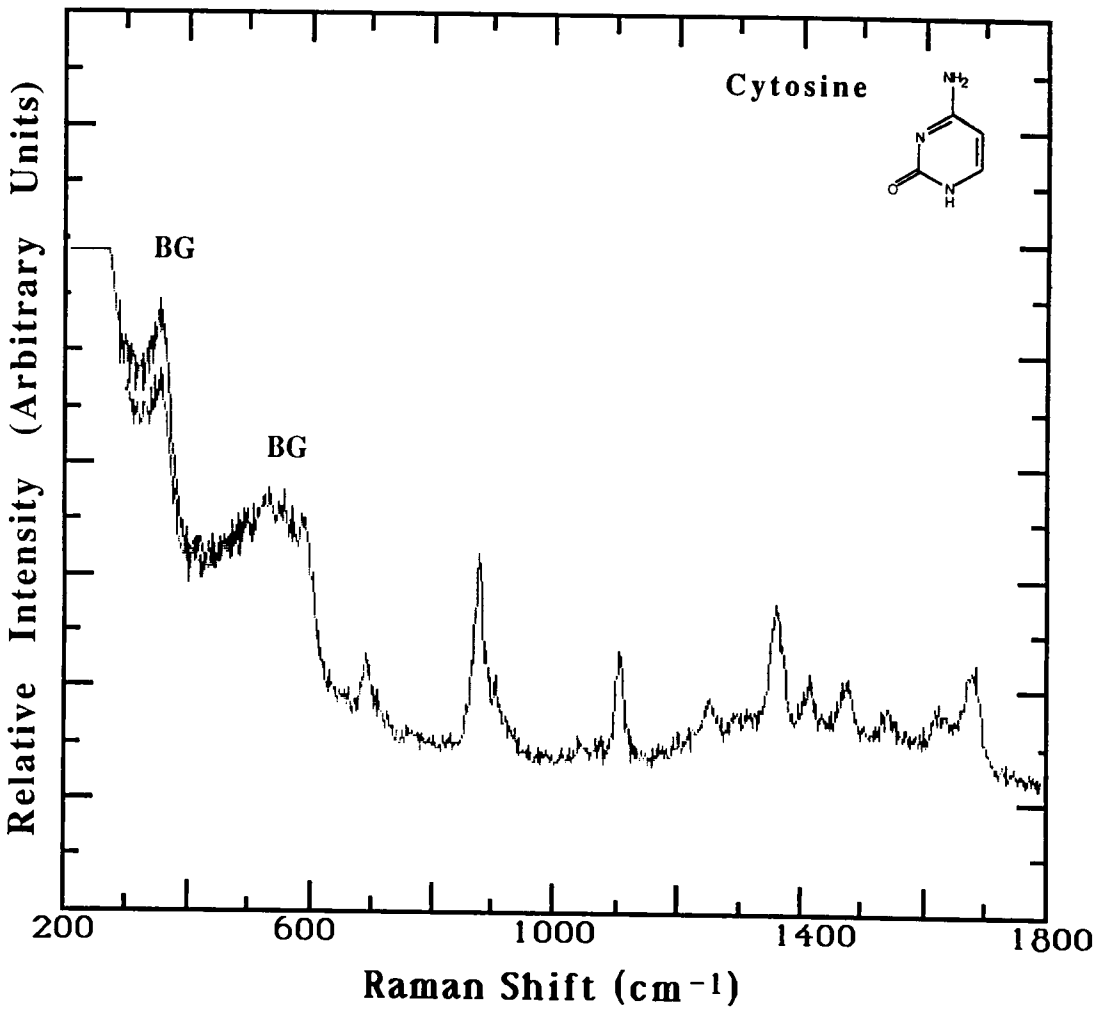
SERS spectra, the conventions listed in Table II-2 were observed when recording and analyzing data. Despite the many variables involved in SERS, spectra were directly comparable when taken with the same excitation wavelength and power, same optical setup, and same series (produced simultaneously, all of one type) of substrates. The validity of this direct comparison is illustrated in the Figure II-1 SERS spectra of cytosine. Note that the two cytosine spectra were coincident to the point of being indistinguishable.

**TABLE II-2**
**Experimental Conventions**

| Problem | Solution |
|---|---|
| Background spectra taken earlier than sample spectra may display different noise levels because of continual PMT and laser warm-up. Warm-up time was especially long for the laser dye head. | One hour warm-up was allowed (twice the manufacturer's recommended warm-up time) before taking data. When possible, background spectra were taken in the middle of an experimental run to avoid problems caused by the laser or PMT not being properly warmed-up, or by the laser interlock being accidentally tripped. |
| Consistency in optical set-up and data-taking was required so that spectra could be directly compared with each other. Optimum sample detectability was desired. If fibers were moved, data was no longer comparable. | Sample spectra were taken after the optics had been aligned using a spectral band of a diffcult-to-detect chemical (sample peak at level of signal-to-noise). The optical fiber was not moved following this alignment. |
| SERS substrate surfaces were not uniform. This resulted from spin-coating microparticles from the center and from evaporating silver at an angle. Substrates prepared with fresh microparticle solutions might differ from earlier substrates. This was especially true with alumina-type substrates, due to manufacturing variations. | When possible, spectra were taken at the same location on the same SERS substrate. Otherwise, studies were performed using substrates produced at the same time with the same microparticle suspension and metal-coated together. Substrates were spun during evaporation to ensure uniform silver coating. |
| Some consistent method of measuring peak intensities had to be used. | "Noise" values were taken as the photon range over a flat portion of a background scan. A five-point smoothing computer program was applied to the data to be sure no false peaks were being mis-assigned. Peaks were measured from the maximum of the smoothed data peak to the maximum of the smoothed background noise. The original, not the smoothed data, is presented in this dissertation. |

**TABLE II-2**
**Experimental Conventions** (cont.)

| Problem | Solution |
|---|---|
| Monochromator grating was subject to variations in temperature, atmospheric pressure, and other factors. | Spectra often included a laser plasma line to check alignment of monochromator and to standardize peak assignments. |




**<FIGURE II-1> Reproducibility of SERS Spectra Demonstrated Using Cytosine** This figure shows two scans of 1.0-μL of 1x10-3-M cytosine in ethanol. Laser excitation was 75-mW at 647.1-nm. Spectra were taken on the same alumina-type substrate and were printed to scale relative to each other.

# CHAPTER 2

# FACTORS AFFECTING THE REPRODUCIBILITY OF A SERS SPECTRUM

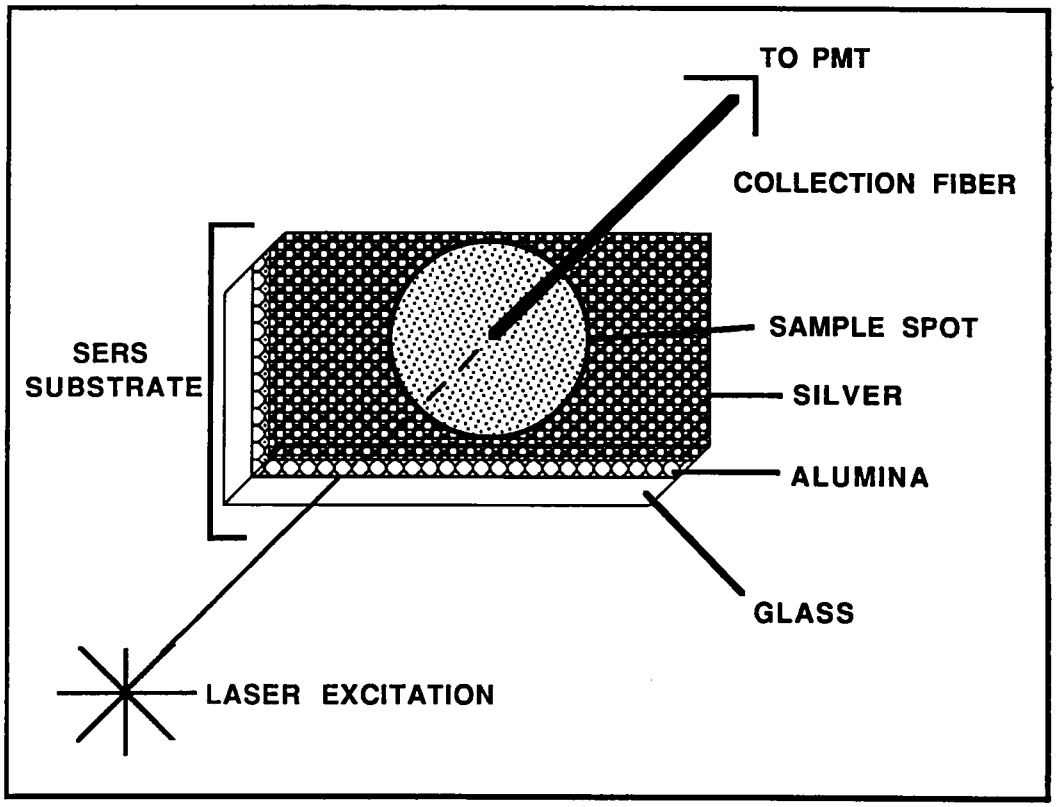
## *SERS Substrates*

In general, the SERS effect requires the presence of a submicron-size roughness (0.01-μm to 0.1-μm) of the electron-conductive substrate surface. One of the earliest SERS substrates was an electrode that had been roughened through repeated oxidation-reduction cycles on the metal surface (1,2). Another type of substrate was a semi-solid aqueous suspension called a *hydrosol* or simply *sol* containing electron-conducting particles. Several procedures have been reported for silver and gold sols that have been shown to possess metal particles of adequate size and shape for SERS (3,4). SERS has also been performed on quartz posts (5,6), metal island films (7), and metal-coated filter paper (8). SERS may even be effective on dielectric surfaces such as polyacetylene (9,10). While there exist SERS substrates in which the plasmon source is non-metallic, these experiments were restricted to metal-covered substrates. The data in this research was obtained using SERS substrates composed of a metal coating on a surface that had been roughened with a thin layer of microparticles such as polystyrene beads (11-14), fumed silica (15-17), titanium dioxide (18), or alumina (19).

Although substrates of several compositions and characteristics may be used for SERS, not every substrate is suited to a given set of experimental conditions. Earlier studies conducted at ORNL determined that the most effective substrate for the particular optical configuration used in the majority of these experiments (krypton-ion excitation at 647.1-nm through

optical fibers) was the silvered, alumina-coated substrate diagrammed in Figure II-2. The most effective substrate for use with argon-ion excitation at 488.0-nm or 514.5-nm was a silvered, polystyrene bead-coated substrate. The optimum bead diameter for SERS had earlier been found to be 0.22-μm. Polystyrene bead-type substrates were produced in the same manner as the alumina-type substrates except that an aqueous suspension of polystyrene spheres was spin-coated onto a glass support in place of an alumina suspension. The thickness of the silver, concentration and particle size of alumina, and the method of application of silver and alumina were optimized for the optical setup and the equipment used to produce the substrates (e.g. evaporators, spin-coating devices). These optimization studies would be of little use to other scientists attempting to duplicate this research because the final characteristics of a substrate are very dependent upon the idiosynchrasies of the apparatus used in its production.

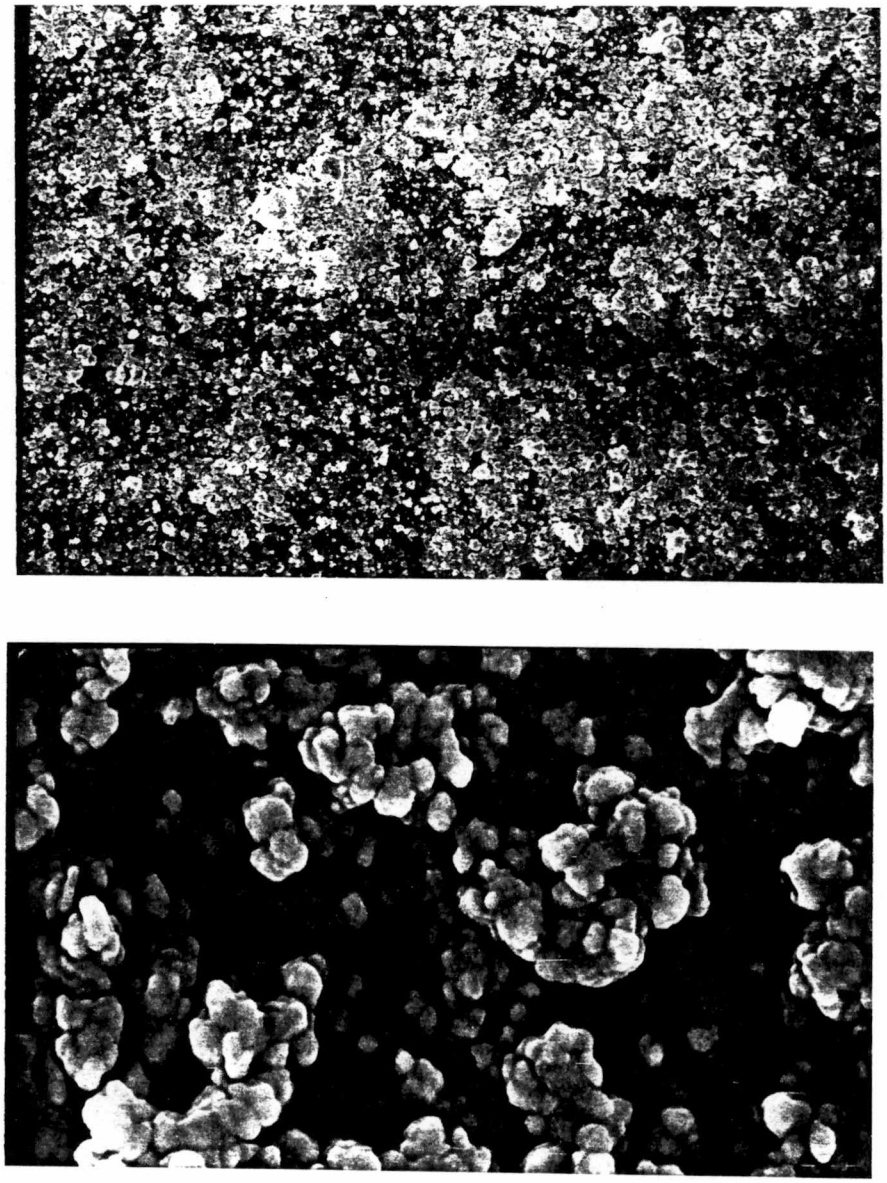
One advantage of the alumina substrate type was that it had an extremely long effective shelf life of approximately one year. Substrates were found to remain effective for an even longer time period when stored in a dessicated, sealed container. (It may be presumed that water and fresh air hasten oxidation of the silver surface.) Polystyrene-type substrates were effective for approximately one month (stored in a sealed compartment) with negligible loss of enhancement properties. Silver island substrates could only be used within a matter of a few hours after their production before becoming ineffectual. These observations indicate a protective quality of the roughening agent for the electromagnetic resonance of the surface plasmon. It may be surmised that the alumina and polystyrene somehow prevent or slow oxidation of the silver.



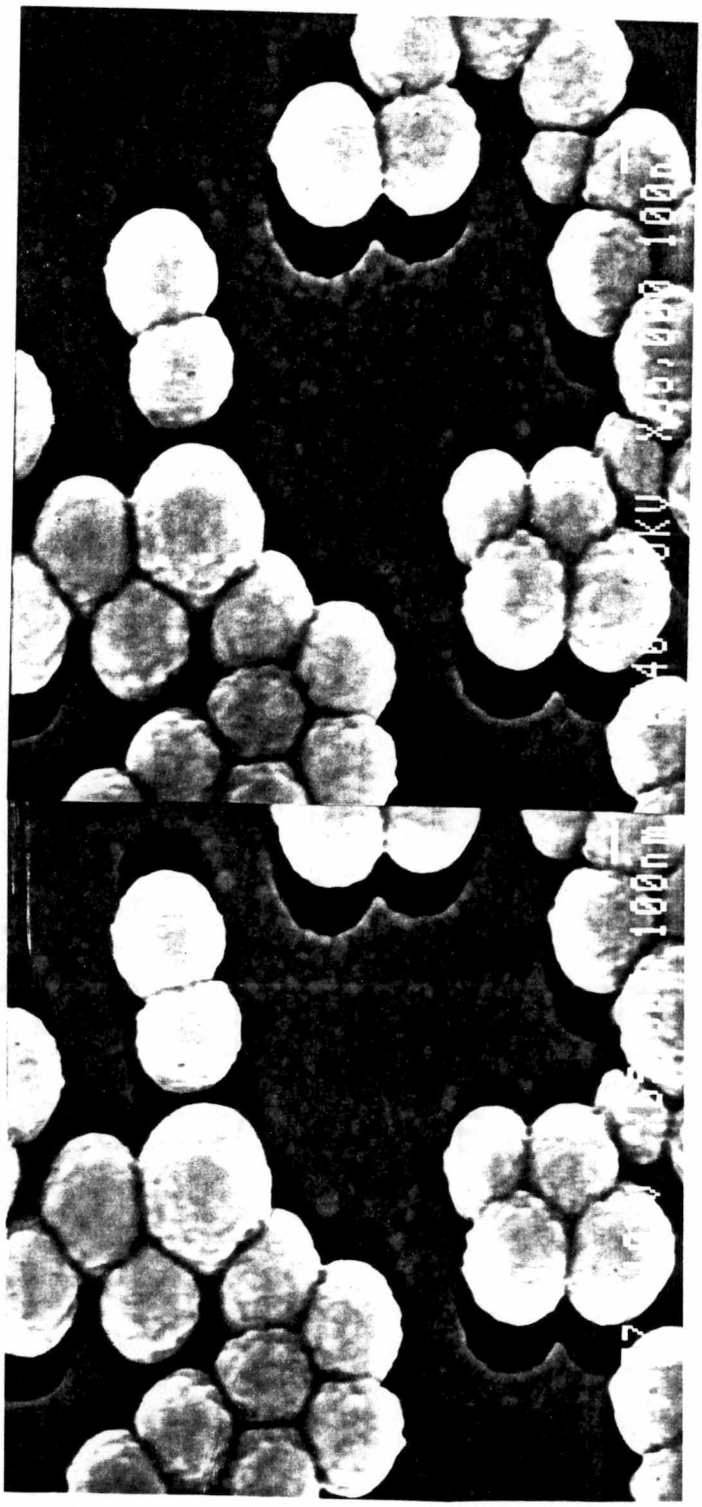

<FIGURE II-2> Diagram of a Silvered, Alumina-Coated SERS Substrate

Scanning electron micrographs were taken of the different routinely-used SERS substrates. It was hoped that analysis of the micrographs could be used to predict the effectiveness of new substrate types for various applications. The micrographs were also used to gauge the reproducibility of substrate production. Substrates that yielded exceptional SERS spectra were compared with substrates that gave substandard spectra for the same chemical. All SEM micrographs were taken by the author with a JEOL Model JSM-890 field-emission scanning electron microscope (JEOL Microscopes Ltd., Tokyo, Japan). Secondary electron images were made with the smallest available aperature and a minimal accelerating voltage of 10-kV to reduce the charging effects from the large amount of dieletric material in these substrates. No rotation of the sample took place except during the production of stereo-micrographs.

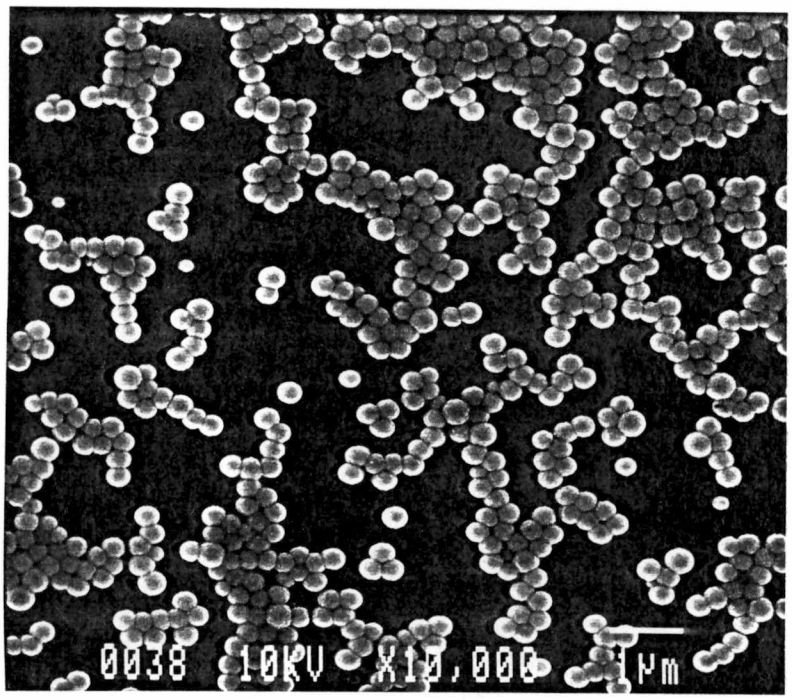
Scanning electron micrographs representative of a silvered, alumina-coated substrate are shown in Figure II-3. A scanning electron stereomicrograph of a polystyrene-type substrate used with argon-ion excitation is shown in Figure II-4. SEM micrographs delineating the general surface characteristics of the silvered, polystyrene bead-coated substrate are shown in Figure II-5. Another type of substrate tested in this study was a silver island substrate (SEM example in Figure II-6). Silver island substrates were quickly and easily prepared by evaporating 1.0-nm to 50.0-nm of silver onto pre-cleaned microscope slide pieces. The silver island substrate was not as practical for use with SERS as the alumina or polystyrene types because the silver oxidized in a matter of hours, rendering the substrate uneffective. Note the dramatic differences in surface characteristics between relatively similar substrate types (alumina, polystyrene bead, and silver island) by comparing Figure II-3 through Figure II-6.

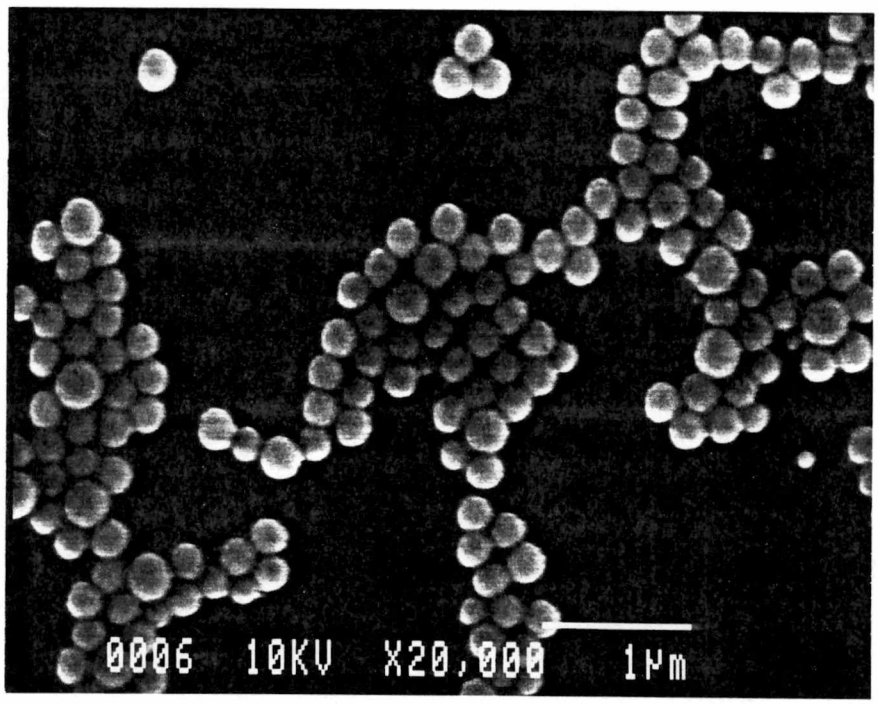


**<FIGURE II-3> Scanning Electron Micrographs of a Silvered, Alumina-Coated SERS Substrate** Substrate was 100-nm silver evaporated onto glass that had been spin-coated with a 5.0% alumina suspension. Magnification was (Top) 1000X and (Bottom) 30,000X.

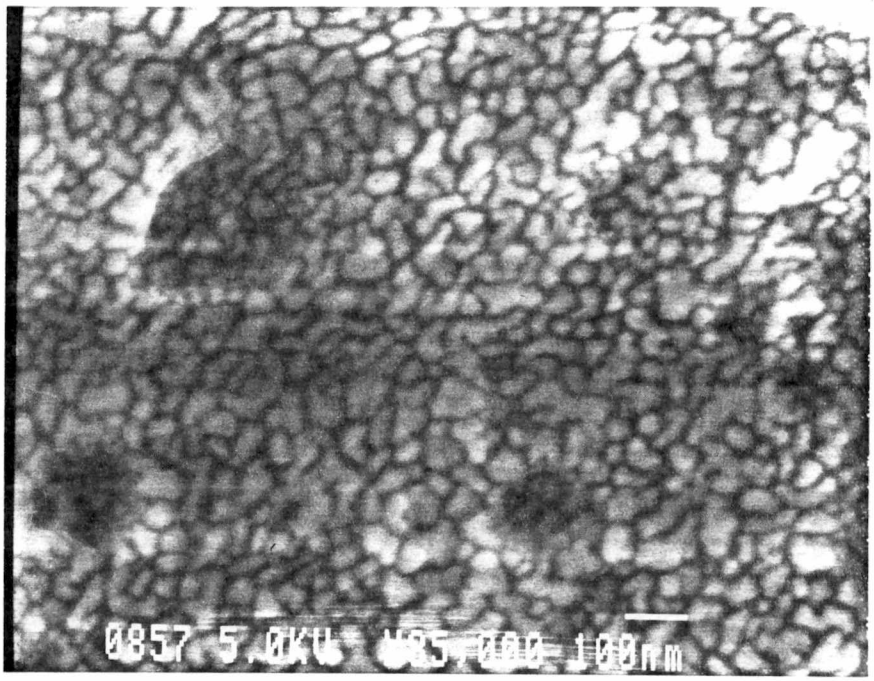


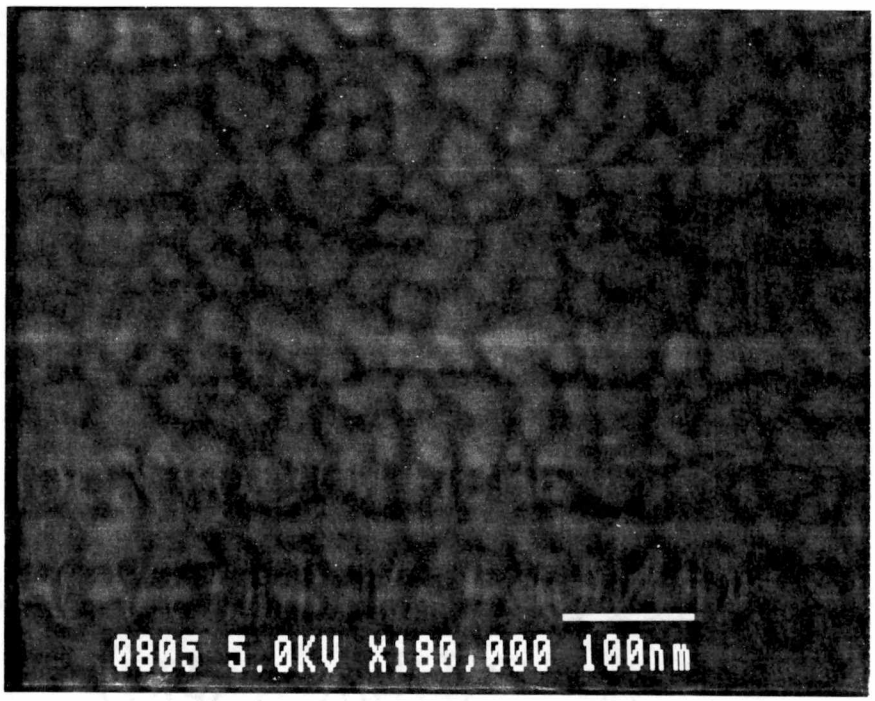
**<FIGURE II-4> Scanning Electron Stereomicrographs of a Silvered, Polystyrene Bead-Coated SERS Substrate** Substrate was 100-nm silver evaporated onto glass that had been spin-coated with a 2.5% suspension of latex beads (diameter 0.22-μm). Angle of rotation was (Left) 0° and (Right) +6°.




**<FIGURE II-5> Low Magnification Scanning Electron Micrographs of a Silvered, Polystyrene Bead-Coated SERS Substrate** Substrate was 100-nm silver evaporated onto glass that had been spin-coated with a 2.5% suspension of latex beads (diameter 0.22-μm).





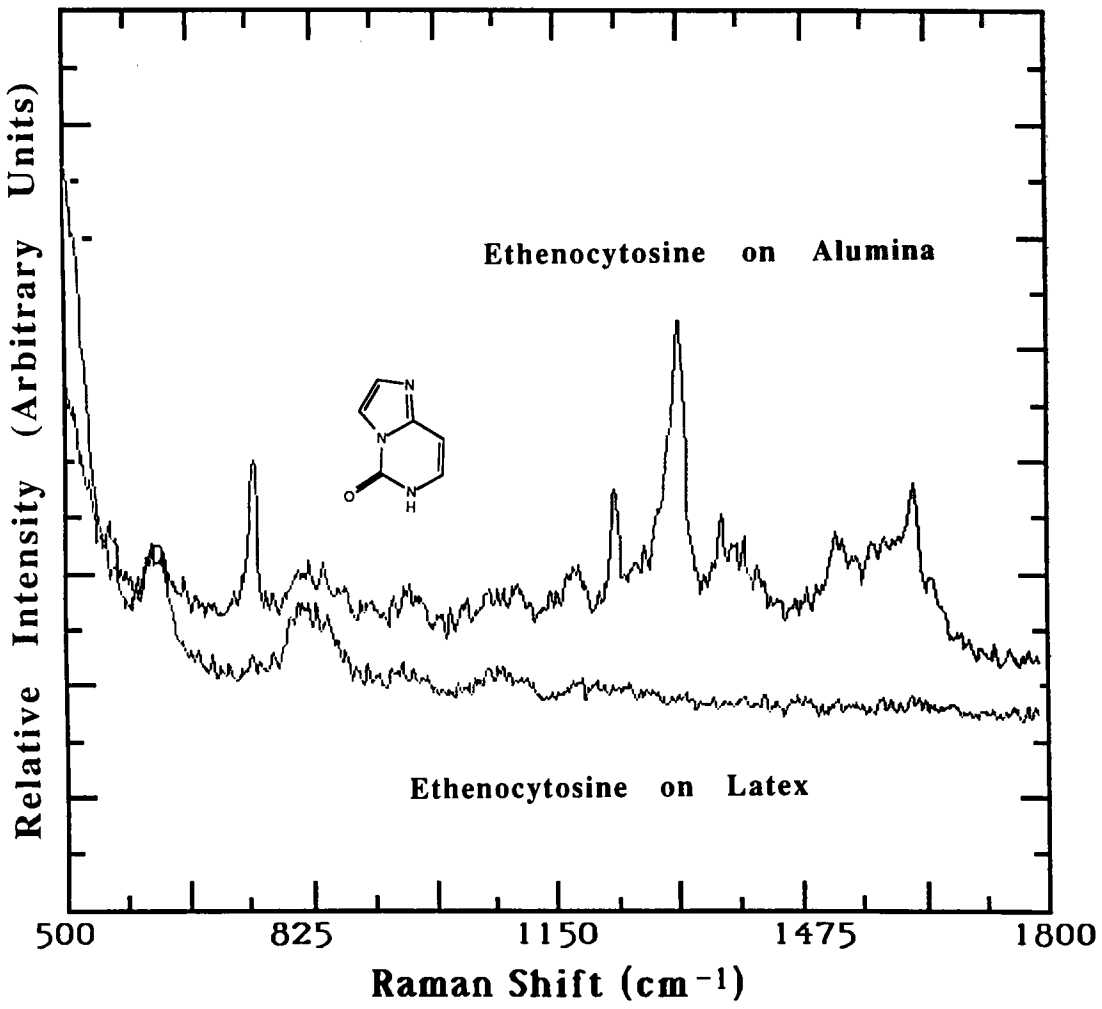

**<FIGURE II-6> Scanning Electron Micrographs of Silver Island SERS Substrates** Evaporated silver thickness was (Top) 16-nm and (Bottom) 10-nm.

The effect of substrate type on sample spectra was usually noticeable, but not predictable for different molecules. Differences between SERS spectra from identical samples on alumina- and polystyrene bead-roughened substrates are illustrated for 3,$N^4$-ethenocytosine (direct laser excitation at 647.1-nm) in Figure II-7 and for benzo[a]pyrene (fiberoptic excitation/collection at 647.1-nm) in Figure II-8, Figure II-9, and Figure II-10. Excellent spectra of all molecules in this study could be taken with the polystyrene-type substrate at 514.5-nm or 488.0-nm, but the relative intensities of peaks from these spectra differed from those taken of the same molecules with excitation at 647.1-nm and could not be directly compared with each other. Also, spectra with higher-energy blue or green (argon-ion) excitation had to be taken at low laser powers to avoid chemical decomposition. This meant that high signal-to-noise ratios were difficult to obtain with SERS using the argon-ion laser.

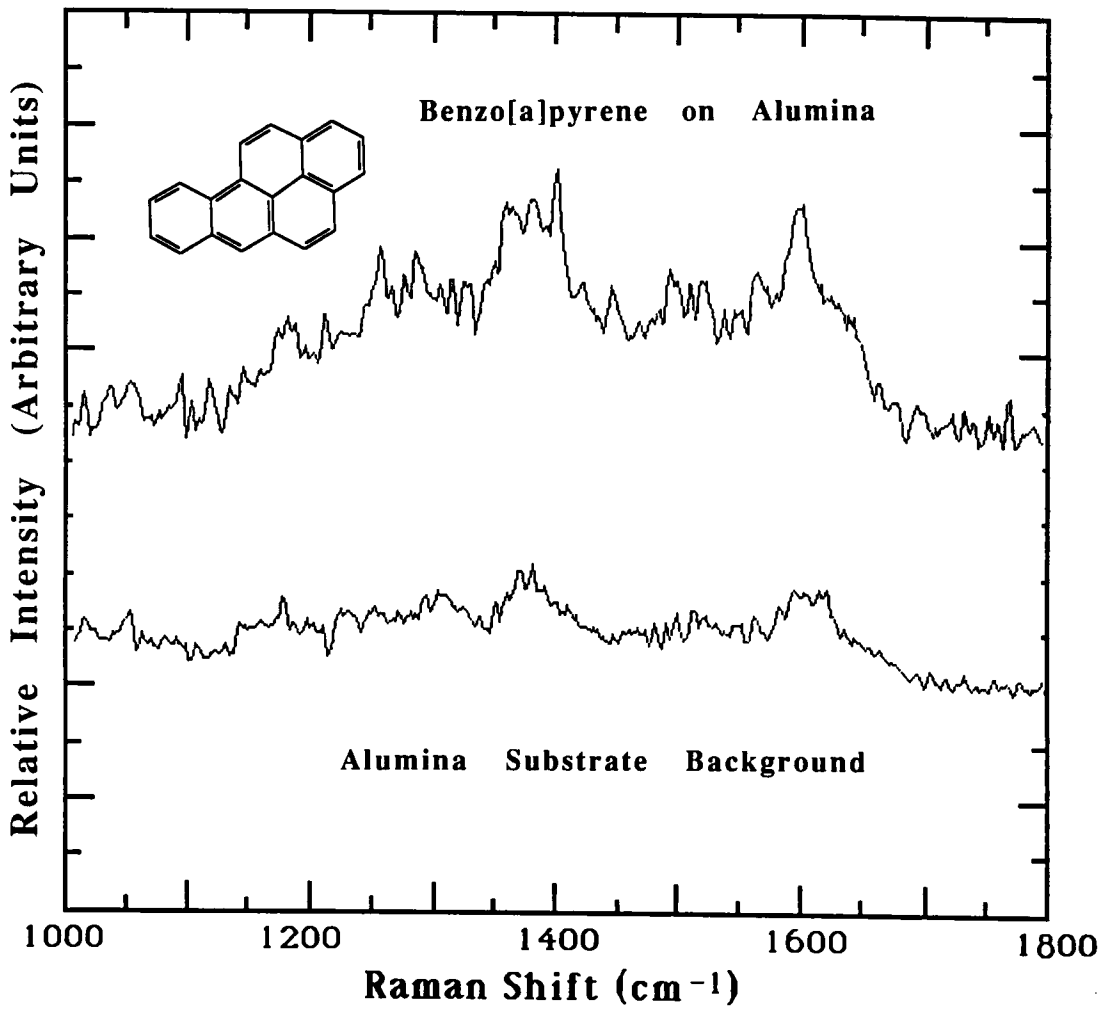
Examples of differences between spectra from alumina-type and silver island-type substrates are shown in Figure II-11 (3,$N^4$-ethenocytosine) and Figure II-12 (para-aminobenzoic acid or PABA, an intense SERS scattering molecule). For both molecules, the alumina-type substrate reliably yielded better spectra than did the silver islands with fiberoptic excitation at 647.1-nm.

### *Selection of Solvents*

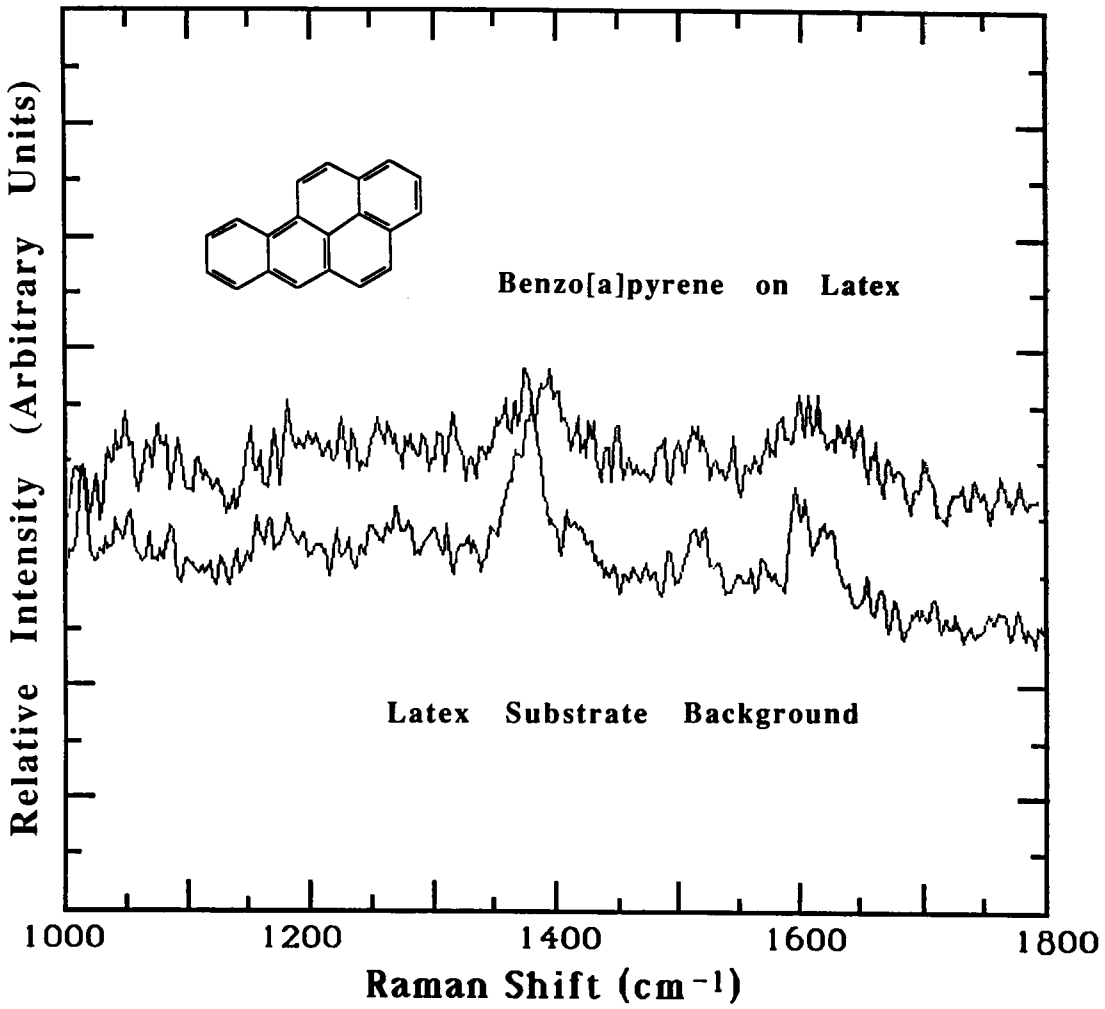
The solvent chosen for a SERS experiment had an important effect on any resultant spectra. Where sample solubility permitted, absolute ethanol was used to produce and dilute solutions. Ethanol did not exhibit a SERS



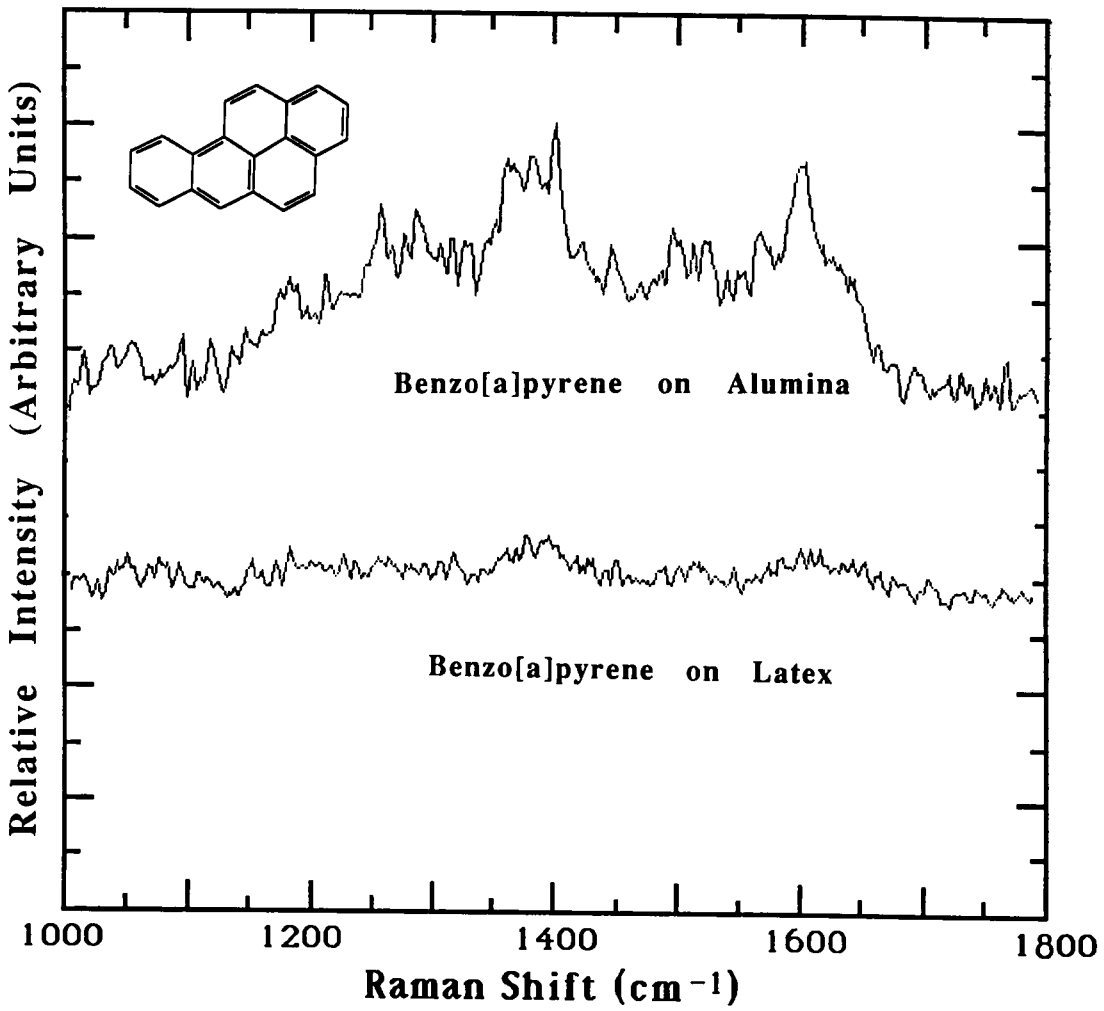

<FIGURE II-7> SERS Spectra of 3,N4-Ethenocytosine on Alumina-Type and Polystyrene-Type Substrates Substrates were (Top) 100-nm silver on 5.0% 0.1-μm diameter alumina and (Bottom) 100-nm silver on 0.22-μm diameter latex beads. Samples were 1.0-μL of 1x10-4-M solution of 3,N4-ethenocytosine in ethanol. Laser excitation was 80-mW at 647.1-nm. Spectra were printed to scale relative to each other. A direct beam optical setup was used.



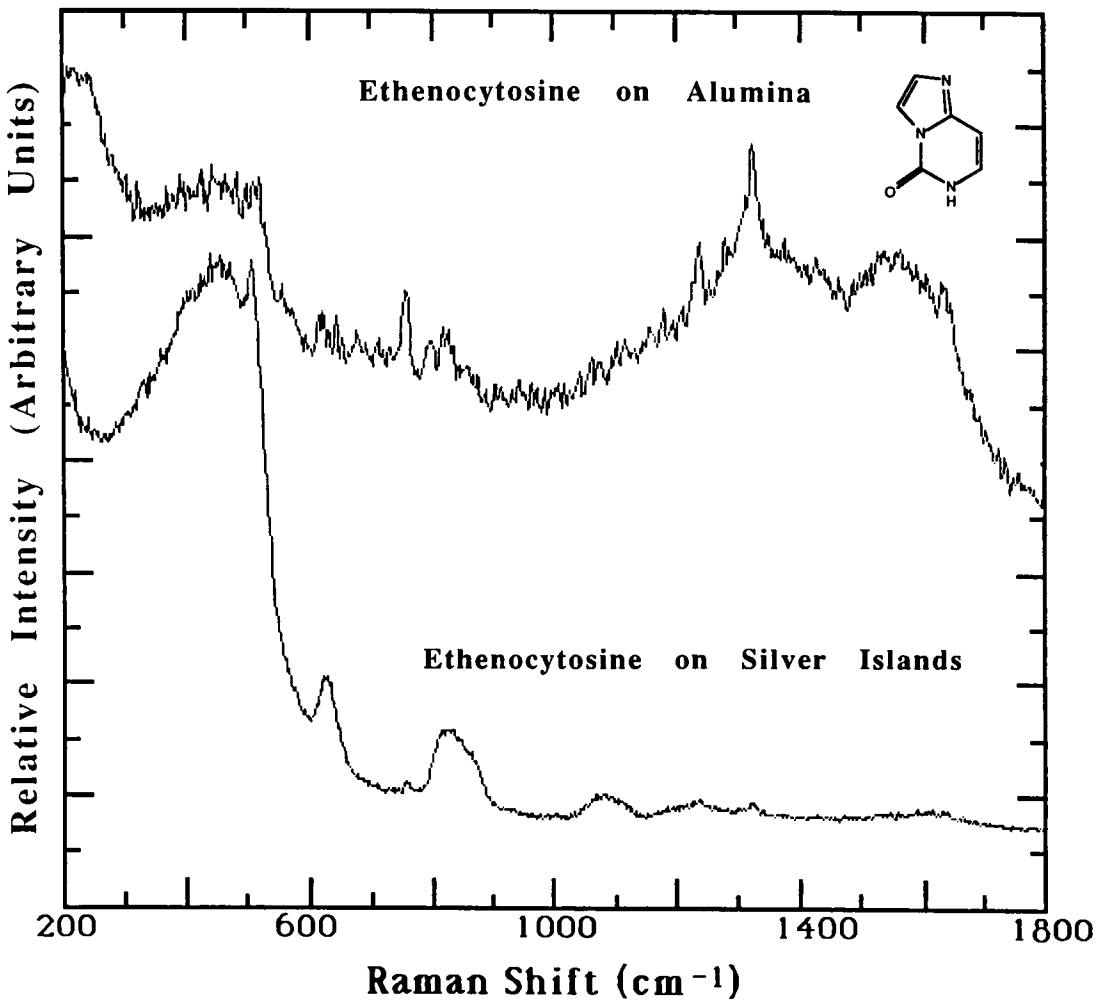

**<FIGURE II-8> SERS Spectra of Benzo[a]Pyrene on an Alumina-Type Substrate** Substrate was 100-nm silver on glass roughened with a 4.0% suspension of 0.1-μm diameter alumina. Spectra are (Top) 3.0-μL of a $1x10^{-4}$-M solution of benzo[a]pyrene in ethanol and (Bottom) the substrate background. Laser excitation was 80-mW at 647.1-nm. Spectra were printed to scale relative to each other.



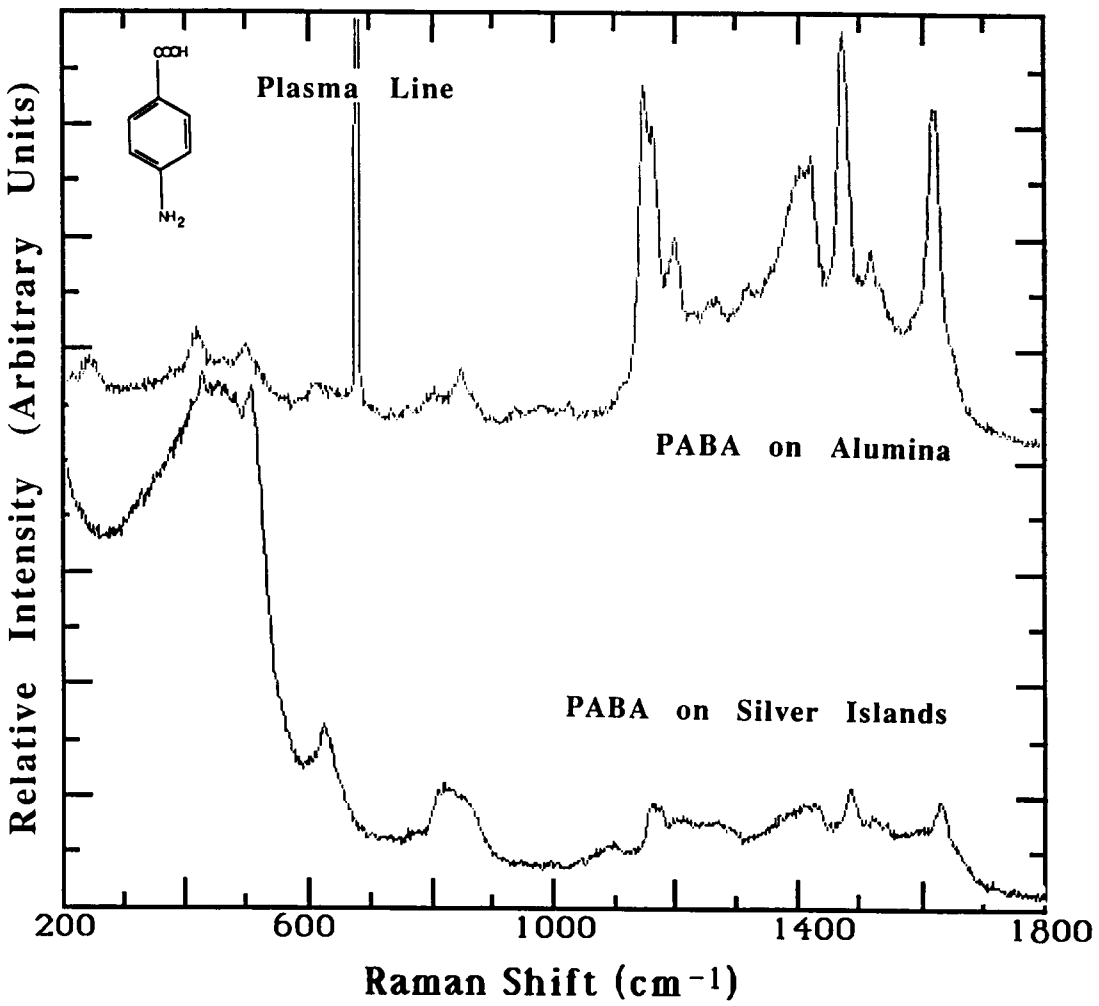

<FIGURE II-9> SERS Spectra of Benzo[a]Pyrene on a Polystyrene Bead-Type Substrate Substrate was 100-nm silver on glass roughened with a 2.5% suspension of 0.22-μm latex beads. Spectra are (Top) 3.0-μL of a $1x10^{-4}$-M solution of benzo[a]pyrene in ethanol and (Bottom) the substrate background. Laser excitation was 80-mW at 647.1-nm. Spectra were printed to scale relative to each other.




**<FIGURE II-10> SERS Spectra of Benzo[a]Pyrene on Alumina-Type and Polystyrene-Type Substrates** Spectra are of 1.0-μL of a 1x10-4-M solution of benzo[a]pyrene in ethanol on a (Top) 4.0% alumina-type substrate and (Bottom) polystyrene-type substrate. Laser excitation was 70-mW at 647.1-nm. Spectra were printed to scale relative to each other.



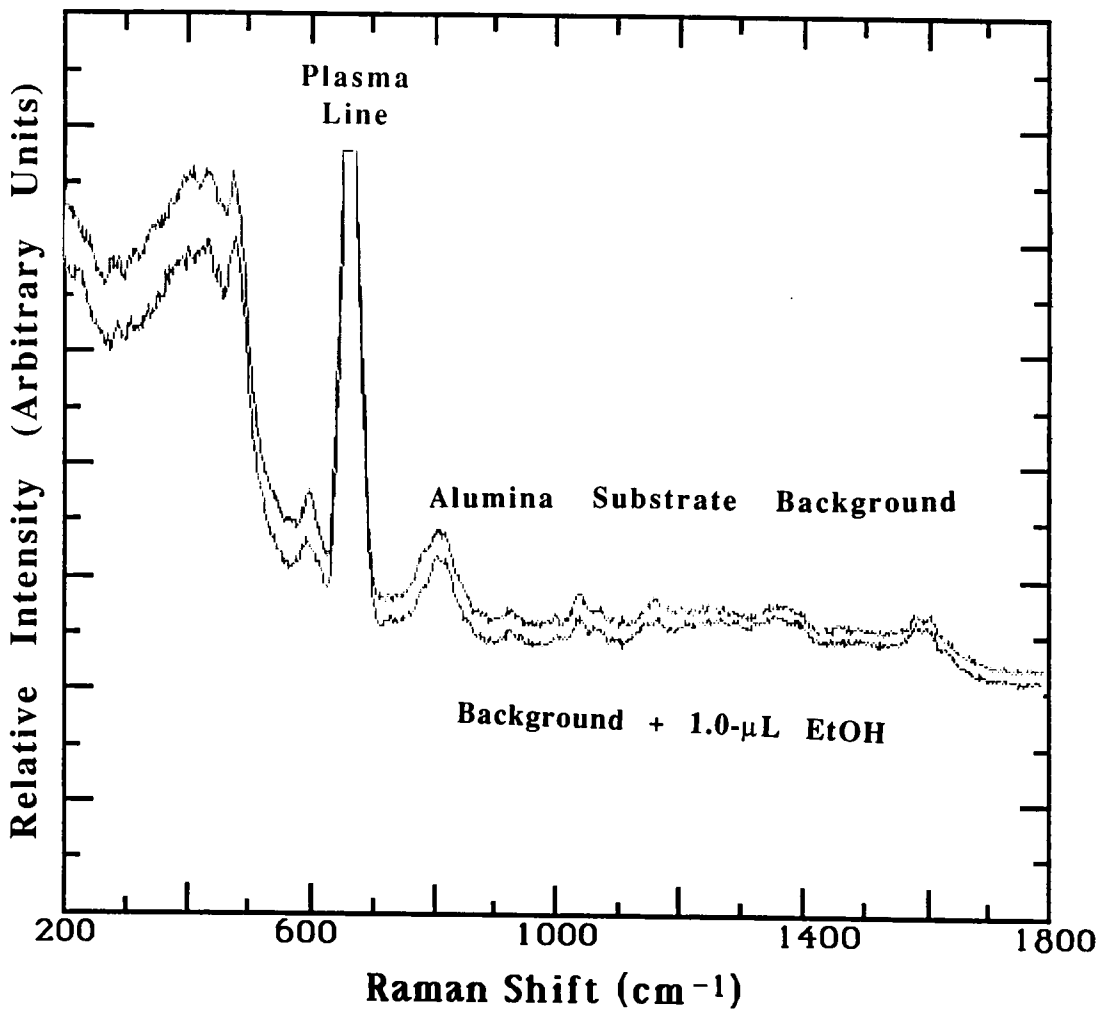

<FIGURE II-11> SERS Spectra of 3,$N^4$-Ethenocytosine on Alumina-Type and Silver Island-Type Substrates Spectra are of 1.0-μL of a 1x10$^{-4}$-M solution of 3,$N^4$-ethenocytosine on a (Top) 5.0% alumina-type substrate and (Bottom) 10-nm silver island-type substrate. Laser excitation for both spectra was 25-mW at 647.1-nm.



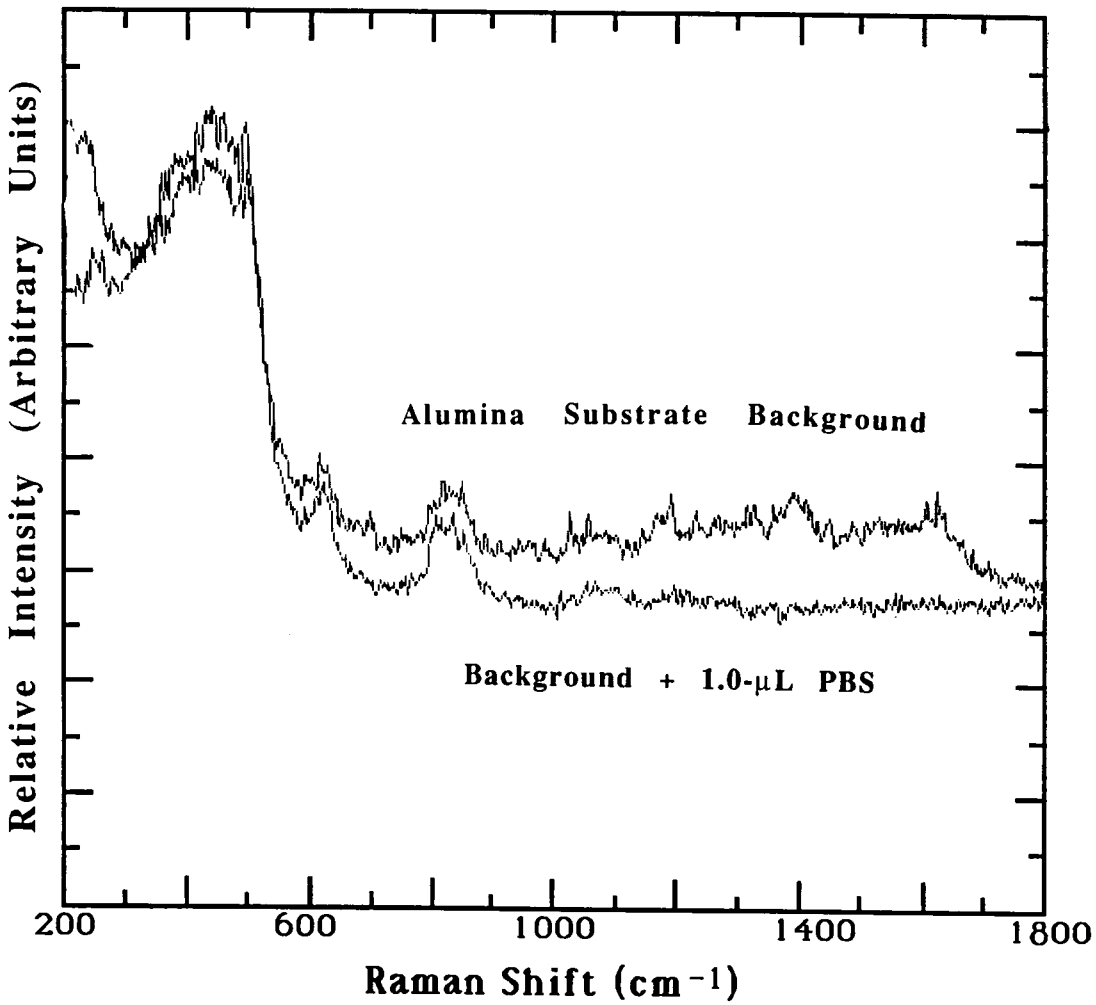

**<FIGURE II-12> SERS Spectra of 4-Para-Aminobenzoic Acid on Alumina-Type and Silver Island-Type Substrates** Spectra are of 1.0-μL of a $1x10^{-3}$-M solution of PABA in ethanol on a (Top) 4.0% alumina-type substrate and (Bottom) 10-nm silver island-type substrate. Note the intense laser plasma line in the top spectrum. Laser excitation for both spectra was 70-mW at 647.1-nm.

spectrum, nor did its use appreciably alter sample spectra (see Figure II-13). Another advantage of ethanol was that ethanol-based solutions spread out uniformly across a SERS substrate (1.0-μL drop spreads into a circle approximately 7-mm in diameter) and evaporated quickly. Water did not especially alter the SERS sample spectra, although the aqueous sample spot was difficult to apply because the drop adhered to the micropipette and because the drop had such high surface tension that it usually rolled off the substrate (did not spread across the substrate surface). In some instances, samples were first dissolved in water and then diluted with ethanol. In these cases, the sample concentration is listed according to the initial volumes of ethanol and water (e.g. 1:1 (v/v) EtOH:$H_2O$) to avoid confusion caused by the complete miscibility of ethanol in water. The ethanol:water mixtures were found to spread better across the substrate surface than the purely aqueous solutions, with the extent of sample application improvement dependent upon the final fraction of ethanol in the solution. Absolute ethanol was preferable to any ethanol:water mixture.

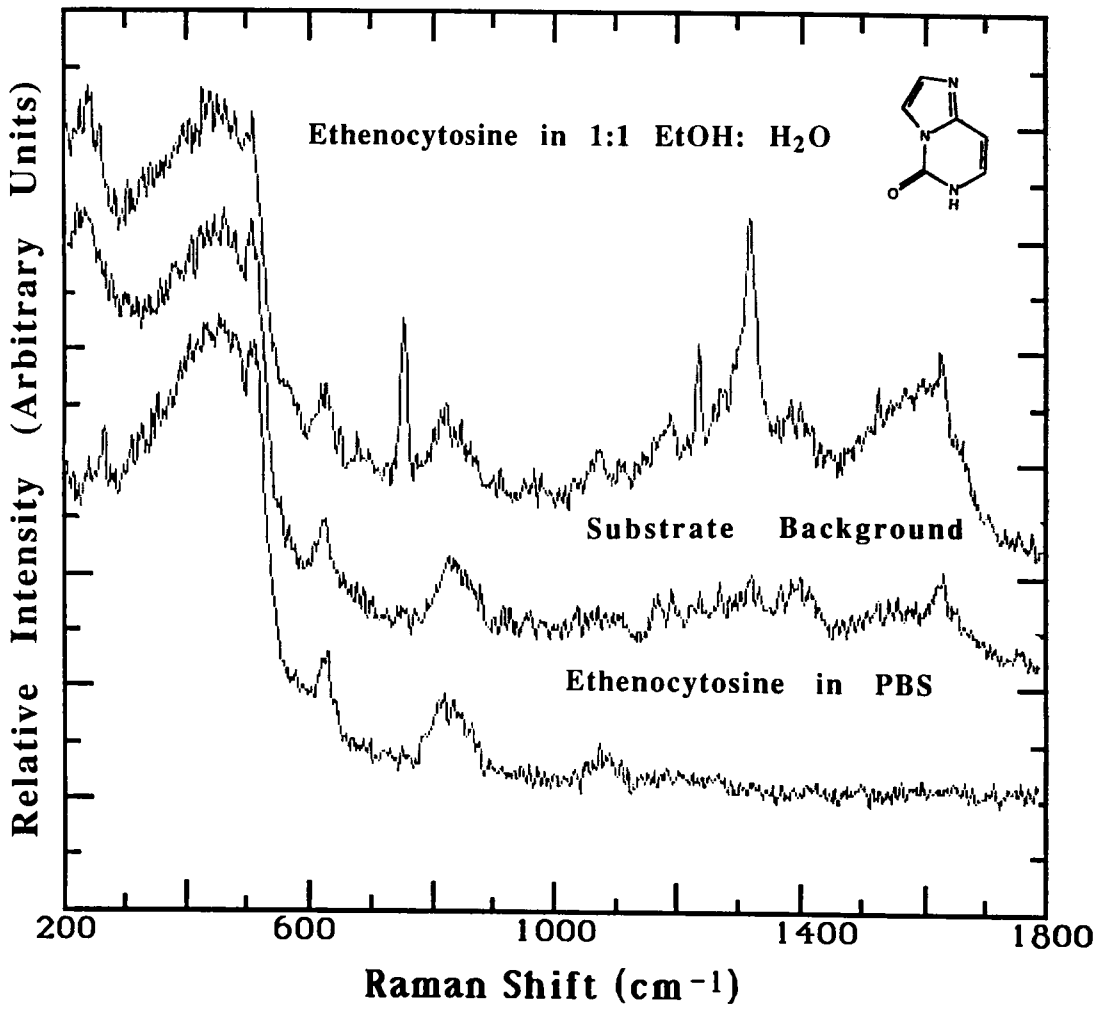
Phosphate-buffered saline (PBS, pH 7.0) was occasionally used for sample preparation, but caused problems with the SERS spectra of most chemicals. As illustrated by data in Figure II-14, a PBS spot applied to a substrate was found to diminish even SERS from the substrate and optical setup. This effect usually translated into poor spectra, as demonstrated in Figure II-15 of SERS of 3,$N^4$-ethenocytosine. The reduction of SERS intensity was probably a result of some interaction between the salt ions in PBS and the silver on the substrate surface. The one exception found to the peak intensity problem with PBS was the molecule 1,$N^6$-ethenoadenine. Figure II-16 and



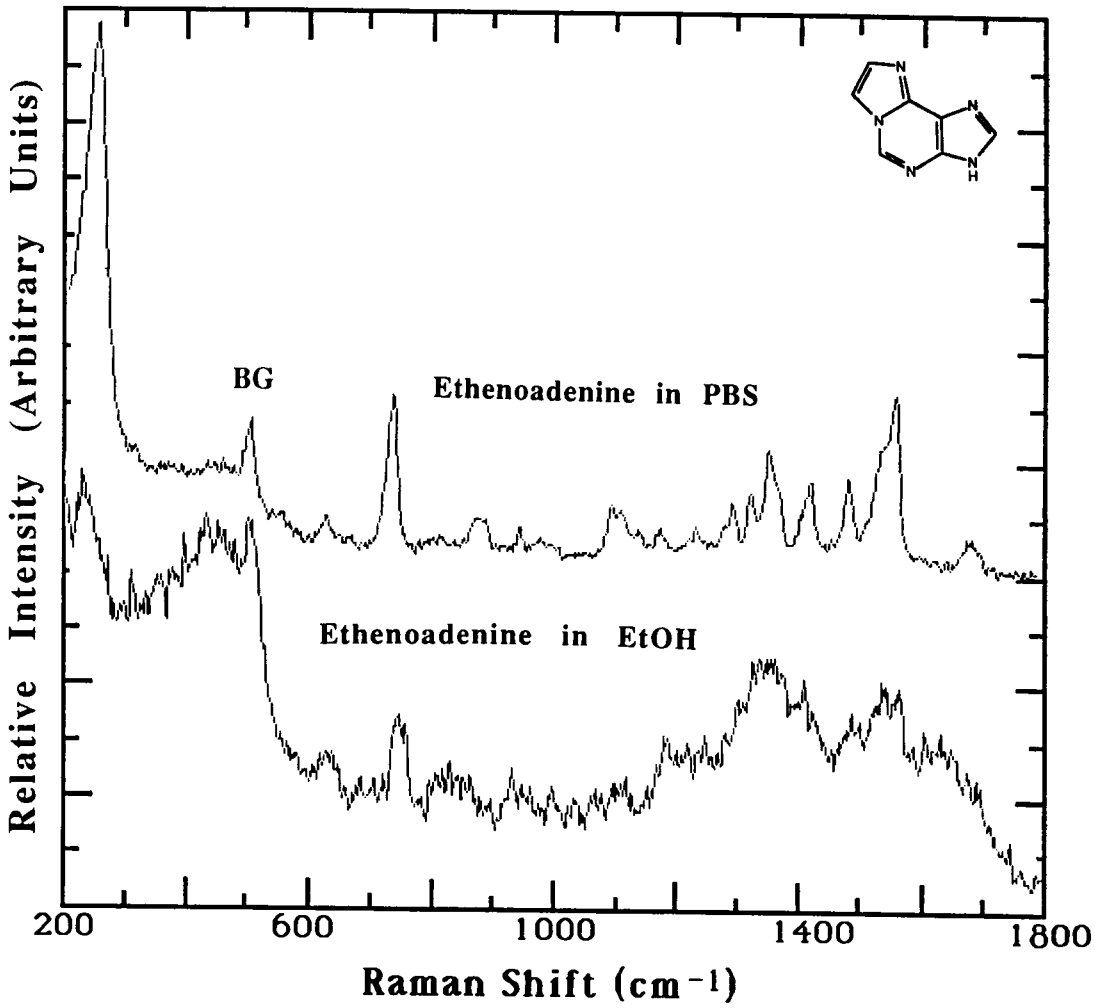

**<FIGURE II-13> SERS Spectra of Ethanol and the Alumina-Type Substrate Background** Spectra are of (Top) 4.0% alumina-type substrate and (Bottom) 1.0-μL of absolute ethanol. Both spectra were taken at the exact same position on the substrate. Note the laser plasma line at 670-$cm^{-1}$. Laser excitation was 70-mW at 647.1-nm. Spectra were printed to scale relative to each other.




**<FIGURE II-14> SERS Spectra of Phosphate-Buffered Saline and the Alumina-Type Substrate Background** Spectra are of (Top) a 4.0% alumina-type substrate and (Bottom) 1.0-μL of a phosphate-buffered saline solution at pH 7.0. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.



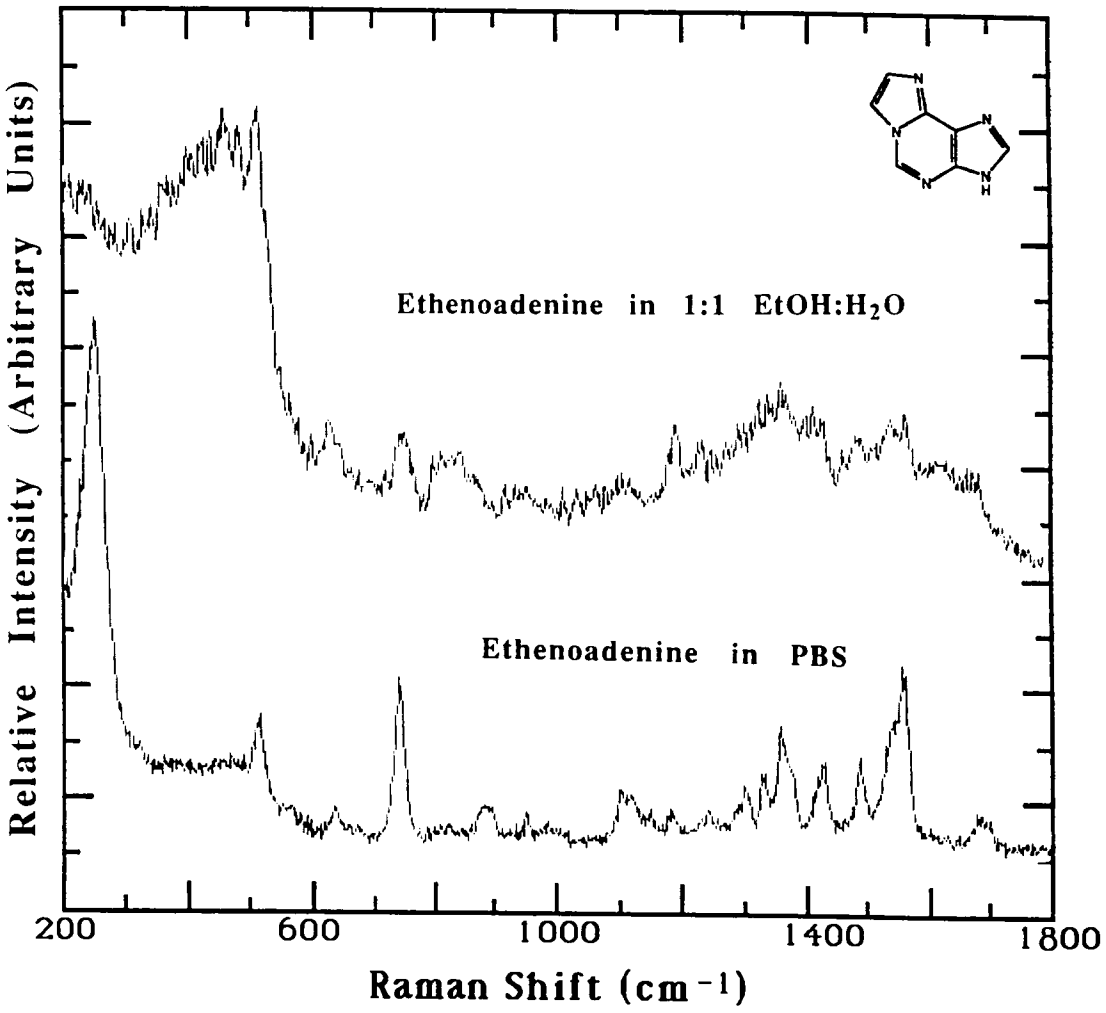

**<FIGURE II-15> SERS Spectra of 3,$N^4$-Ethenocytosine in Different Solvents** Spectra are of (Top) 1.0-μL of a 1x$10^{-2}$-M solution of 3,$N^4$-ethenocytosine in 1:1 (v/v) EtOH:$H_2O$, (Center) a 4.0% alumina-type substrate, and (Bottom) 1.0-μL of 1x$10^{-4}$-M solution of 3,$N^4$-ethenocytosine in PBS. All spectra were taken on the same substrate. Laser excitation was 50-mW at 647.1-nm.



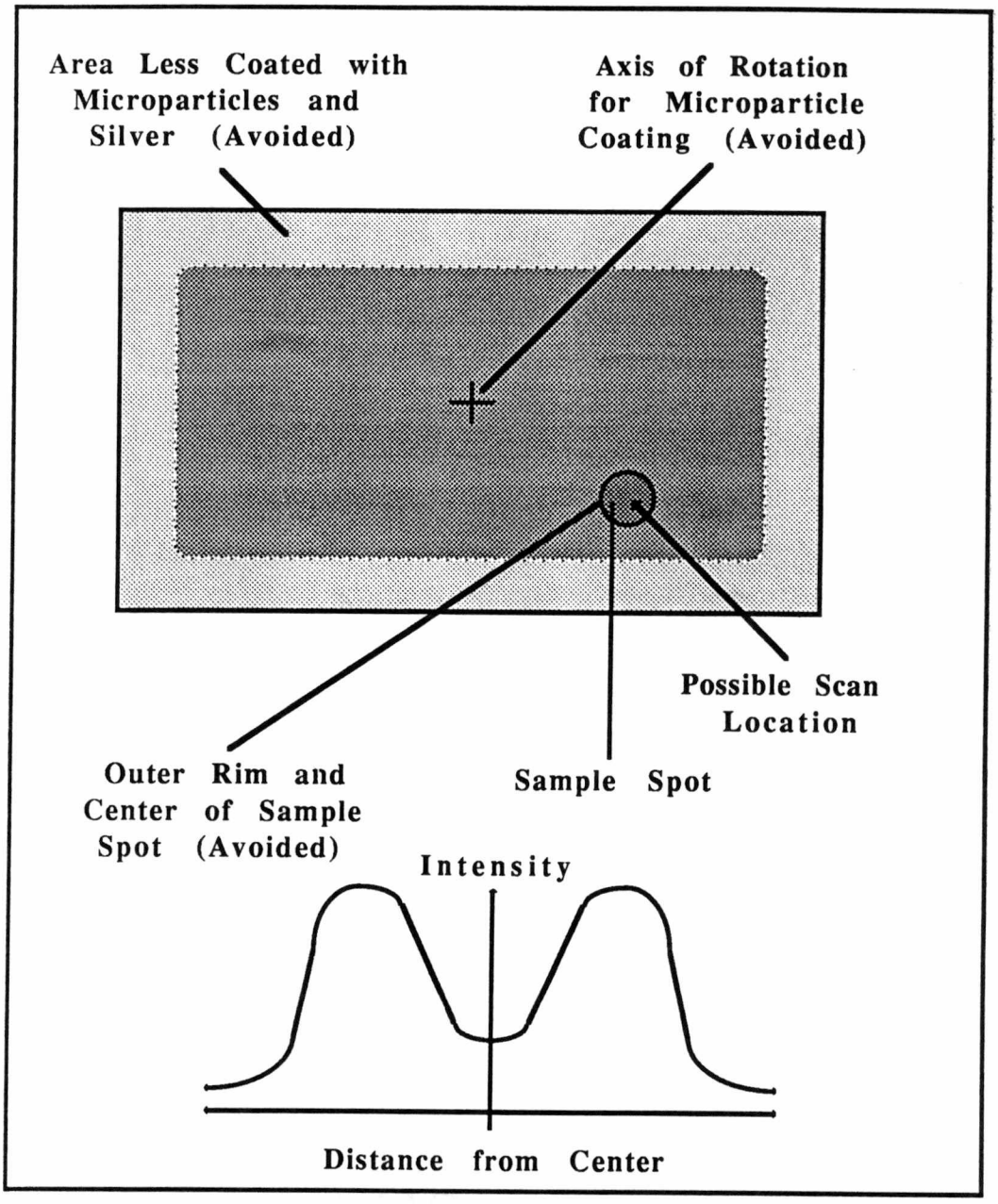

**<FIGURE II-16> SERS Spectra of 1,N⁶-Ethenoadenine in PBS and EtOH Solutions** Spectra are of (Top) 1.0-μL of a 8.9x10$^{-4}$-M solution of 1,N⁶-ethenoadenine in PBS and (Bottom) 1.0-μL of a 1x10$^{-3}$-M solution of 1,N⁶-ethenoadenine in ethanol. Note that the maximum absolute photon count was (Top) 7978.9 photons and (Bottom) 2836.7 photons; these spectra were not printed to scale. Laser excitation was 50-mW at 647.1-nm.

Figure II-17 clearly demonstrate the excellent SERS spectra that could be obtained when PBS was used as the solvent for the preparation of ethenoadenine samples. No explanation is offered for the anomolous behavior of ethenoadenine in PBS.

No matter which solvent was used, there was an apparent chromatographic effect as the sample spot spread across the substrate. This effect was not quantified and may have been different for each molecule in this study. The chromatographic effect was sometimes visible with highly concentrated samples when crystalline rings would appear on the sample spot perimeter. The exact center of a sample spot was avoided for SERS scanning because occasionally the micropipette used to apply the sample would nick the substrate surface. As listed in Table II-2, samples were scanned in regions of the substrate more likely to be similar between different substrates and optimal for securing SERS spectra. Figure II-18 is presented to clarify this point. Included in Figure II-18 is a rough plot of the expected enhanced Raman scattering intensity for the sample (number of scattered photons) versus the distance from the center of the sample spot. Note that even if the substrate was damaged by touching the micropipette tip to the surface, some SERS signal would still be obtained. This effect likely results from the collection fiber or lens gathering some photons from the region around the disfigured center. SERS intensity reaches a fairly uniform maximum value between the spot center and the outer rim of the spot. Signal intensity decreases sharply as a scan moves beyond the perimeter of the spot.




**<FIGURE II-17> SERS Spectra of 1,N$^6$-Ethenoadenine in Different Solvents** Spectra are of (Top) 1.0-μL of a 1x10$^{-3}$-M solution of 1,N$^6$-ethenoadenine in 1:1 (v/v) EtOH:$H_2O$ and (Bottom) 1.0-μL of a 8.9196x10$^{-4}$-M solution of 1,N$^6$-ethenoadenine in PBS. Note that the maximum absolute photon count was (Top) 2458.5 photons and (Bottom) 7965.0 photons; these spectra were not printed to scale. Laser excitation was 50-mW at 647.1-nm.




<FIGURE II-18> Diagram of the Optimum Scan Location on a SERS Substrate

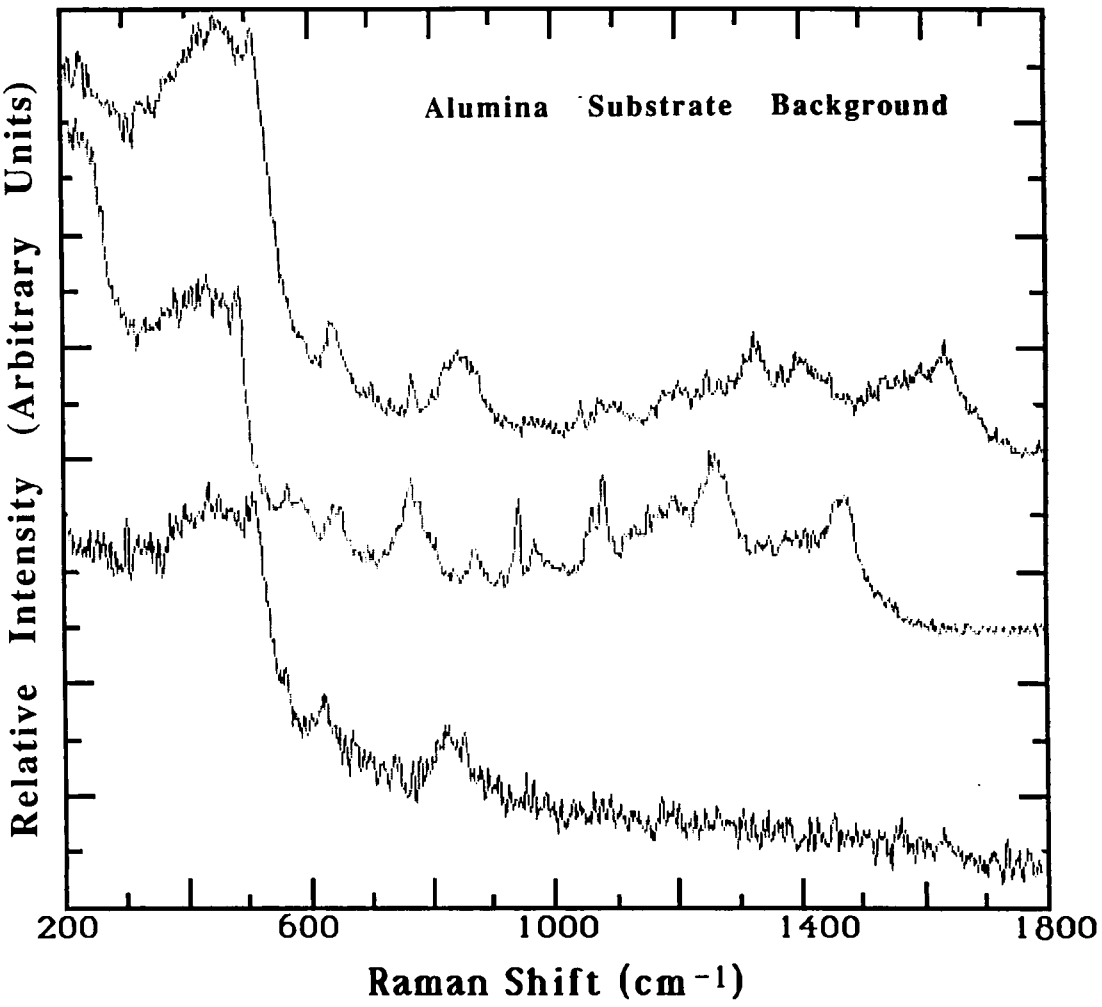
*Excitation Power and the Sample*

Several experiments included studies on the effects of excitation power on SERS spectra and on molecular integrity. The results of these studies will be dealt with in the context of the experiments presented later on, but it is worth noting here that proper excitation power was also important for obtaining quality SERS spectra from a sample. When too low power was applied, little or no SERS bands were detected because the quantity of Raman scattered radiation was lower or comparable to the amount scattered off the substrate and optics (background noise). When the excitation power was too intense, the molecules underwent thermal- or photo-decomposition.

In most instances, it was relatively simple to determine optimum excitation power for a specimen. An intermediate power (50-75 mW at 647.1-nm) was first tried to determine whether or not the substance would yield any SERS spectrum under normal operating conditions. Next, the power was increased until the SERS signal diminished under exposure to the laser beam. This was done by scanning a strong peak detected at low power, and watching for a quick drop in scattered light intensity. The optimum power was an experimentally determined value just less than the peak diminishment level -- where the quantity of scattered radiation was highest, yet under which the specimen was stable in the laser beam indefinetely and could be rescanned repeatedly.

*Background Noise*

The enhanced Raman signals from the optical setup and substrates affected the reproducibility of SERS spectra. An example of this variation is illustrated in Figure II-19. Figure II-19 depicts three sample background spectra taken at the same laser excitation power at the same wavelength and with substrates prepared in the same manner, but on separate days (all alumina-type substrates). While sample spectra taken with different optical setups will be similar, bands that may be attributed to background noise from the optics, substrate, and environment will not remain constant. Caution should be used when attempting to compare SERS spectra taken at different times or on different substrates. As will become obvious from the experimental results, the general SERS background bands remained similar for a given substrate type, but did vary somewhat from day to day. Background noise was affected primarily by the choice of optical setup. Direct laser excitation/collection and fiber excitation/collection exhibited differently-shaped background spectra. This may be seen by comparison of Figure II-7 (3,$N^4$-ethenocytosine, direct excitation/collection) and Figure II-11 (3,$N^4$-ethenocytosine, fiberoptic excitation/collection). When attempting to detect and identify SERS bands shifted a particular distance from the excitation wavelength, it will be important to keep the shape of the background spectra in mind. For example, the fiberoptic SERS setup invariably yielded intense SERS background bands in the 200 $cm^{-1}$ to 500 $cm^{-1}$ regions of the Raman trace. Most of these bands may be attributed to the silica collection fiber, since the bands appeared when no substrate was in place and also when direct laser excitation was used. Had the samples in this study also had SERS bands in this




**<FIGURE II-19> SERS Substrate Background Spectra** Substrates were 100-nm silver evaporated onto glass that had been roughened with 4.0% alumina. Laser excitation was 80-mW at 647.1-nm. All spectra were taken within a one week period.

region, they may have been rendered undetectable by the fiber background. The excitation wavelength did not appreciably alter the background, except that different SERS substrates were best for different excitation wavelengths.

# CHAPTER 3

# CONCLUSIONS

The greatest flexibility may be exercised by the researcher by using a variety of types of SERS substrates. Alteration of the substrate type affects the relative intensities of SERS bands for the sample and the background. Certain prominent substrate background bands are due to the presence of fiberoptics in the experimental setup. Other bands are specific for the plasmon source (usually silver). Since sample molecular orientation and the cross-scattering enhancement are dependent upon the characteristics of the substrate, the choice of substrate greatly affects the experimental results. By testing several substrates for a particular substance, additional structural information may be obtained about the molecules.

In this section, multiple factors were clearly shown to affect SERS spectra from a specimen. The major variables accountable for differences between spectra were substrate type, solvent, and excitation power. Optical setup and substrate type usually determined the shape of the background spectrum. The advantages of sample site specificity and remote sensing available with a fiberoptic SERS setup were countered by the loss of detectable sample signal through scattering inside the collection fiber and overwhelming Raman signal from the silica fibers. The intense fiberoptic SERS bands in the 200 $cm^{-1}$ to 500 $cm^{-1}$ Raman shift region may prohibit the application of this type of optical setup to certain chemicals exhibiting SERS in this range.

As shown in Figure II-1, SERS spectra from a chemical (cytosine) were directly comparable when consideration was made for the parameters listed in

Table II-1. The importance of optimization of these variables for SERS cannot be overstated. The power the researcher can exercise over experimental parameters is one of the advantages of SERS over other spectroscopic techniques. Different combinations of these variables may be used and the results integrated to obtain unique structural information about a molecule.

# SECTION II

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## REFERENCES


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# PART III: SERS OF GENOTOXIC CHEMICALS

# CHAPTER 1

# SERS OF ETHENO ADDUCTS

## *Introduction*

Measurement of DNA adduct levels may be used to assess low-level exposure to genotoxic agents. In these experiments, the effectiveness of SERS was investigated for the analysis of etheno adducts of adenine and cytosine, cyclic base adducts produced from the metabolism of vinyl chloride.

Since 1974, vinyl chloride has been known to act as a potent carcinogen in both animals and humans (1-6). Vinyl chloride is mutagenic in both prokaryotic and eukaryotic cells upon metabolic activation via microsomal monooxygenases into chloroethylene oxide (CEO) and chloroacetaldehyde (CAA) (7-9). The primary DNA adduct formed from the reaction of CEO with genetic material is 7-oxoethylguanine. CAA reacts with DNA, nucleic acid polymers, and deoxyribonucleosides to form etheno adducts of deoxycytidine, deoxyadenosine, and deoxyguanosine (10-14). Both 3,$N^4$-etheno-2'-deoxycytidine and 1,$N^6$-etheno-2'-deoxyadenosine direct incorporation of noncomplementary nucleosides. The 3,$N^4$-ethenocytidine species are believed to be responsible for the most significant quantity of base misincorporation (15).

This investigation focused on the analysis of the *etheno* DNA/RNA adducts, since it is probable that they are the promutagenic species (15,16) in the liver cell population which, with the lungs, is a common site for angiosarcomas resulting from exposure to vinyl chloride. The SERS technique was used for the study of the etheno adducts because SERS is a technique with

very high sensitivity and chemical group selectivity and because other spectroscopic techniques have proven inadequate for comparing bases with their modified counterparts. Fluorescence studies have been carried out on the etheno adducts (16), however, fluorescence could not be used to compare the etheno adducts to unaltered bases and nucleosides because they do not fluoresce. Almost every chemical species detected in this study could be distinguished from the other chemicals. The results of this study indicated that the SERS technique may be used to monitor both unmodified bases and the etheno adducts, and so may yield original insights into the biochemical properties of the adducts.

## *Materials and Methods*

### Instrumentation

Instrumentation for these experiments was identical to that used in the optimization studies presented in Section II. The primary optical setup used for these experiments has been depicted in Figure I-4 (p. 18).

### Chemicals

Purines and pyrimidines were supplied by the Sigma Chemical Company (St. Louis, MO) and were used without further purification. Solutions were made by dissolving the bases in highly purified, deionized water and diluting to the desired concentration with absolute ethanol (1:1 v/v unless otherwise noted), or by dissolving and diluting the bases directly in absolute ethanol. Some solutions were also prepared using phosphate-buffered saline.

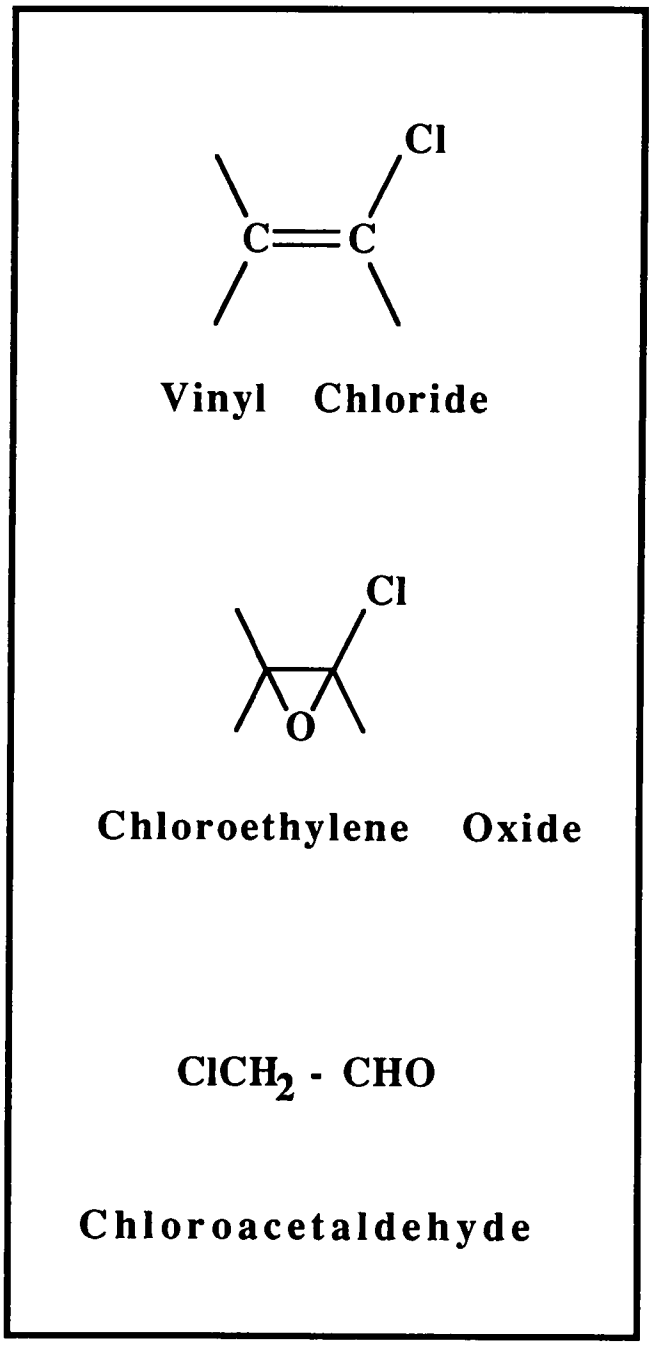
The chemical structures of vinyl chloride, chloroethylene oxide, and

chloroacetaldehyde are shown in Figure III-1. The structures of 1,$N^6$-ethenoadenine, adenine, and adenosine are given in Figure III-2. 3,$N^4$-ethenocytosine, cytosine, and cytidine are shown in Figure III-3.

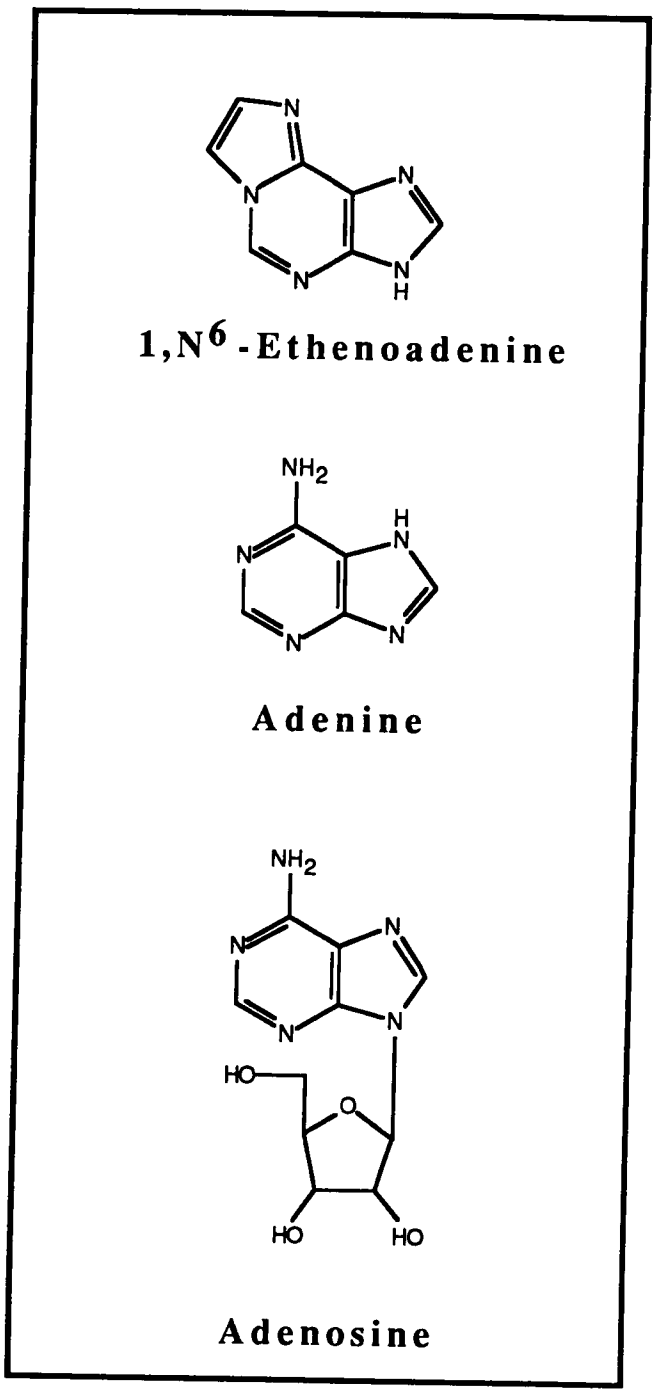
The chemical concentrations listed with the spectra in this section (especially when limit-of-detection studies were performed using serial dilutions) are given with a large number of significant figures. Note that if the accumulated rounding errors and propagated errors caused by the addition and multiplication of concentration values were accounted for, the numerical concentrations would be very imprecise. Extremely small quantities of chemicals were handled in these experiments. Solutions were made in volumetric flasks and were thoroughly mixed prior to examination to evenly disperse the molecules. The given concentration values are not intended as an exact measure of the sample concentrations, but to give the reader a general idea of how dilute the samples were. It is the author's opinion that molarity and molality labels are of little real value when the number of molecules present in a sample could be measure in terms of thousands or hundreds of thousands. Labelling concentration in terms of parts per million, parts per billion, etc. may give the reader an unwarranted confidence in sample concentrations. A better nomenclature may need to be developed when analysis of exceedingly dilute samples becomes commonplace.

## Procedures

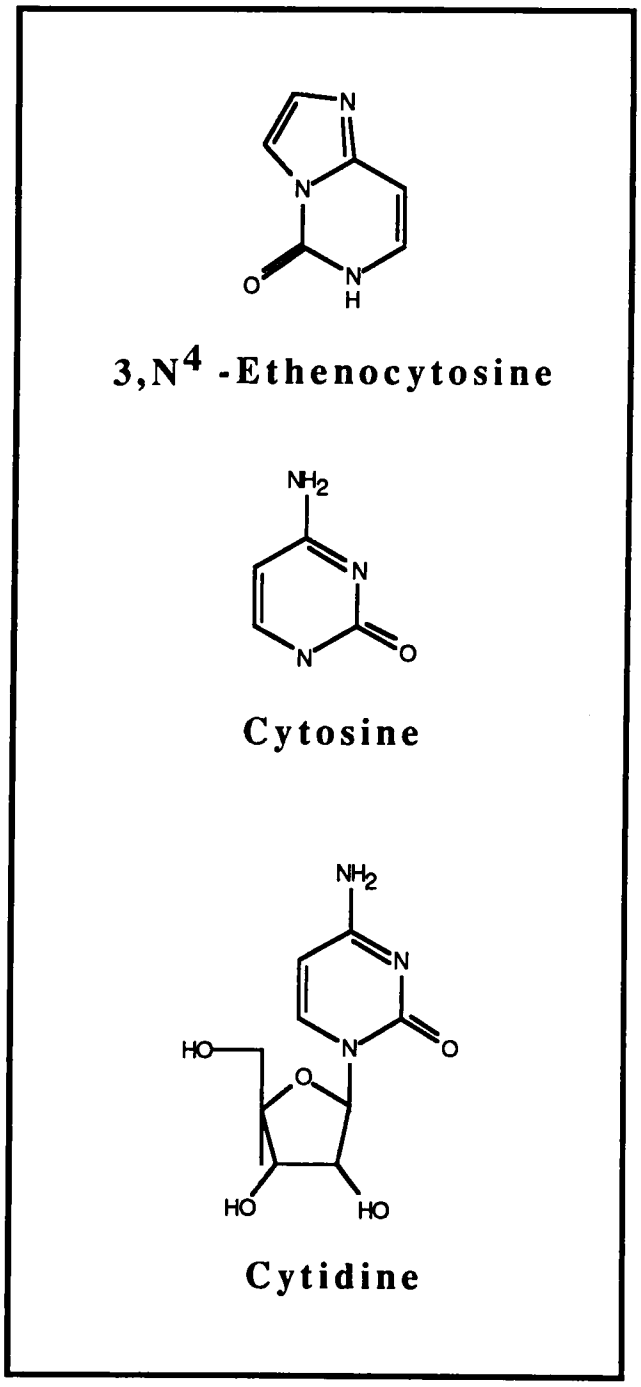
Unless otherwise noted, SERS substrates were prepared by spin-coating (Headway Research Inc., Model EC101 photoresist spin-coating device) 4.0% aqueous alumina for 25 seconds at 2000-rpm onto pieces of clean miscoscope slide glass. In more recent studies, 4.0% alumina was used rather than a 5.0%




<FIGURE III-1> Chemical Structures of Vinyl Chloride, Chloroethylene Oxide, and Chloroacetaldehyde




<FIGURE III-2> Chemical Structures of 1,N6-Ethenoadenine, Adenine, and Adenosine




<FIGURE III-3> Chemical Structures of 3,N4-Ethenocytosine, Cytosine, and Cytidine

alumina suspension because the 4.0% suspension was experimentally determined to be more effective for Raman enhancement and because its use resulted in more uniformly-coated substrates. 100-nm of silver was then thermally evaporated onto the alumina-coated glass plates. Substrates were used within two weeks of preparation, with no noticeable loss in sensitivity during that time.

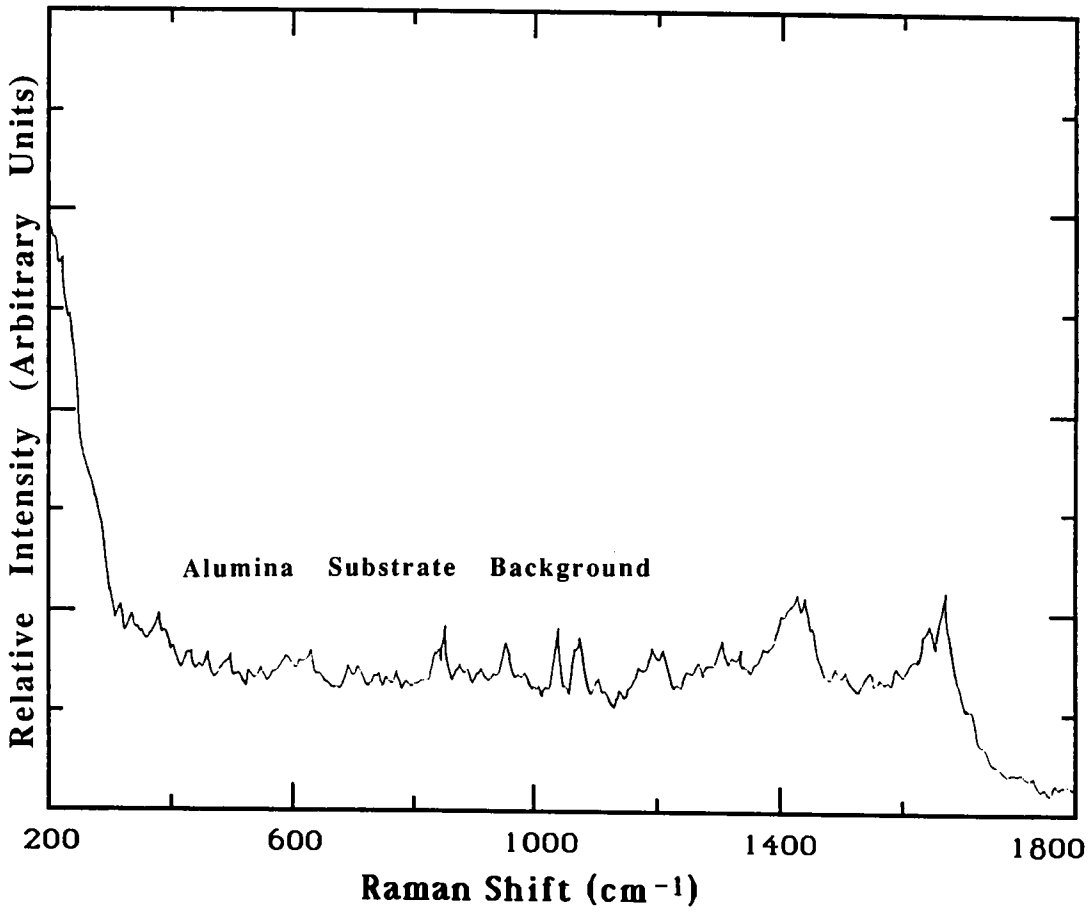
Most sample excitation was through the back of a silvered, alumina-coated substrate by the 647.1-nm line of a krypton-ion laser (Coherent Inc., Palo Alto, CA, Model Innova 70) at a power of 25-mW to 140-mW. A 600-μm diameter soft plastic-clad silica optical fiber with a numerical aperture of 0.26 (General Fiber Optics, Inc., Cedar Grove NJ) was used to collect the scattered light. The fiber was positioned as close to the sample/substrate surface as possible without disrupting the silver film. Some sample excitation was with the 514.5-nm line of an argon-ion laser, using a direct excitation/collection optical setup.

Samples of the bases were prepared for SERS by depositing 1.0-μL or 3.0-μL of an analyte solution onto a silvered, microparticle-coated substrate. The solvent was allowed to evaporate at room temperature before exposure of the molecules to the laser beam. The experimental setup and the production of the silvered, alumina-coated substrates have been described in detail previously (17-19).

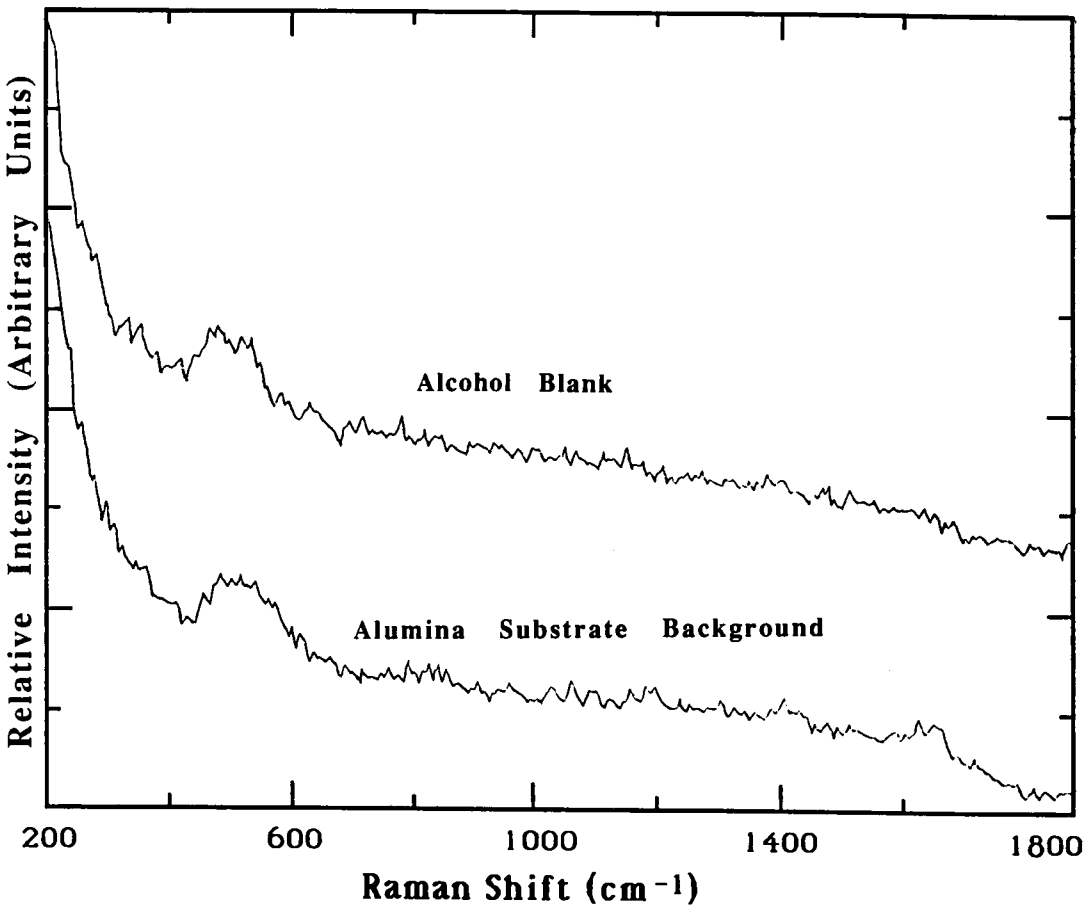
Unaltered Purines and Pyrimidines

SERS has previously been performed on purines and pyrimidines (20-25). SERS spectra of unaltered molecules were taken under the same experimental conditions as the etheno adduct study as a preliminary test to determine if altered molecules could be expected to exhibit SERS bands and for comparison with the spectra later observed for the etheno adducts. Spectra taken using 647.1-nm excitation of adenine, guanine, cytosine, and thymine, as well as of SERS background from the substrate and solvent are shown in Figure III-4 (5.0% alumina substrate background at 647.1-nm, same plate as samples), Figure III-5 (solvent and another example of a substrate background), Figure III-6 (adenine and cytosine), and Figure III-7 (guanine and thymine). A spectrum is available for comparison purposes of adenine on a *polystyrene* substrate (Figure III-8) at 647.1-nm. These spectra may be contrasted with spectra at 514.5-nm in Figure III-9 (5.0% alumina substrate background at 514.5-nm, same plate as samples), Figure III-10 (thymine and cytosine), and Figure III-11 (adenine and guanine). A SERS spectrum from a sample containing a mixture of bases is shown in Figure III-12 (adenine, thymine, and cytosine). This spectrum was taken with the same background and substrate as Figure III-9, Figure III-10, and Figure III-11.

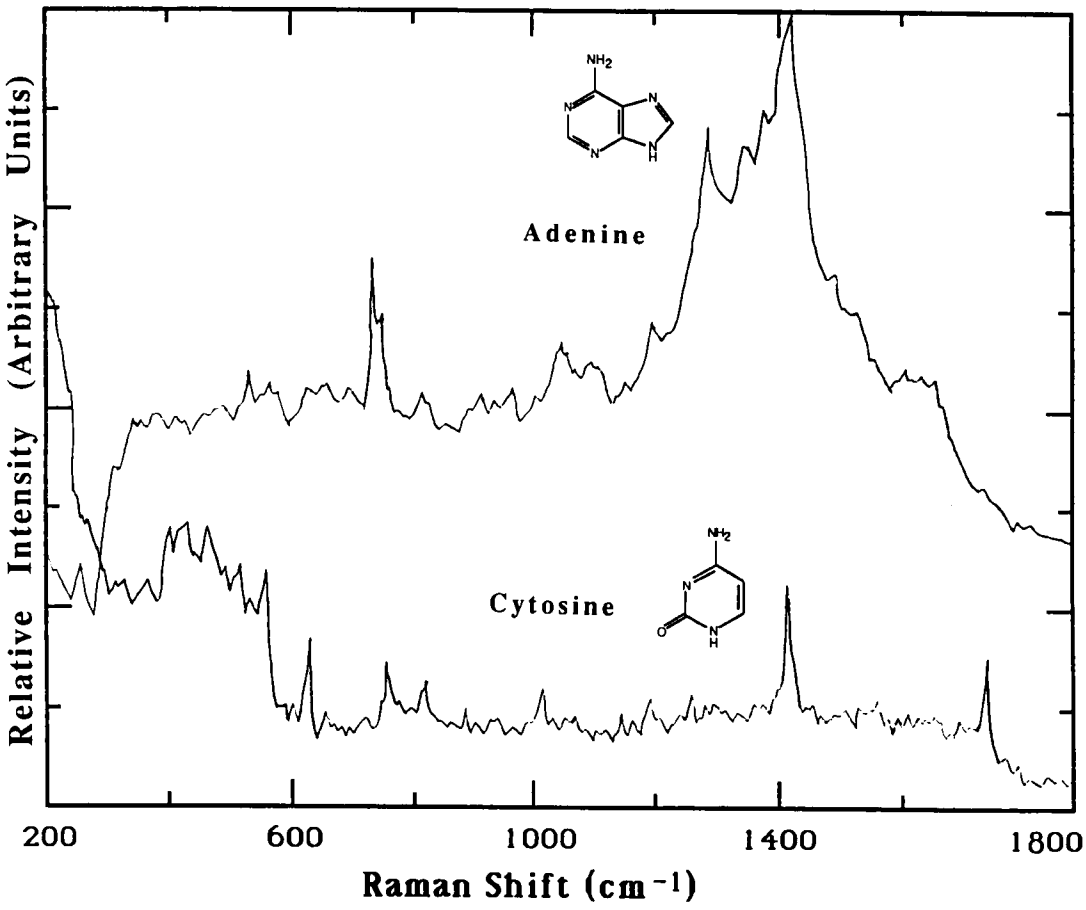
Note that SERS spectra for the purine/pyrimidine samples exhibit bands with similar Raman shifts and relative peak intensities for excitation at either 647.1-nm or 514.5-nm. This is evidence that the excitation wavelength is not an influencing factor in peak location or the relative intensity of peaks. The spectra taken at 647.1-nm and 514.5-nm were both taken on 5.0% alumina-type



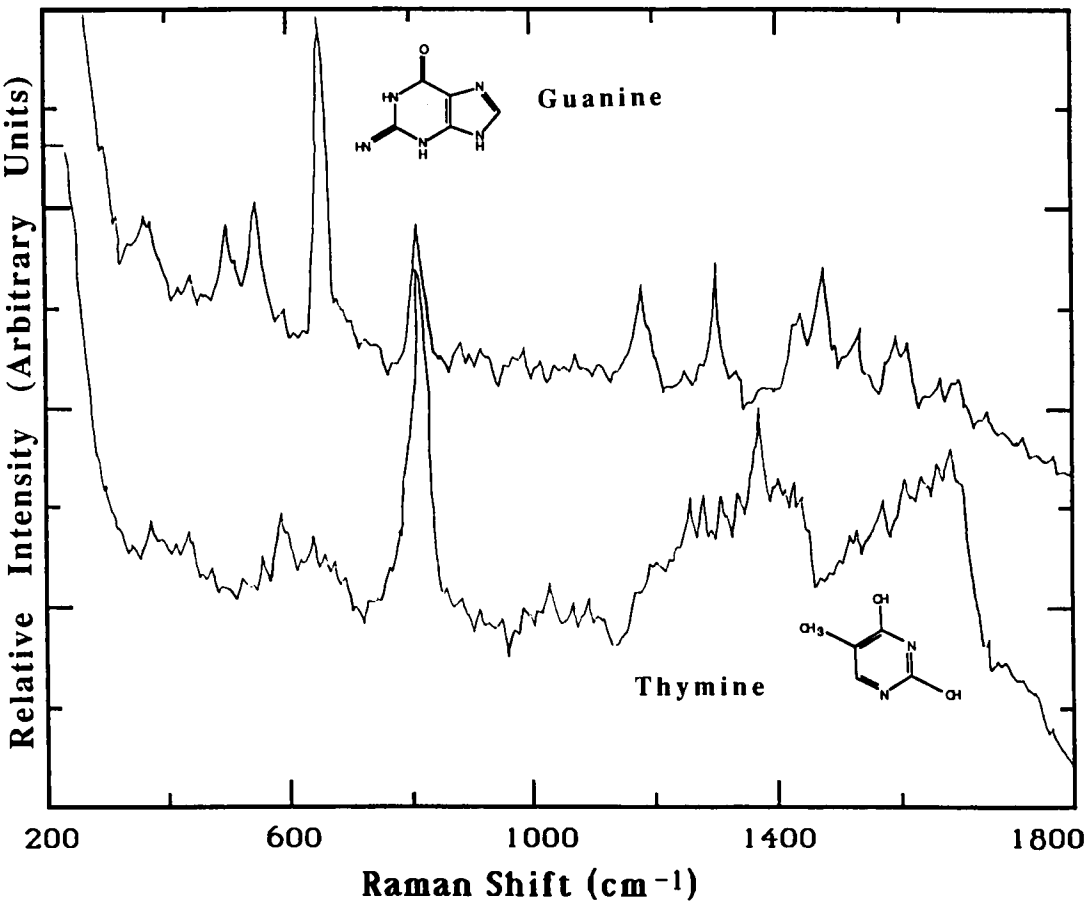

**<FIGURE III-4> SERS Substrate Background for Purine/Pyrimidine Study Using Krypton Laser Excitation** Substrate was 100-nm silver on glass roughened with a 5.0% alumina suspension. Laser excitation was 50-mW at 647.1-nm using a direct excitation/collection optical setup.



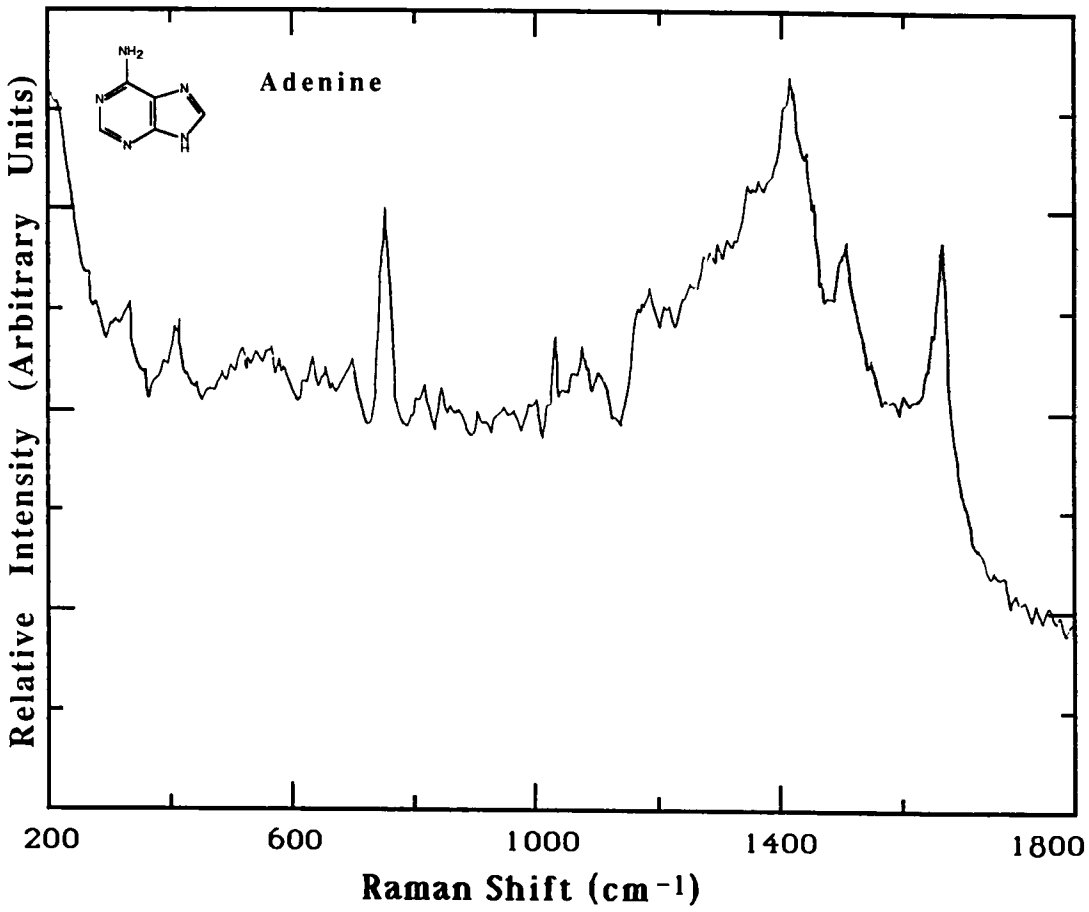

<FIGURE III-5> Solvent and Background SERS Spectra for Purine/Pyrimidine Study Using Krypton Laser Excitation Spectra are of (Top) 3.0-μL of the alcohol used to prepare the sample solutions and (Bottom) 5.0% alumina-type substrate background. Laser excitation was 50-mW at 647.1-nm using a direct excitation/collection optical setup.



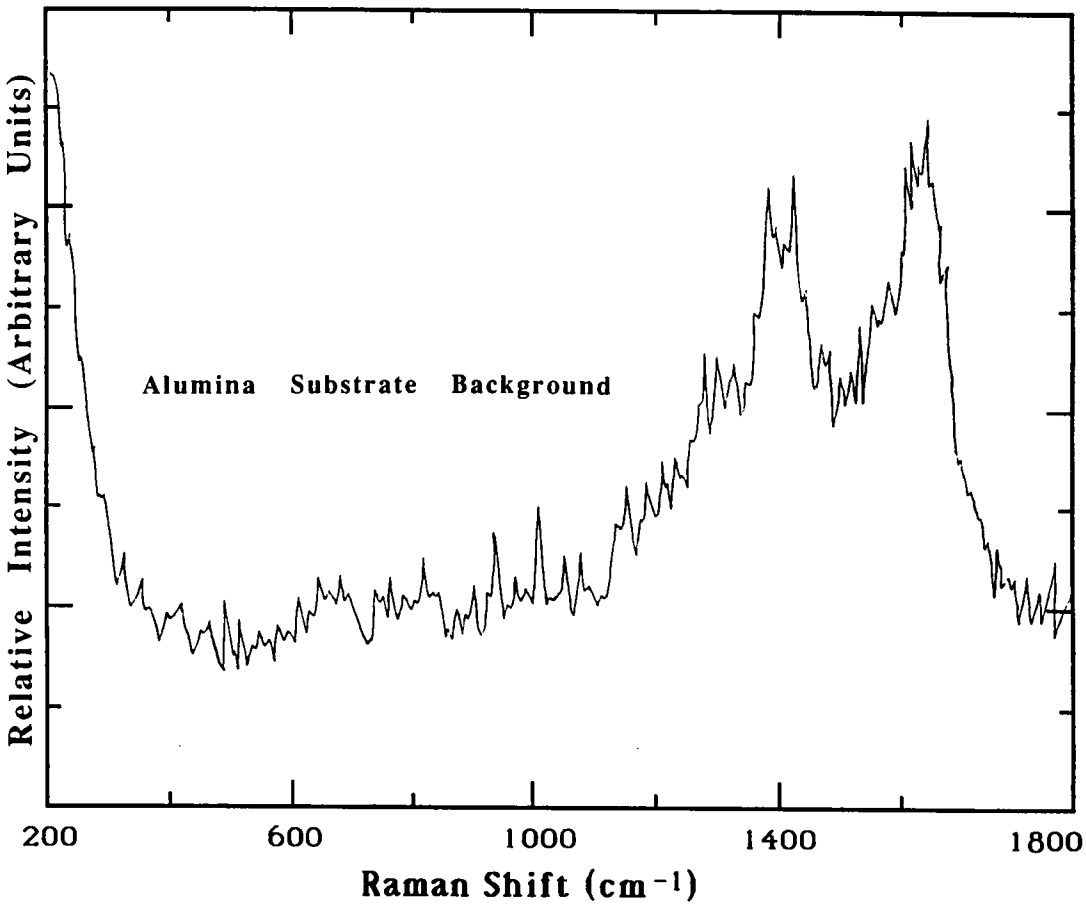

**<FIGURE III-6> SERS Spectra of Adenine and Cytosine Using Krypton Laser Excitation** Spectra are of (Top) 3.0-μL of a 2.799x10-2-M solution of adenine in alcohol and (Bottom) 3.0-μL of a 2.354x10-2-M solution of cytosine in alcohol. Laser excitation was 50-mW at 647.1-nm using a direct excitation/collection optical setup.



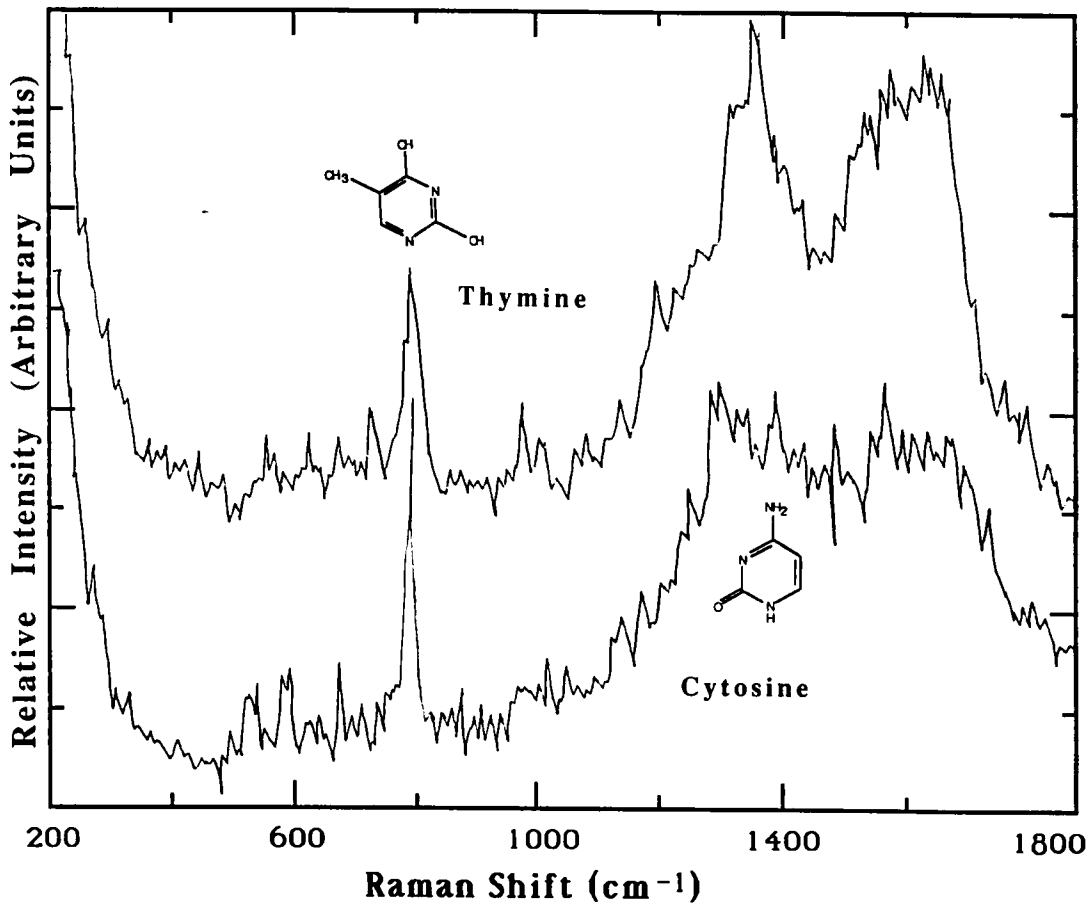

<FIGURE III-7> **SERS Spectra of Guanine and Thymine Using Krypton Laser Excitation** Spectra are of (Top) 3.0-μL of a 1x10-2-M solution of guanine in alcohol and (Bottom) 3.0-μL of a 1x10-2-M solution of thymine in alcohol. Laser excitation was 50-mW at 647.1-nm using a direct excitation/collection optical setup.



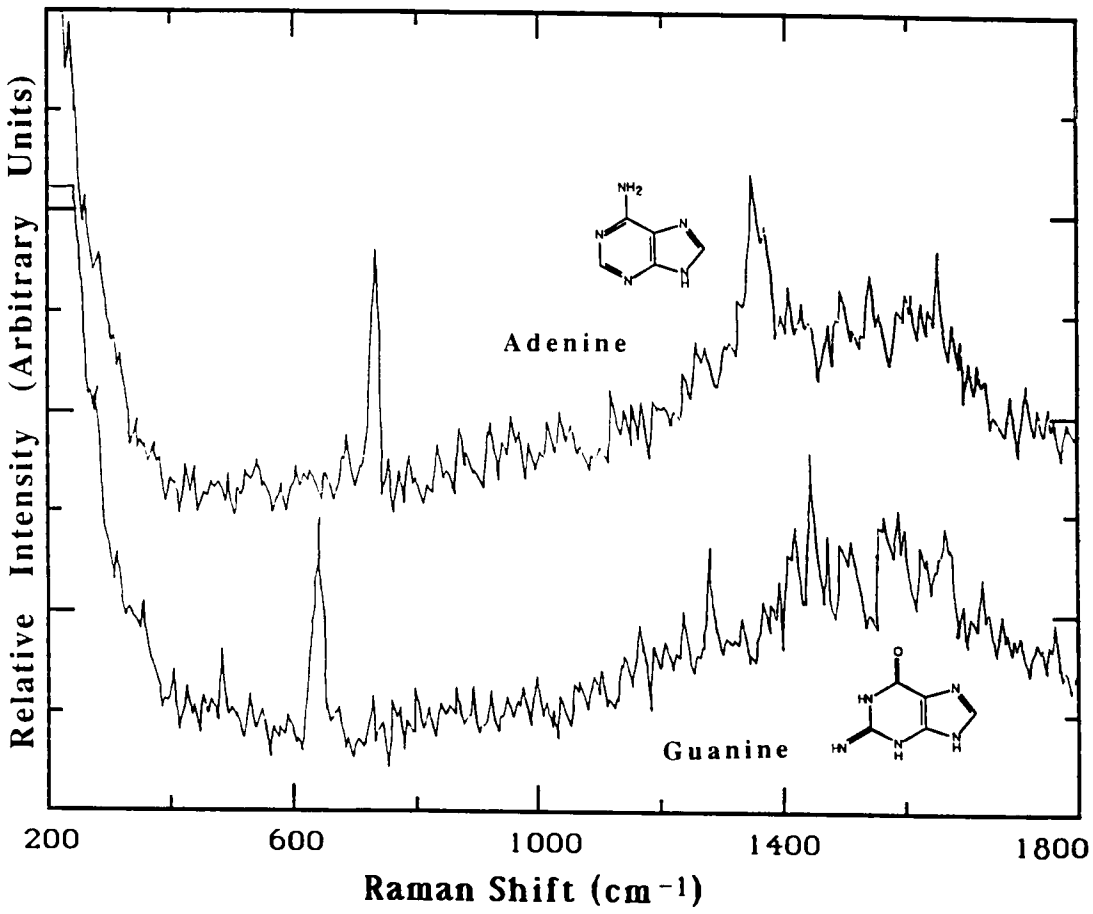

<FIGURE III-8> SERS Spectrum of Adenine on a Polystyrene-Type Substrate Sample was 3.0-μL of a 2.799x10-3-M solution of adenine. Substrate composition was 100-nm silver evaporated onto glass roughened with a 2.5% suspension of polystyrene beads (diameter 0.22-μm). Laser excitation was 50-mW at 647.1-nm using a direct excitation/collection optical setup.



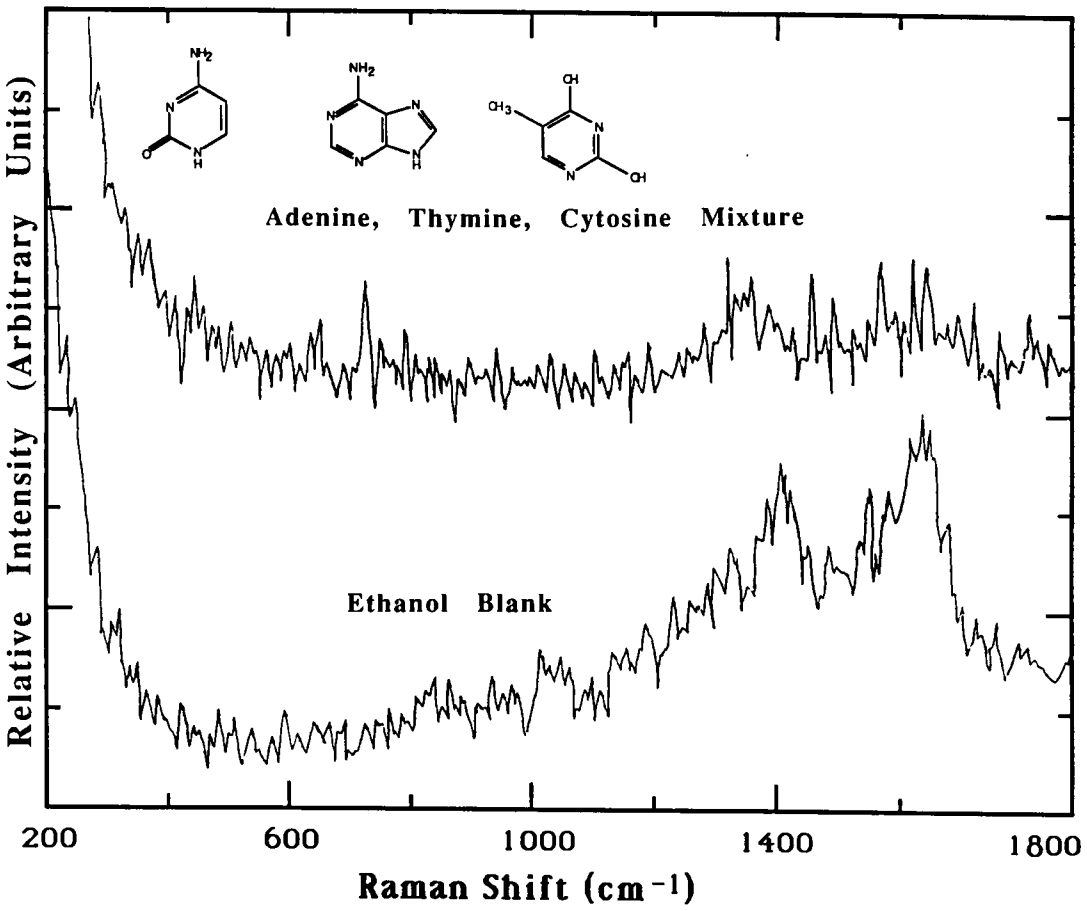

**<FIGURE III-9> SERS Substrate Spectrum for Purine/Pyrimidine Study Using Argon Laser Excitation** Substrate composition was 100-nm silver evaporated onto glass roughened with a 5.0% alumina suspension. Laser excitation was 50-mW at 514.5-nm using a direct excitation/collection optical setup.




**<FIGURE III-10> SERS Spectra of Thymine and Cytosine Using Argon Laser Excitation** Spectra are of (Top) 3.0-μL of a 3.70-mg/mL solution of thymine in 1:1 (v/v) EtOH/$H_2O$ and (Bottom) 3.0-μL of a 3.72-mg/mL solution of cytosine in 1:1 (v/v) EtOH/$H_20$. Spectra were taken on the same substrate and had approximately the same maximum photon counts. Laser excitation was 50-mW at 514.5-nm using a direct excitation/collection optical setup.




**<FIGURE III-11> SERS Spectra of Adenine and Guanine Using Argon Laser Excitation** Spectra are of (Top) 3.0-μL of a 3.26-mg/mL solution of adenine in 1:1 (v/v) $EtOH/H_2O$ and (Bottom) 3.0-μL of a 3-mg/mL solution of guanine in 1:1 (v/v) $EtOH/H_2O$ to which 10-μL concentrated HCl was added to improve sample solubility. Spectra were taken on the same substrate and had similar maximum photon counts. Laser excitation was 50-mW at 514.5-nm using a direct excitation/collection optical setup.




**<FIGURE III-12> SERS Spectra of a Mixture of Adenine, Thymine, and Cytosine** Spectra are of (Top) 5.0% alumina-type substrate background and (Bottom) 3.0-μL of a $1x10^{-4}$-M solution of equal parts of adenine, thymine, and cytosine in 1:1 (v/v) $EtOH/H_2O$. Guanine was omitted because the sample solution was very acidic. Laser excitation was 50-mW at 514.5-nm using a direct excitation/collection optical setup.

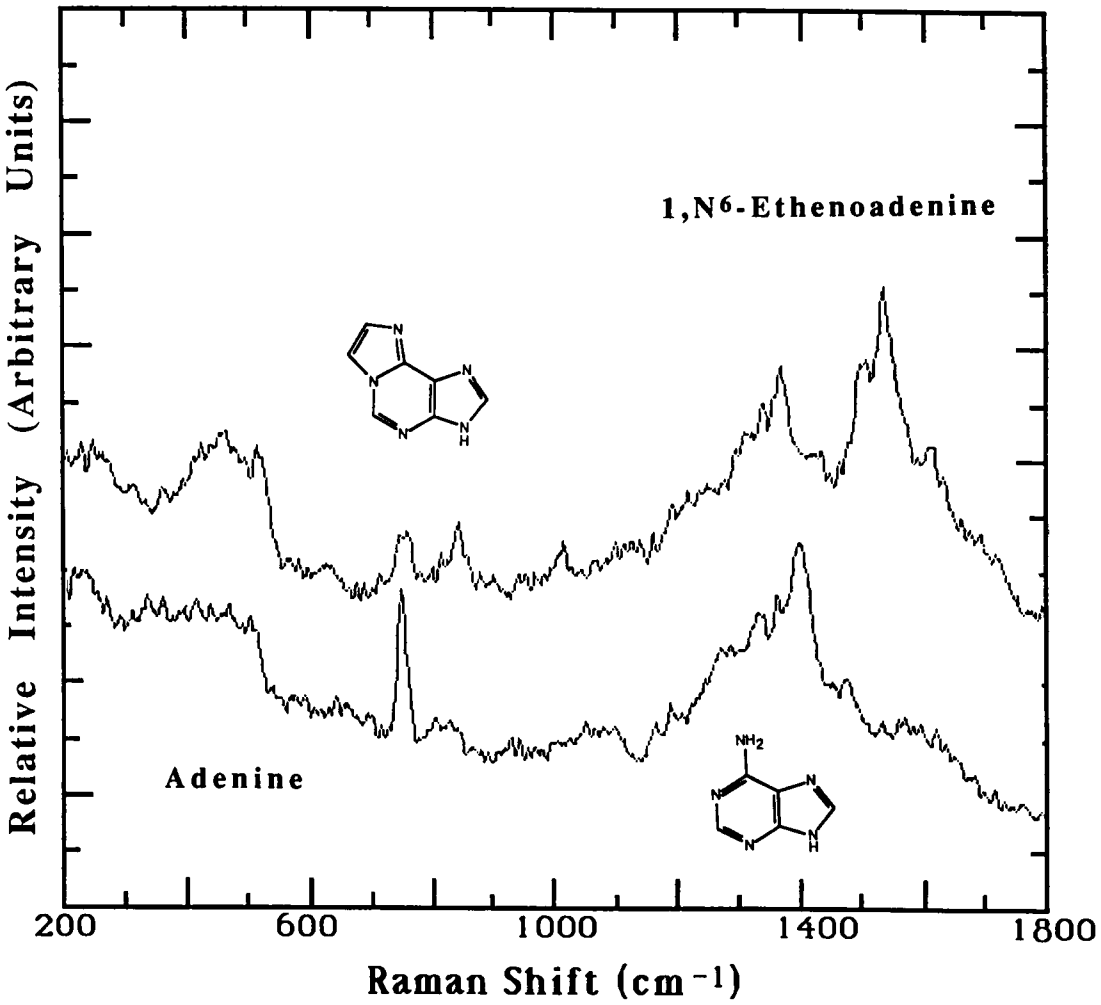
substrates. The adenine spectrum shown in Figure III-8 was taken on a polystyrene-type substrate and exhibited a different peak structure from the adenine spectra taken on the alumina-type substrates. Thus, substrate composition has a marked effect on spectral band location and relative intensity.

Ethenoadenine Derivatives

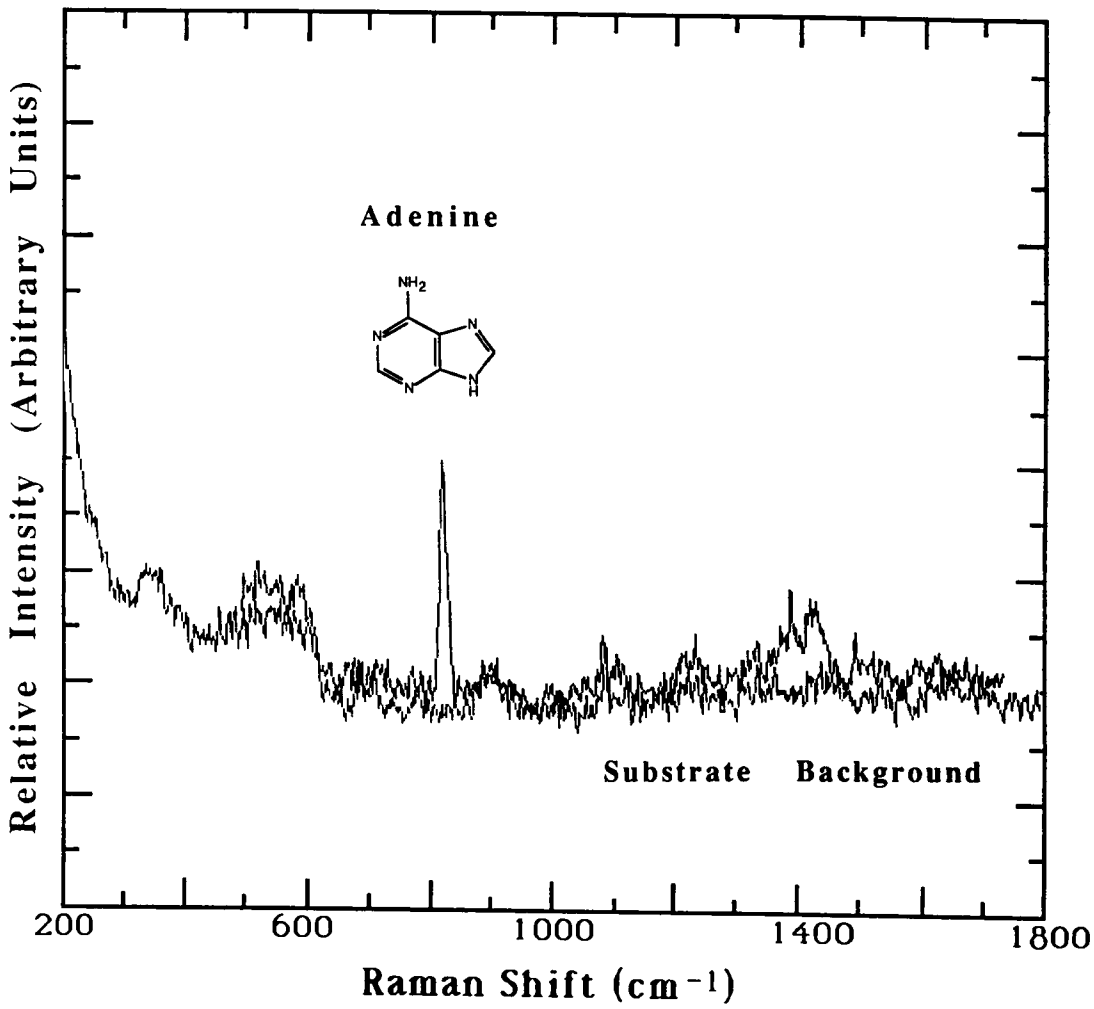
A filtered laser excitation of 25-mW to 50-mW proved adequate for signal detection from the etheno adducts of adenine with acceptable signal-to-noise ratios. Spectra could be recorded repeatedly with samples studied within this excitation power range without chemical decomposition or diminished SERS peak intensities. Laser excitation power above 75-mW resulted in photodegradation of the bases and these overexposed samples could not be re-analyzed by SERS.

Sample SERS spectra of 1,$N^6$-ethenoadenine and adenine adsorbed on the silver-coated alumina are displayed in Figure III-13. For comparison purposes, a substrate background spectrum is included with an adenine spectrum in Figure III-14. The background spectrum may be compared directly with the adenine spectrum, since the samples were examined on the same SERS substrate and the spectra were printed to scale relative to each other.

Many reports have appeared in the literature describing the adsorption of adenine on various SERS substrates such as silver sols and electrochemically-roughened silver surfaces (20-25). No previous SERS analysis had been performed on 1,$N^6$-ethenoadenine. The SERS spectrum of adenine (Figure III-13) exhibited two prominent bands at 738 $cm^{-1}$ and 1372 $cm^{-1}$. The 738 $cm^{-1}$ band corresponds within 10 $cm^{-1}$ to the most intense SERS band observed for




**<FIGURE III-13> SERS Spectra of 1,N$^6$-Ethenoadenine and Adenine** Spectra are of (Top) 1.0-μL of a 1x10$^{-3}$-M (nanomole) solution of 1,N$^6$-ethenoadenine in ethanol and (Bottom) 1.0-μL of a 1x10$^{-3}$-M solution of adenine in ethanol. Laser excitation was 70-mW (1,N$^6$-ethenoadenine) and 130-mW (adenine) from a krypton-ion laser (647.1-nm), using a 5.0% alumina-type substrate.




<FIGURE III-14> **SERS Spectra of Adenine and the Substrate Background** Spectra are of 1.0-μL of a 1.2028x10$^{-3}$-M solution of adenine and the 4.0% alumina-type substrate background. Laser excitation was 75-mW at 647.1-nm using a direct excitation/collection optical setup. Spectra were printed to scale relative to each other.

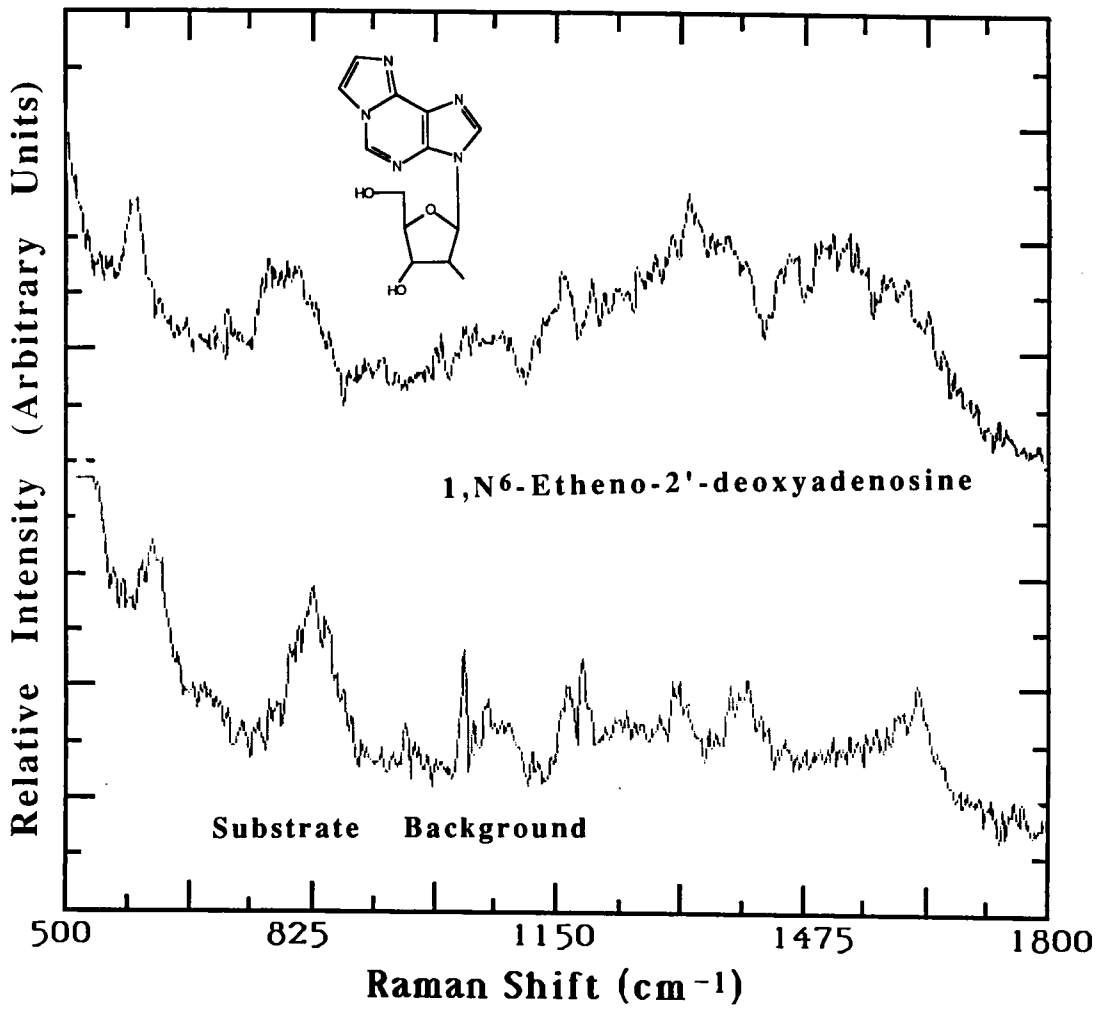
adenine on silver sols (20-22) and roughened metal electrodes (23-25). This band may be assigned to ring-breathing vibration. It is unlikely that this band resulted from coupled $-NH_2$ deformation and ring vibration as has been suggested for a metal-adenine complex (26). This is because 1,$N^6$-ethenoadenine does not have a primary amino group. It is noteworthy that the relative intensity of the prominent 738 $cm^{-1}$ band for adenine (Figure III-13) was weak when high laser power was used for excitation. This may have arisen from either a change of the molecular orientation of the analyte molecule on the silver film or from thermal decomposition of adenine.

Vibrational assignments for 1,$N^6$-ethenoadenine were essentially based on the assumption that force fields around the adenine ring did not vary appreciably upon the formation of adducts. A change in the molecular orientation on the surface might affect spectral features. Several characteristic bonds of 1,$N^6$-ethenoadenine may be used to differentiate it from adenine. As shown in Figure III-13, the most prominent SERS spectral band of 1,$N^6$-ethenoadenine at 1500 $cm^{-1}$ was not present in the SERS spectrum of adenine. This peak was assigned to a double-bond stretching mode. The medium intensity band at 1472 $cm^{-1}$ may correspond to the weaker band at 1454 $cm^{-1}$ in adenine. This band could be assigned to $N_1$-H in-plane bending. The 1372 $cm^{-1}$ band ($C_2$-H in-plane bending) was prominent in adenine, but was not observed in the SERS spectrum of 1,$N^6$-ethenoadenine. Also absent from the 1,$N^6$-ethenoadenine spectrum were the 1300 $cm^{-1}$ (in-plane ring vibration) and 1252 $cm^{-1}$ (Kekule vibration) bands of adenine. Several weak peaks at 994, 826, and 798 cm-1 appeared in 1,$N^6$-ethenoadenine spectra, but not in the adenine spectra.

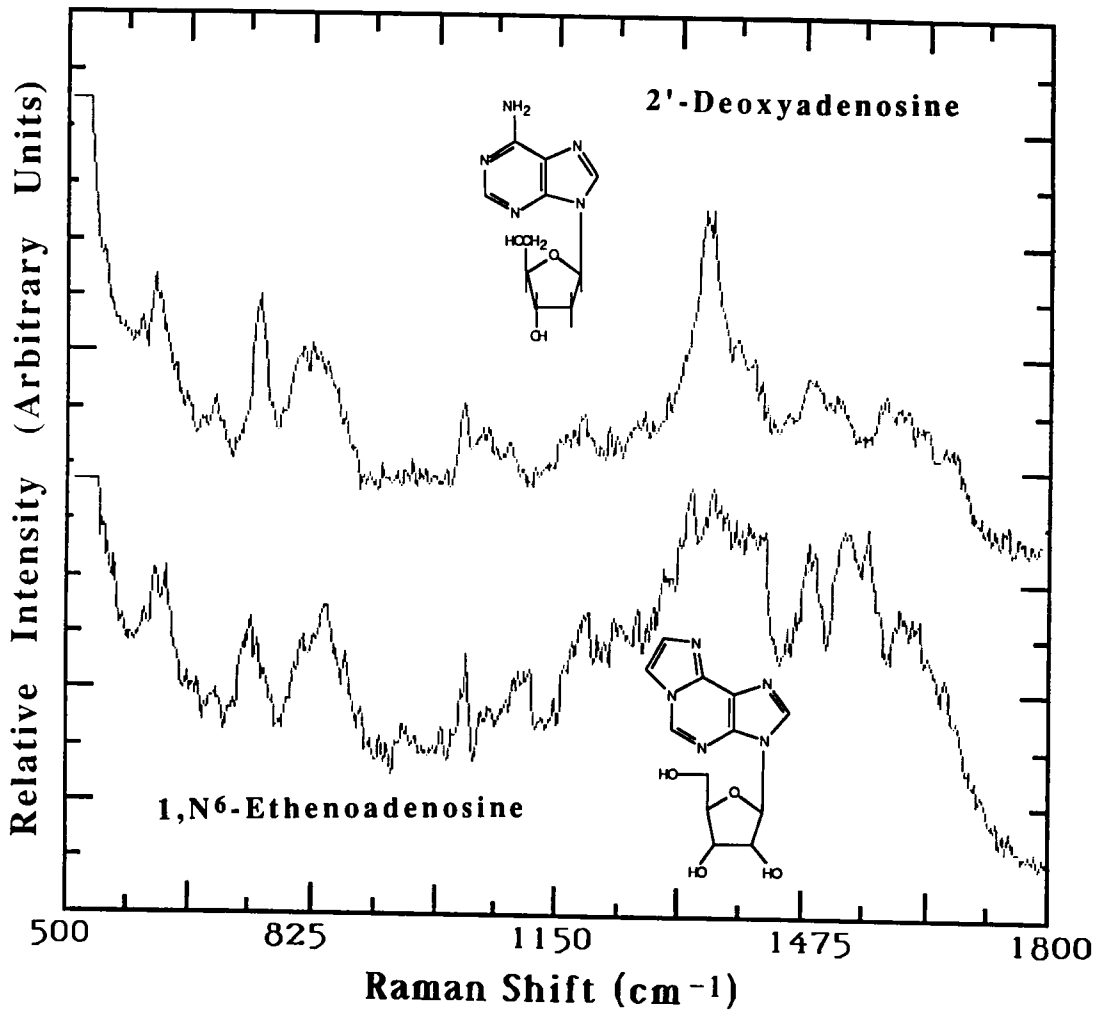
Results comparable to those for adenine and 1,$N^6$-ethenoadenine were obtained for 1,$N^6$-ethenoadenosine, 2'-deoxyadenosine, and 1,$N^6$-etheno-2'-deoxyadenosine. SERS spectra for the substrate background and 1,$N^6$-etheno-2'-deoxyadenosine are presented in Figure III-15. Spectra of 2'-deoxyadenosine and 1,$N^6$-ethenoadenosine (same background as in Figure III-15) are shown in Figure III-16.

The measured SERS frequencies and tentative assignments for the peaks of adenine, 1,$N^6$-ethenoadenine, 1,$N^6$-ethenoadenosine, 2'-deoxyadenosine, and 1,$N^6$-etheno-2'-deoxyadenosine are given in Table III-1. Table III-1 assignments were made for the average band positions in all spectra studied. An average band position was defined because the relatively low signal-to-noise ratio of the spectra resulted in uncertainty in the maximum photon count at any wavenumber. Some peaks which appear in the sample spectra but were not accounted for in Table III-1 were due to background SERS bands from the optical setup and the silvered, alumina-coated substrates. While background SERS from a fiberoptic setup may be intense enough to obscure chemically induced SERS peaks from 200-500 $cm^{-1}$, the selected optical setup gave excellent detection in the 500-1800 $cm^{-1}$ region where the majority of SERS bands from these samples were located. The intense background bands found from 200 $cm^{-1}$ to 500 $cm^{-1}$ were attributed to the all-silica optical fibers, since these bands were not present when a direct excitation/collection setup was used, but were present when a fiberoptic setup was used even when no substrate was in place.

Most vibrational assignments in Table III-1 were in good agreement with assignments made for adenine by Hirakawa *et al.* (27). In Suh and Moskovits' study of adenine adsorbed onto silver colloid particles (21), the




**<FIGURE III-15> SERS Spectra of 1,N6-Etheno-2'-Deoxyadenosine and Substrate Background** Spectra are of (Top) 1.0-μL of a 1x10-4-M solution of 1,N6-etheno-2'-deoxyadenosine in ethanol and (Bottom) 4.0% alumina-type substrate background. Laser excitation was 70-mW at 647.1-nm.




<FIGURE III-16> SERS Spectra of 2'-Deoxyadenosine and 1,N6-Ethenoadenosine Spectra are of (Top) 1.0-μL of a 1.363-mg/mL solution of 2'-deoxyadenosine in ethanol and (Bottom) 1.0-μL of a 1.613-mg/mL solution of 1,N6-ethenoadenosine in ethanol. Substrate background is shown in Figure III-15. Laser excitation was 70-mW at 647.1-nm.

**TABLE III-1**
**Chemical Group Assignments to SERS Spectral Bands for Ethenoadenine and Related Chemical Species**

| ADENINE | EADENINE | EADENO | dADENO | EdADENO | ASSIGNMENT |
|---|---|---|---|---|---|
| | | 1650 w,sh | | 1642 w,sh | *Not Given*[1] |
| 1574 w,b | 1582 sh | | 1568 w,b | | A[double bond str.] |
| | | 1536 S,sh | | 1542 S,sh | *Not Given* |
| | 1500 S | 1508 S,sh | | 1510 S,sh | I[double bond str.] |
| 1454 w | 1472 m,sh | 1472 S,sh | 1468 m,b | 1472 S,sh | A[$N_1$-H in-plane ben.] |
| | | 1460 S,sh | | 1464 S,sh | *Not Given* |
| 1372 S | | | | | A[$C_2$-H in-plane ben.] |
| 1338 m,sh | 1338 m,sh | 1346 m,b | 1340 S | 1338 S,vb | A[in-plane ring vib.] |
| | | | 1332 S | | *Not Given* |
| 1300 m,sh | | | | | A[in-plane ring vib.] |
| 1252 w,sh | | | | | A[Kekule vibration] |
| | | 1090 m | | 1100 m,b | *Not Given* |
| | 994 w | | | | I vibration |
| | 826 w | | | | I vibration |
| | 798 vw | | | | I vibration |
| 738 S | 740 w | 730 m | 748 S | 736 S | A[ring breathing] |

**TABLE III-1** (cont.)
**Chemical Group Assignments to SERS Spectral Bands for Ethenoadenine and Related Chemical Species**

| ADENINE | EADENINE | EADENO | dADENO | EdADENO | ASSIGNMENT |
|---|---|---|---|---|---|
| | | | 694 m | 688 m | *Not Given*[1] |
| | | 598 w | | | *Not Given* |

*Abbreviations*

| | |
|---|---|
| EADENINE | 1,$N^6$-ethenoadenine |
| EADENO | 1,$N^6$-ethenoadenosine |
| dADENO | 2'-deoxyadenosine |
| EdADENO | 1,$N^6$-etheno-2'-deoxyadenosine |
| S | strong |
| m | medium |
| w | weak |
| s h | shoulder |
| b | broad |
| v | very |
| A | adenine |
| I | $C_6N_{10}C_{11}C_{12}N_1$ ring |
| ben. | bending |
| str. | stretching |
| vib. | vibration |

[1] Many assignments listed as *Not Given* correspond to background spectral bands. Other assignments could not be attributed to a specific chemical group with a high degree of certainty.

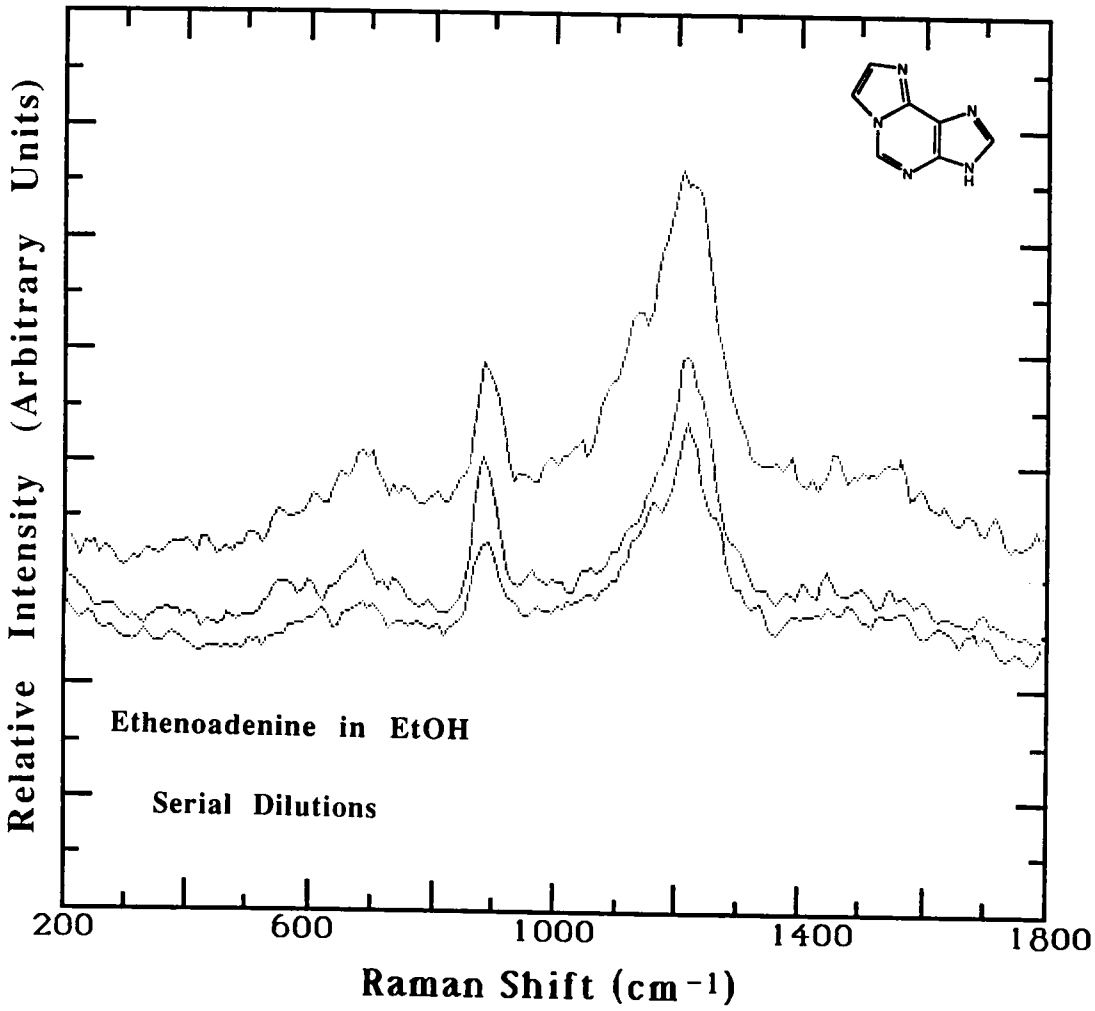
adenine molecule was shown to be flat on the metal surface for the weak band at 1317 $cm^{-1}$ expected for the $C_6$-$N_{10}$ stretching mode. In another SERS study of adenine on silver sols, Kim *et al.* (22) observed a frequency decrease of ring-breathing vibration from 746 $cm^{-1}$ to 735 $cm^{-1}$ when the sample concentration was increased from $7.5x10^{-7}$-M to $9.0x10^{-6}$-M. Kim *et al.* suggested that the molecular adsorption was "ring-on" at low adenine concentrations, but "end-on" at high concentrations.

Several different vibrational analyses have been used to derive the molecular orientations of analytes on the surfaces of SERS-active substrates. In a SERS study of adenine on a silver electrode, Watanabe *et al.* (24) assigned the strong band at 1328 $cm^{-1}$ to $N_7$-$C_5$ stretching vibration. From this assignment and from the intensity of the band, it followed that when using a $5.0x10^{-3}$-M solution, the molecule was adsorbed "end-on" via the $N_7$-atom. The SERS peak observed around 1330 $cm^{-1}$ when adenine was adsorbed onto silver particles (20-22) and electrodes (23-25) was generally strong. In this study, however, the corresponding band at 1338 $cm^{-1}$ appeared as a shoulder on an intense band at 1372 $cm^{-1}$. This may suggest that the adsorption of adenine on the substrate was not in the "end-on" position via the $N_7$ atom. One possible assignment of the strong band at 1372 $cm^{-1}$ is to the $C_2$-H in-plane bending. Assuming this assignment is correct, the molecule would be expected to "stand" or "tilt" on the surface via the $N_3$ and $N_9$ atoms. It should be noted, however, that knowledge of the true orientation of the molecule is dependent upon correct vibrational assignment of the bands and on information about whether the molecules are adsorbed in a monolayer or are stacked upon each other. The concentration of adenine used in this study may have resulted in

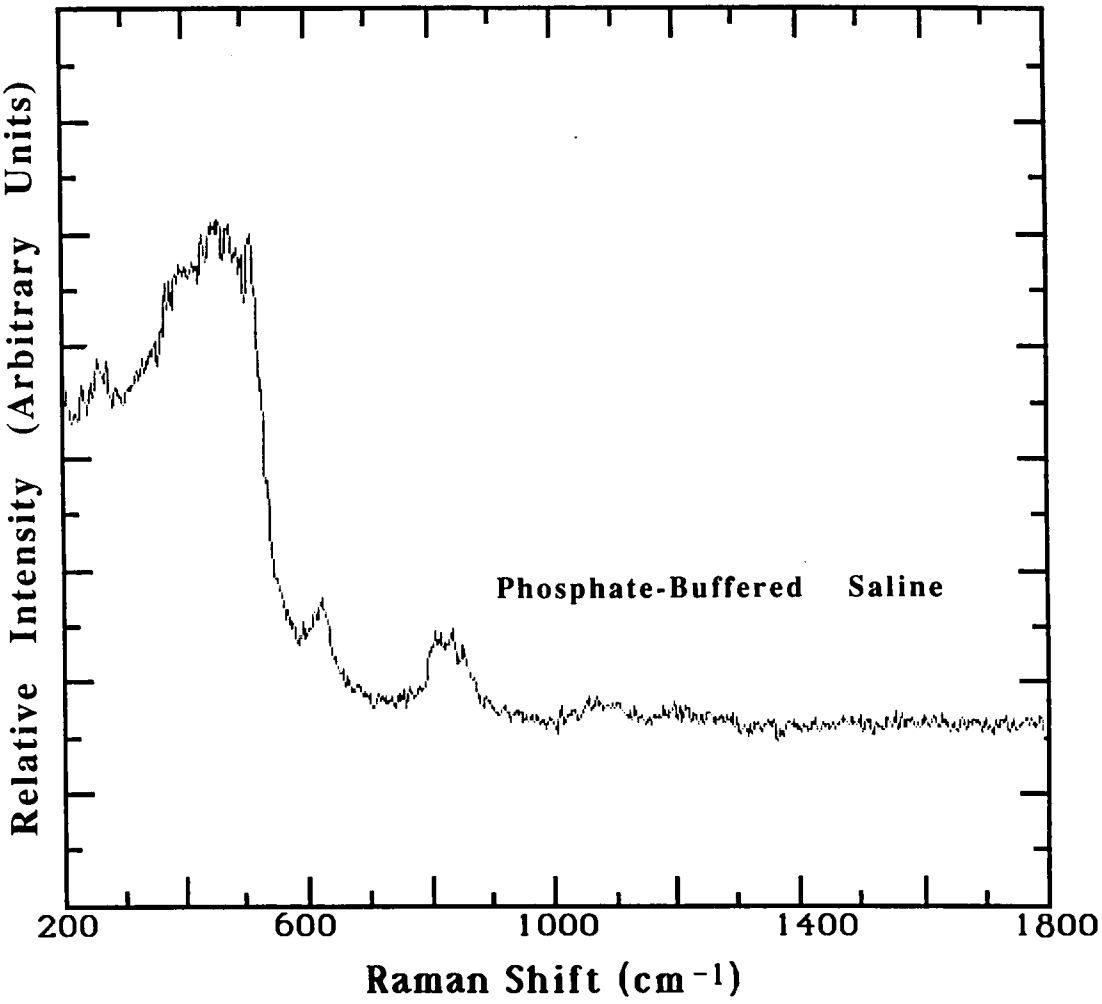
the stacking of molecules and not in a monolayer. Samples preparations of $1x10^{-3}$-M and more concentrated probably resulted in molecularly-stacked sample spots; samples less the $1x10^{-4}$-M in concentration probably resulted in monolayer spots. Samples at $1x10^{-4}$-M were borderline cases and their stacking potential was largely determined by the final sample spot size (spreading of the spot was dependent on the solvent).

A SERS limit-of-detection study was performed using serial dilutions of 1,$N^6$-ethenoadenine samples prepared in absolute ethanol. Some spectral results at 50-mW excitation at 647.1-nm are shown in Figure III-17. Ethanol was selected as the solvent for this experiment because it spread more evenly on the substrate and was less damaging to the silver surface than was an ethanol:water mixture. Because adenine has a very low solubility in pure ethanol and because 1,$N^6$-ethenoadenine was to be compared with adenine, absolute ethanol was not used for most other experiments in the adenine study. Under these experimental conditions, SERS could be used to detect 1,$N^6$-ethenoadenine from a dried 1.0-μL spot of $1x10^{-5}$-M (10 picomoles) analyte. Since the laser beam did not impinge on the entire sample spot, but only on approximately one percent of the sample area, the limit of detection was in actuality better than *100 femtomoles.* Similar limits of detection for SERS were observed for the other compounds used in this study.

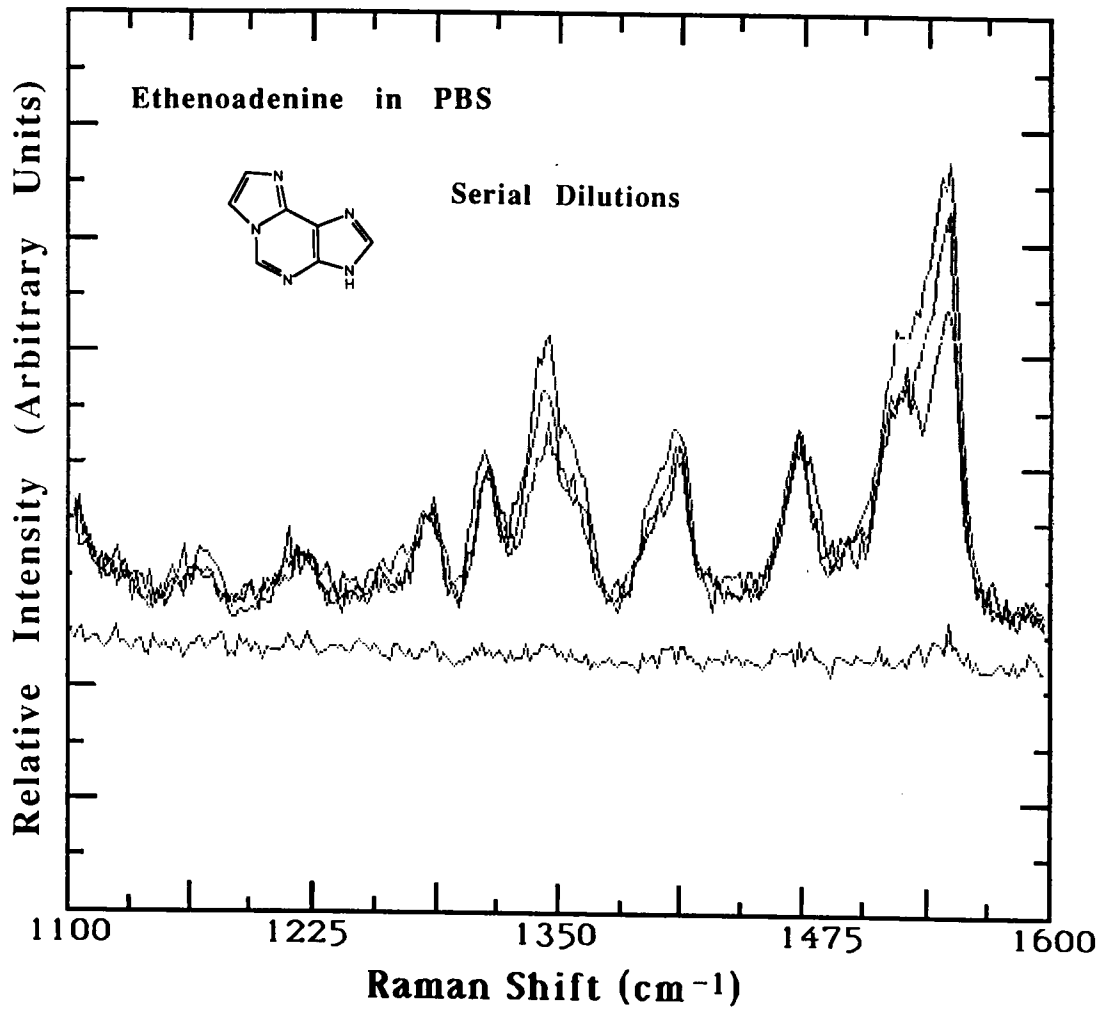
A SERS limit-of-detection study for 1,$N^6$-ethenoadenine was also performed in phosphate-buffered saline (PBS). Results at 50-mW at 647.1-nm are given in Figure III-18 (solvent background) and Figure III-19 (serial dilutions in PBS). 1,$N^6$-ethenoadenine was the only sample in these studies that could be routinely detected by SERS in PBS.




<FIGURE III-17> SERS Spectra of Serial Dilutions of 1,$N^6$-Ethenoadenine in Ethanol Spectra are of (Top) 1.0-μL of a $1x10^{-4}$-M, (Center) 1.0-μL of a $1x10^{-6}$-M, and (Bottom) 1.0-μL of a $1x10^{-5}$-M solution of 1,$N^6$-ethenoadenine in ethanol. Laser excitation was 30-mW at 647.1-nm. Spectra were printed to scale relative to each other.




**<FIGURE III-18> SERS Spectrum of Phosphate-Buffered Saline** Sample was 1.0-μL at pH 7.0. Laser excitation was 50-mW at 647.1-nm.

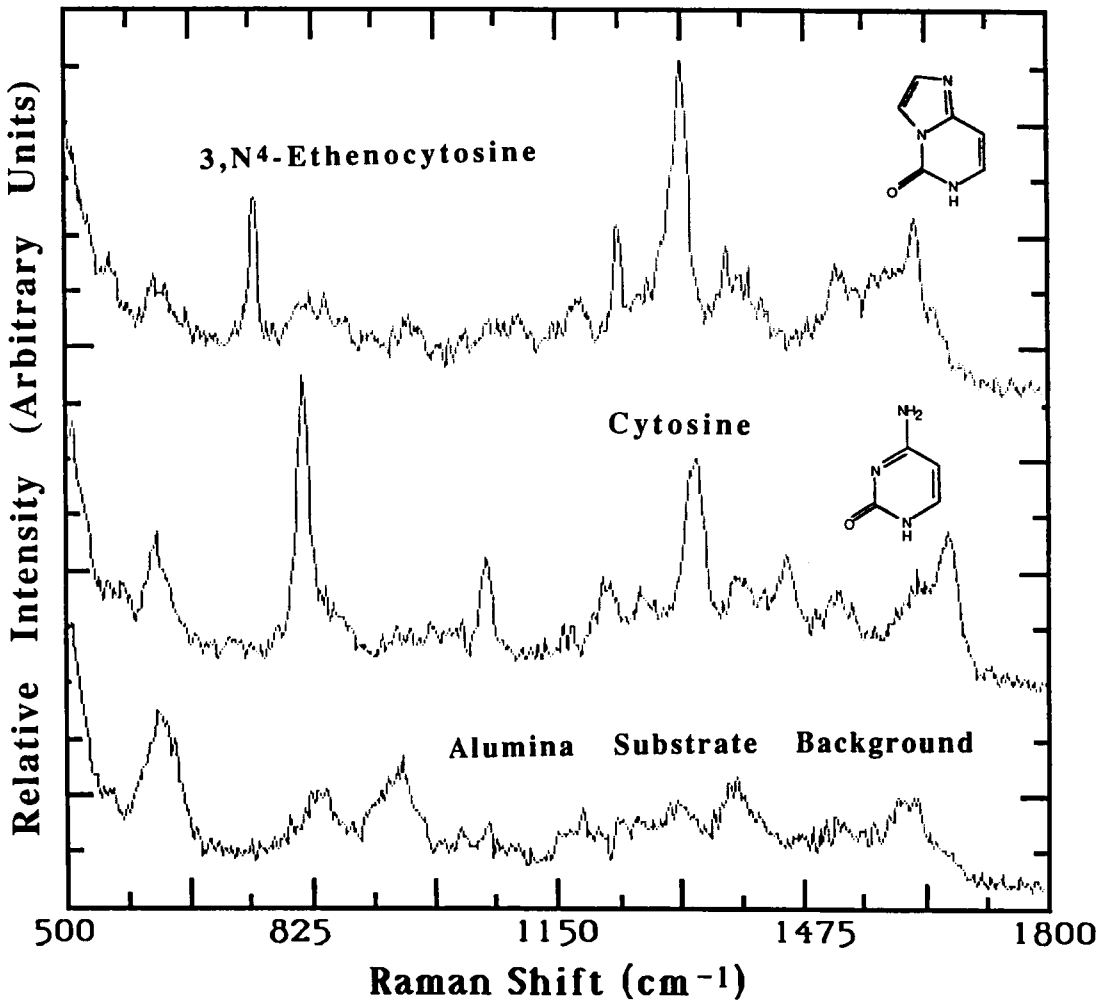



<FIGURE III-19> SERS Spectra of Serial Dilutions of 1,N$^6$-Ethenoadenine in PBS Spectra are of 1.0-µL of a (top to bottom) 8.9196x10$^{-4}$-M, 8.9196x10$^{-4.5}$-M, 8.9196x10$^{-5}$-M, and 8.9196x10$^{-5.5}$-M solution of 1,N$^6$-ethenoadenine in PBS. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.

Ethenocytosine Derivatives

SERS spectra of 3,$N^4$-ethenocytosine, cytosine, and substrate background are compared in Figure III-20. Listed in Table III-2 are the observed SERS frequencies for these molecules. Optimum excitation power for cytosine and 3,$N^4$-ethenocytosine on the silvered, alumina-coated substrate was found to be 75-mW to 100-mW at 647.1-nm. Below this power range SERS signals could be detected, although the intensity of the spectral peaks was the same order of magnitude as the background noise. Using 75-mW to 100-mW excitation power, samples could be scanned indefinitely with no change in peak intensity. Above this power range photo-decomposition of the chemicals appeared to occur and SERS spectra were not obtained when the overexposed chemicals were rescanned.

SERS spectra of cytidine, 2'-deoxycytidine, and 3,$N^4$-ethenocytidine are given in Figure III-21. Note that cytosine and 3,$N^4$-ethenocytosine could be distinguished from each other and from the cytidine-based molecules (Table III-2). Additionally, all cytosine-type molecules could be distinguished from all adenine-type molecules, with the exception of 2'-deoxycytidine and cytidine, which could not be distinguished from each other. The inability of the SERS technique to distinguish 2'-deoxycytidine from cytidine was not unexpected, since the molecules differ only by an oxygen in the 2'-position of the ribose ring. Spectral peak assignments corresponding to chemical groups in the sample molecules could usually be used to identify analytes. For example, although Kekule vibration caused a SERS band in both cytosine and 3,$N^4$-ethenocytosine spectra, the peaks resulting from the Kekule stretching may be distinguished from each other because they occurred at slightly




<FIGURE III-20> SERS Spectra of 3,$N^4$-Ethenocytosine, Cytosine, and Substrate Background Spectra are of (Top) 1.0-μL of a $1x10^{-4}$-M solution of 3,$N^4$-ethenocytosine, (Center) 1.0-μL of a $1x10^{-4}$-M solution of cytosine, and (Bottom) 4.0% alumina-type substrate substrate background. Laser excitation was 70-mW at 647.1-nm.

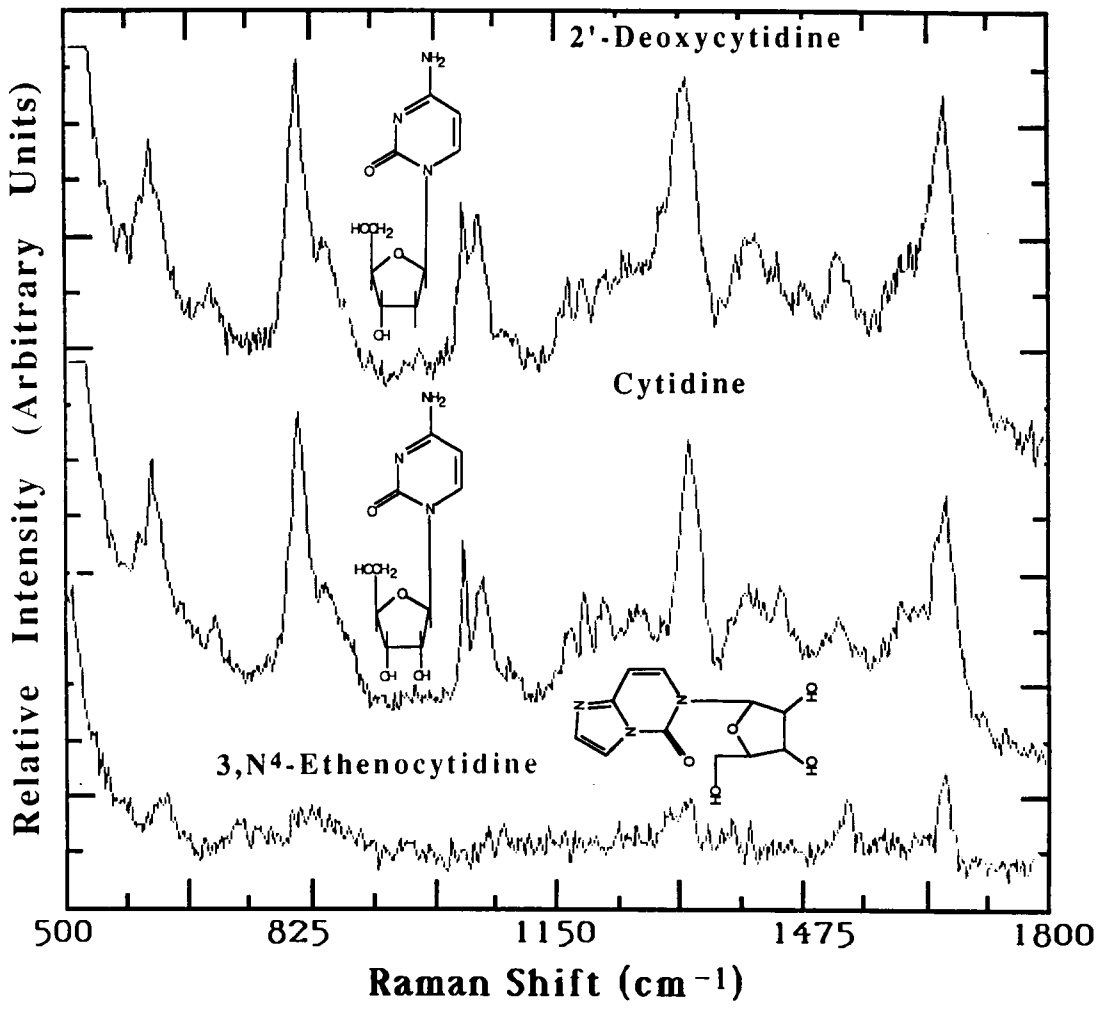
TABLE III-2
Chemical Group Assignments to SERS Spectral Bands for Ethenocytosine and Related Chemical Species

| BG | CYTOSINE | ECYTOSINE | ECYTIDINE | CYTIDINE | dCYTIDINE | ASSIGNMENT |
|---|---|---|---|---|---|---|
| | 1650 m | 1630 w,sh | 1640 S | 1648 m | 1646 S | C=O stretching |
| | | | | 1588 w | | $C_2$=O-$C_2N_3$ str. |
| 1612 m | 1610 w,sh | 1608 m | | | | C=C stretching |
| | | 1572 w,sh | | | | C=N stretching |
| | | 1554 w,sh | | | | *Not Given*[1] |
| | | | 1514 m | | | *Not Given* |
| | 1508 w | 1506 w | | 1506 m | 1504 m | -$N_3C_4$-$N_1C_2$ str. |
| | | | | | 1458 w | *Not Given* |
| | 1434 w | | | 1436 m | 1428 w | δin-pl($N_1$-H) |
| | 1406 vw | 1404 vw | | 1390 m,b | | δ+in-pl($C_5H/C_6H$) |
| 1392 m | | | | | | Background SERS |
| | | 1388 vw | 1386 w | | 1398 m,b | *Not Given* |
| 1368 m | 1366 w | 1372 vw | 1362 w | | 1374 w,sh | $C_4N$-$C_5C_6$ str. |
| | | 1350 w | | | | *Not Given* |
| 1302 m | 1318 S | 1302 S | 1308 m | 1318 S | 1312 S | Kekule vibration |
| 1294 m | | | | | | Background SERS |
| | | | 1284 w,sh | | 1276 sh | ribose |
| | 1244 vw | 1220 m | | 1244 m | | δ-in-pl($C_5H/C_6H$) |
| | | | | 1200 m | 1206 w | *Not Given* |
| 1176 m | 1196 w | 1172 w | | 1174 m | 1176 m | $C_6H$+$C_4N_{11}$ ben. |
| 1154 m | 1156 vw | | | 1156 w | 1156 m | Background SERS |
| 1078 m | | | | 1076 vw | | Background SERS |
| 1052 m | 1046 m | | | 1046 m | 1038 S | Background SERS |
| 1020 S | | | | 1018 S | 1018 S | Background SERS |
| 942 w | | 946 w | | | 958 w | Background SERS |
| 836 S,b | | 834 w | | 838 m,b | 834 m,sh | Background SERS |
| 822 S,b | | | | | | Background SERS |
| | | 816 w | | | | *Not Given* |
| | 806 S | 744 m | 728 w | 806 S | 804 S | ring breathing |
| 694 w | | | | 694 m | 688 w | Background SERS |
| 612 S | 614 m | 620 w | 628 m | 614 s | 612 S | $C_2$=O+$N_1$R+$C_4N_{11}$ ben. |
| 598 m | | | | 594 w,sh | | Background SERS |
| | | 556 w | | | 576 w | *Not Given* |

*Abbreviations*

| | |
|---|---|
| BG | Background |
| ECYTOSINE | 3,$N^4$-ethenocytosine |
| ECYTIDINE | 3,$N^4$-ethenocytidine |
| dCYTIDINE | 2'-deoxycytidine |
| S | strong |
| m | medium |
| w | weak |
| sh | shoulder |
| b | broad |
| v | very |
| str. | stretching |
| ben. | bending |
| in-pl | in-plane |

[1] Assignments listed as *Not Given* could not be attributed to a specific chemical group with a high degree of certainty.




**<FIGURE III-21> SERS Spectra of 2'-Deoxycytidine, Cytidine, and 3,N4-Ethenocytidine** Spectra are of (Top) 1.0-μL of a 2.079-mg/mL solution of 2'-deoxycytidine, (Center) 1.0-μL of a 1.380-mg/mL solution of cytidine, and (Bottom) 1.0-μL of a solution of 3,N4-ethenocytidine in ethanol. Laser excitation was 70-mW at 647.1-nm.

different wavenumbers.

Optimum excitation power at 647.1-nm was 25-mW to 50-mW for cytidine, 2'-deoxycytidine, and 3,$N^4$-ethenocytidine. Note that this was a lower power range than was used for cytosine and 3,$N^4$-ethenocytosine, presumably because the ribose attached to the bases caused the molecules to become unstable under the higher excitation power. At laser excitation powers slightly higher than 50-mW, photo-degradation of the chemicals appeared to have taken place. This was observed in real time by measuring the SERS signal intensity which maximized and then rapidly fell back to background levels. At excitation powers greater than 100-mW, the laser beam rapidly damaged the silvered alumina substrate. This damage could be verified by positioning the laser beam over the affected area and noting that the number of photons passing through it was higher than for the same substrate under a lower excitation power. Scanning electron microscope studies have shown that small holes were being burned through the silvered alumina substrate under high laser power (not shown because of severe charging of the exposed glass).

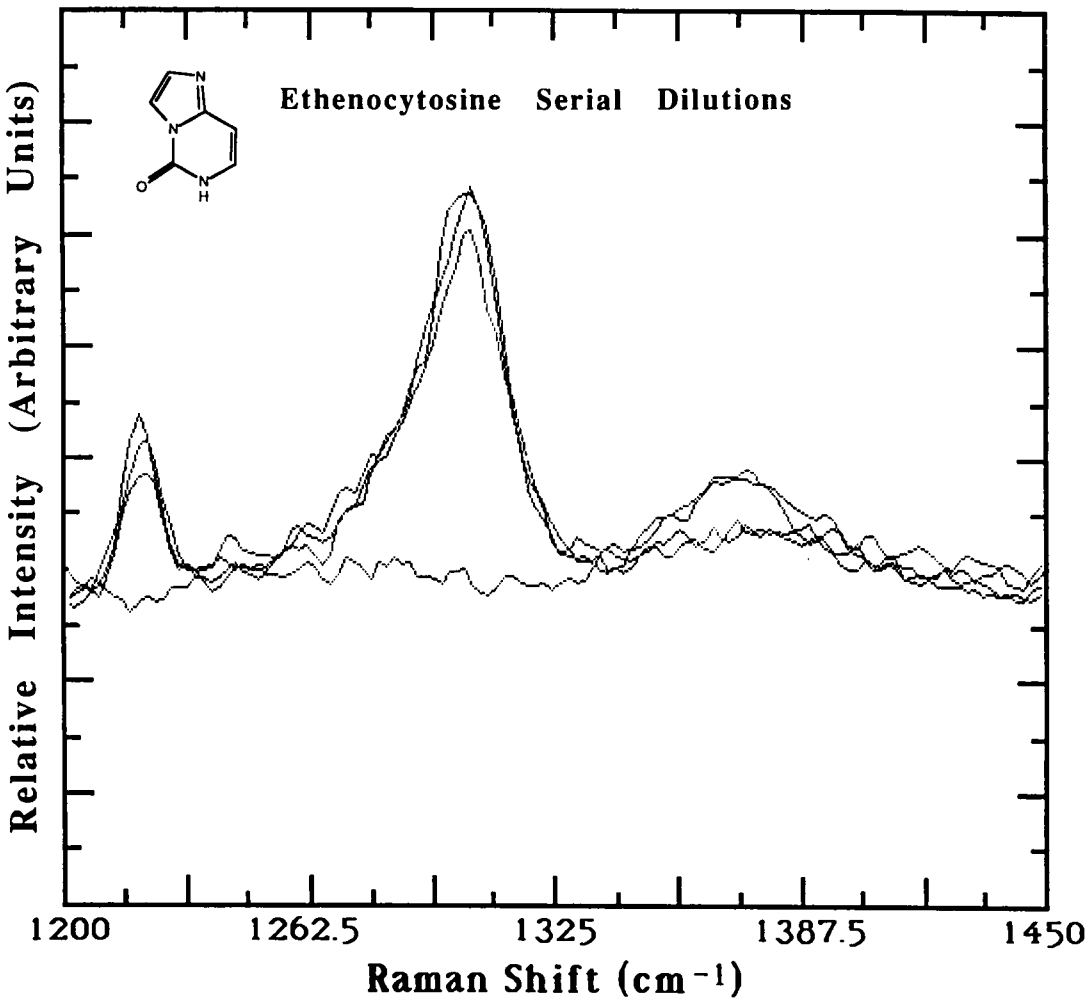
The spectral band frequencies of the bases were in good agreement with those previously published for SERS. For example, the 1650 $cm^{-1}$ band for cytosine and the equivalent bands for the other sample spectra correspond to the 1657 $cm^{-1}$ and 1658 $cm^{-1}$ bands assigned to C=O stretching vibration as reported in a SERS investigation of 1,5-dimethylcytosine on silver and copper sols (28-30).

SERS limit-of-detection studies were performed for 3,$N^4$-ethenocytosine. The limit of detection for 3,$N^4$-ethenocytosine was reproducibly better than for the other samples, which could rarely be detected in a solution less dilute than $1x10^{-5}$-M. A 1.0-μL spot of $1x10^{-6}$-M solution (1 picomole) could sometimes

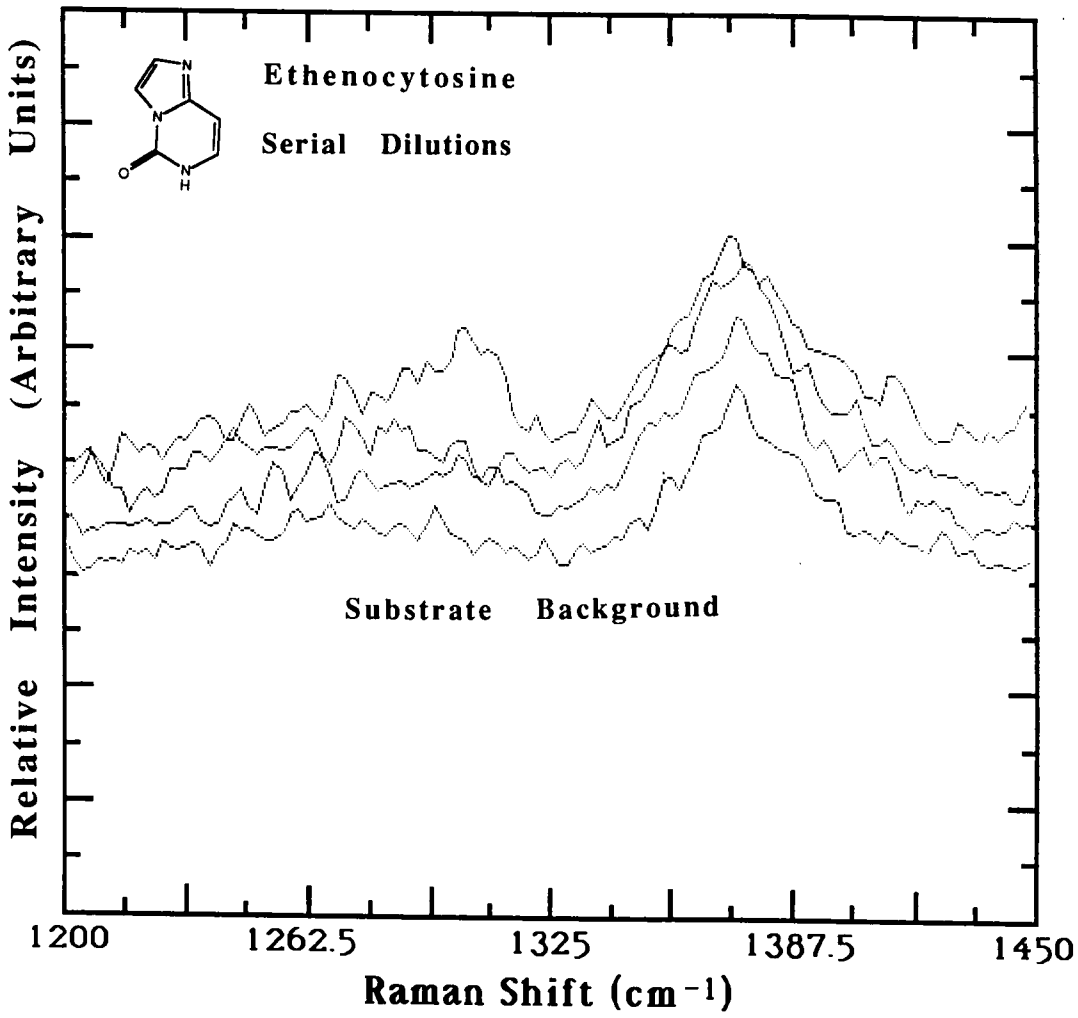
be detected for all of the chemicals. SERS spectra of 3,$N^4$-ethenocytosine are shown in Figure III-22, Figure III-23, Figure III-24, and Figure III-25. When the optics and substrates were optimized, the limit of optical detection for 3,$N^4$-ethenocytosine was nominally 1.0-µl of a $1x10^{-9}$-M solution (1 femtomole), although the SERS bands for this concentration were very near the noise level of the background spectrum. As in the ethenoadenine limit-of-detection experiment, the entire sample spot was not illuminated by the excitation beam. SERS detection of 3,$N^4$-ethenocytosine may have been considerably better than one femtomole.

2'-deoxycytidine-5'-monophosphate was also examined (Figure III-26), but exhibited no discernable sample bands under these conditions. No SERS signal was obtained for either L-ribose or D-ribose (Figure III-27, D-ribose compared with substrate background in Figure III-28) in these experiments, although 1.0-µL of a $1x10^{-4}$-M (aqueous) ribose sample tended to diminish any bands appearing in the background spectra in the 1000 $cm^{-1}$ to 1800 $cm^{-1}$ region. Although SERS signals were observed for bases with and without the ribose, the sugar-containing bases tended to be more difficult to observe, requiring especially fine optical alignment and substrates. No difference was detected between ribose-containing and 2'-deoxyribose-containing samples. No SERS bands were detected for 2'-deoxy-D-ribose as seen in Figure III-29. Two other sugars, D-glucose and D-fucose, were also examined by SERS, but could not be distinguished from the substrate background (Figure III-30).

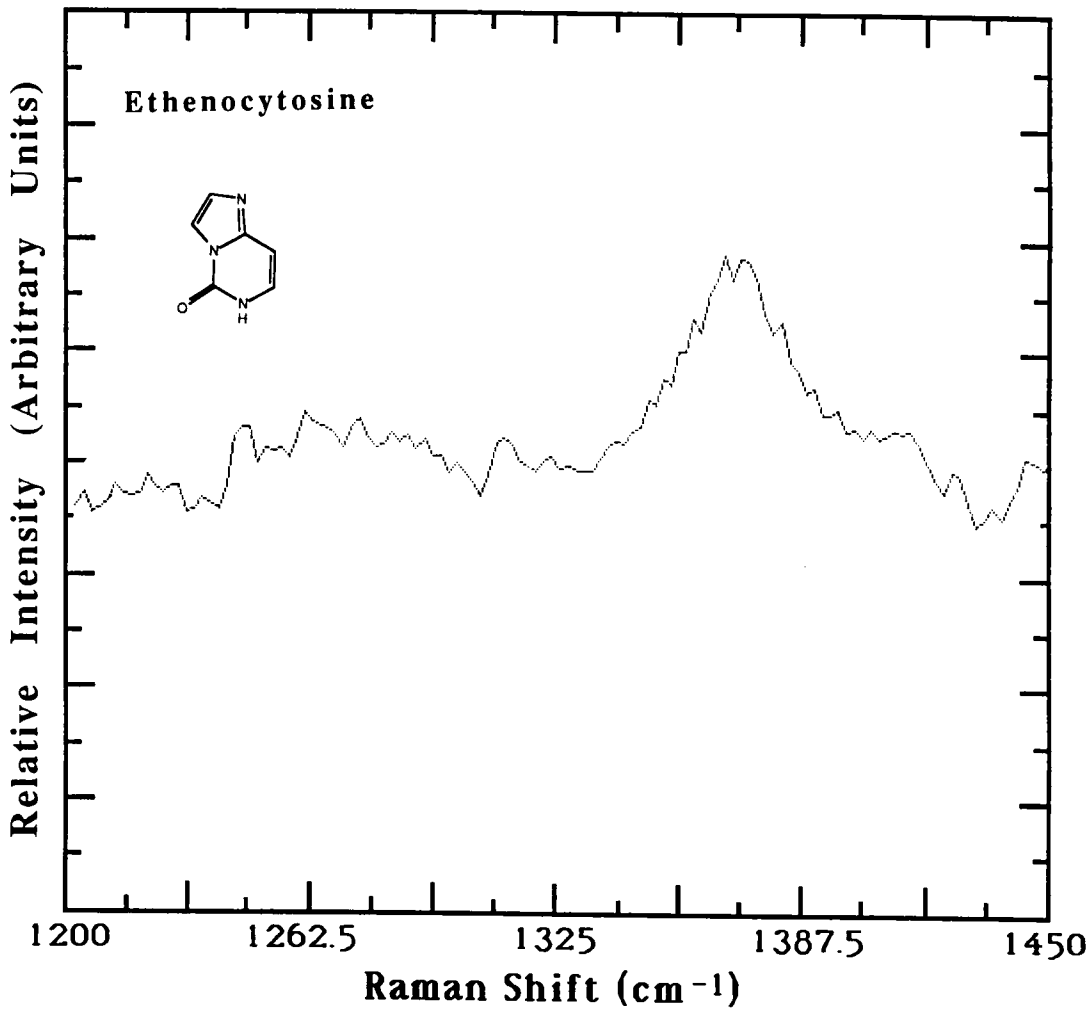
The sample substances were easiest to detect with the SERS technique in either ethanol or water. When phosphate-buffered saline (PBS, pH 7.0) was used as solvent the non-ribose containing bases (cytosine and 3,$N^4$-ethenocytosine) were difficult to observe, while 1,$N^6$-ethenoadenine (Figure



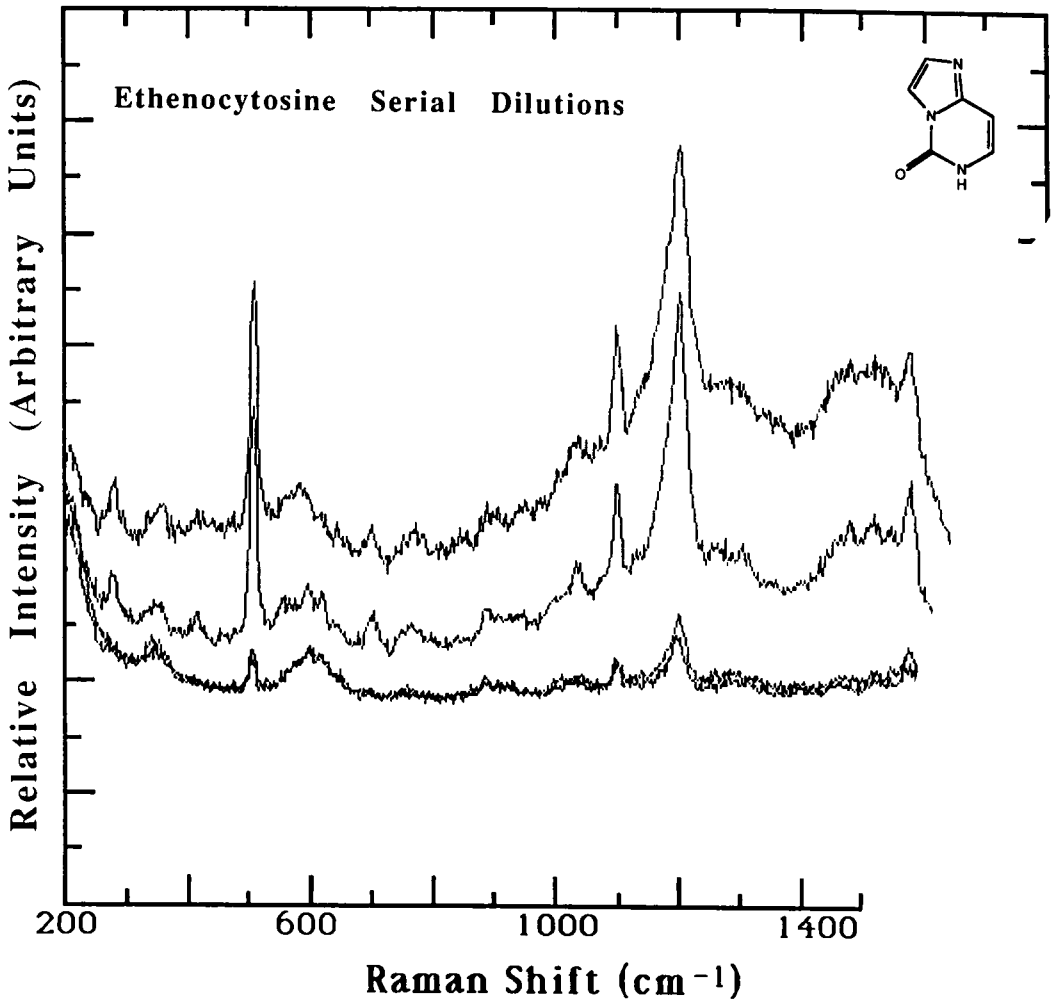

<FIGURE III-22> SERS Spectra of Concentrated Samples of 3,N4-Ethenocytosine Spectra are of (Top to Bottom) 1.0-μL of 1.3990x10-3-M, 1.3990x10-4-M, 1.3990x10-5-M solutions of 3,N4-ethenocytosine in ethanol, and the 4.0% alumina-type substrate background. Laser excitation was 75-mW at 647.1-nm. Spectra were printed to scale relative to each other.



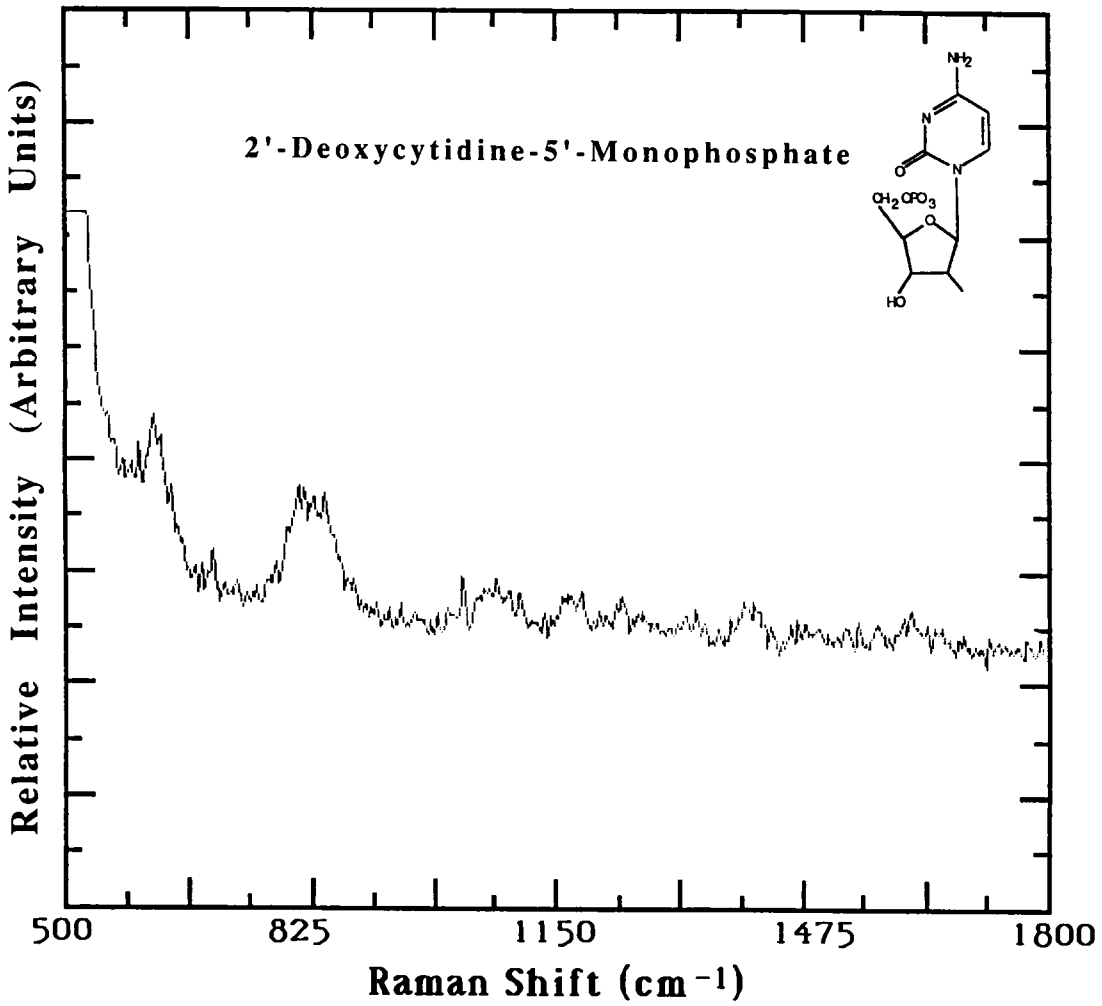

<FIGURE III-23> SERS Spectra of Dilute Samples of 3,$N^4$-Ethenocytosine Spectra are of (Top to Bottom) 1.0-µL of $1.3990 \times 10^{-6}$-M, $1.3990 \times 10^{-7}$-M, $1.3990 \times 10^{-8}$-M solutions of 3,$N^4$-ethenocytosine in ethanol, and the 4.0% alumina-type substrate background. Laser excitation was 75-mW at 647.1-nm. Spectra were printed to scale relative to each other.



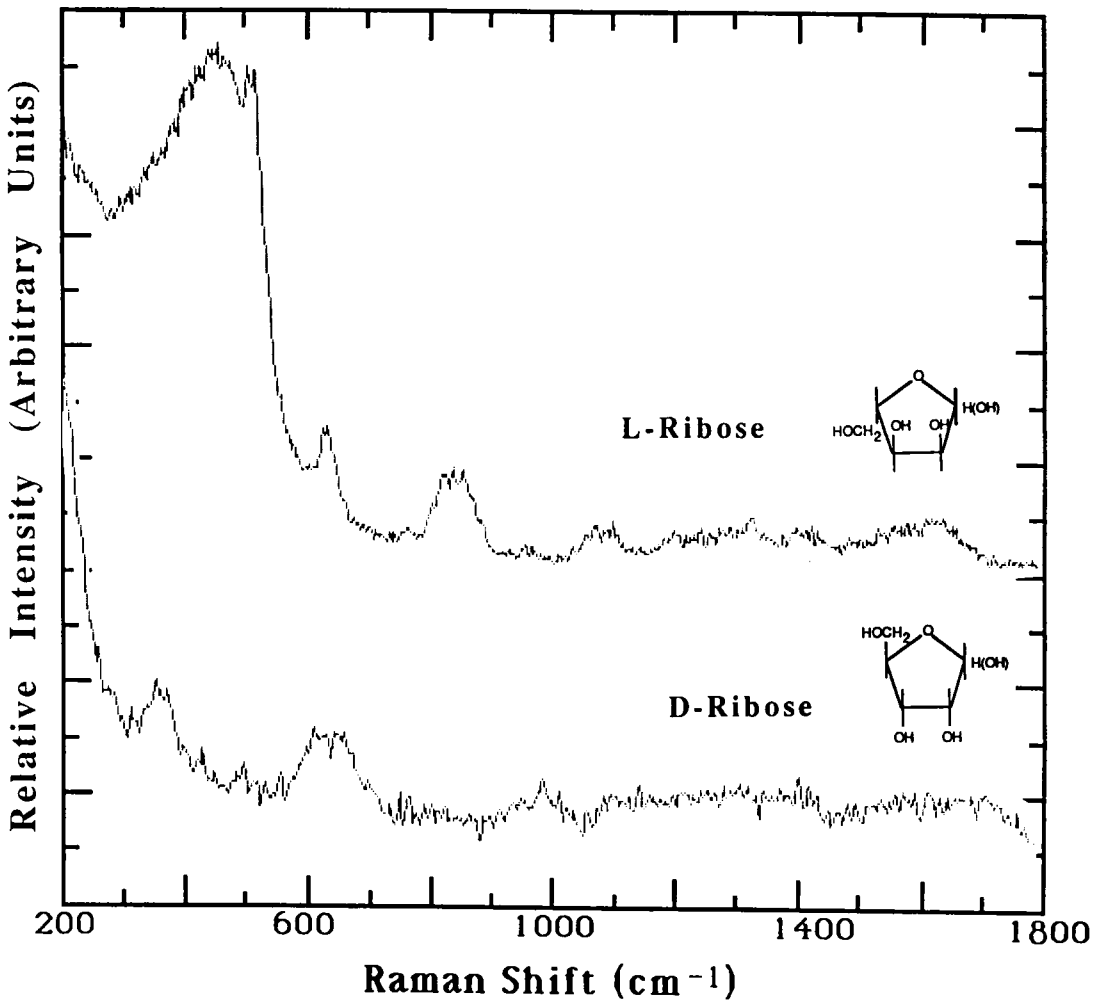

<FIGURE III-24> SERS Spectra of 1.0-μL of a 1.3990x10-9-M Solution of 3,N4-Ethenocytosine Laser excitation was 75-mW at 647.1-nm. Background spectra are given in Figure III-22 and Figure III-23.



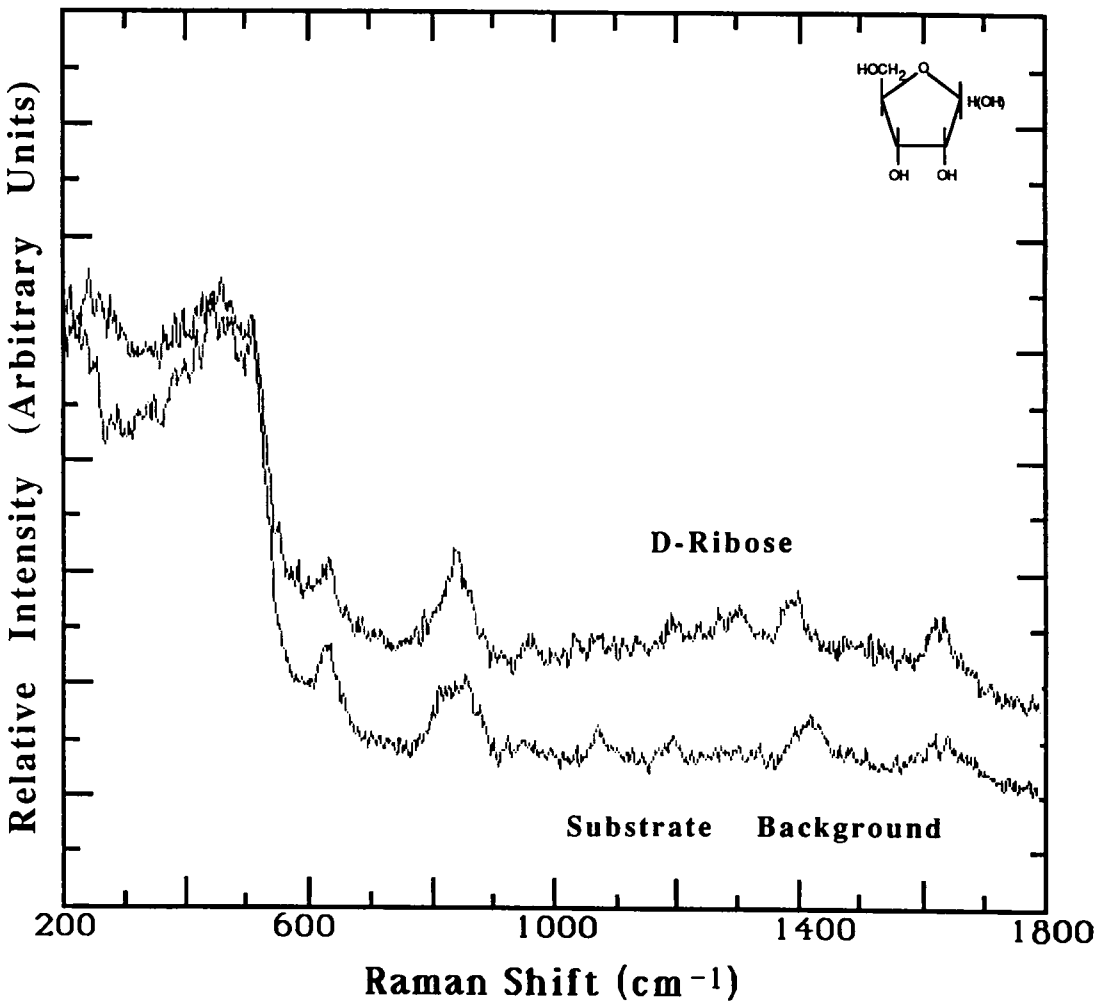

**<FIGURE III-25> SERS Spectra of Serial Dilutions of 3,N⁴-Ethenocytosine** Spectra are of 1.0-µL of (top to bottom) 9.252x10$^{-5}$-M, 9.252x10$^{-4}$-M, 9.252x10$^{-8}$-M, and 9.252x10$^{-10}$-M solutions of ethenocytosine in ethanol. Laser excitation was 80-mW at 647.1-nm. Spectra were printed to scale relative to each other.



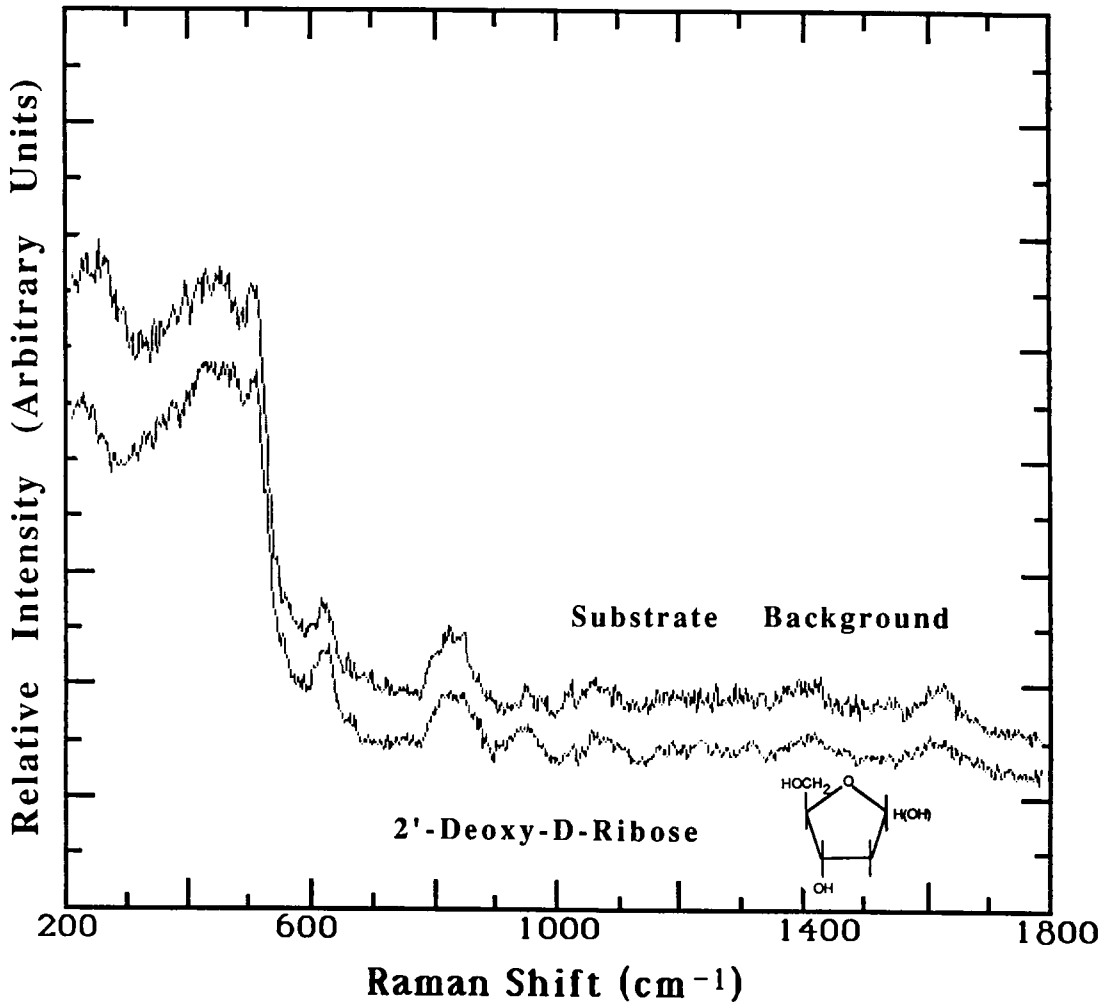

<FIGURE III-26> SERS Spectrum of 1.0-μL of a 0.283-mg/mL Solution of 2'-Deoxycytidine Monophosphate in Water Laser excitation was 70-mW at 647.1-nm.



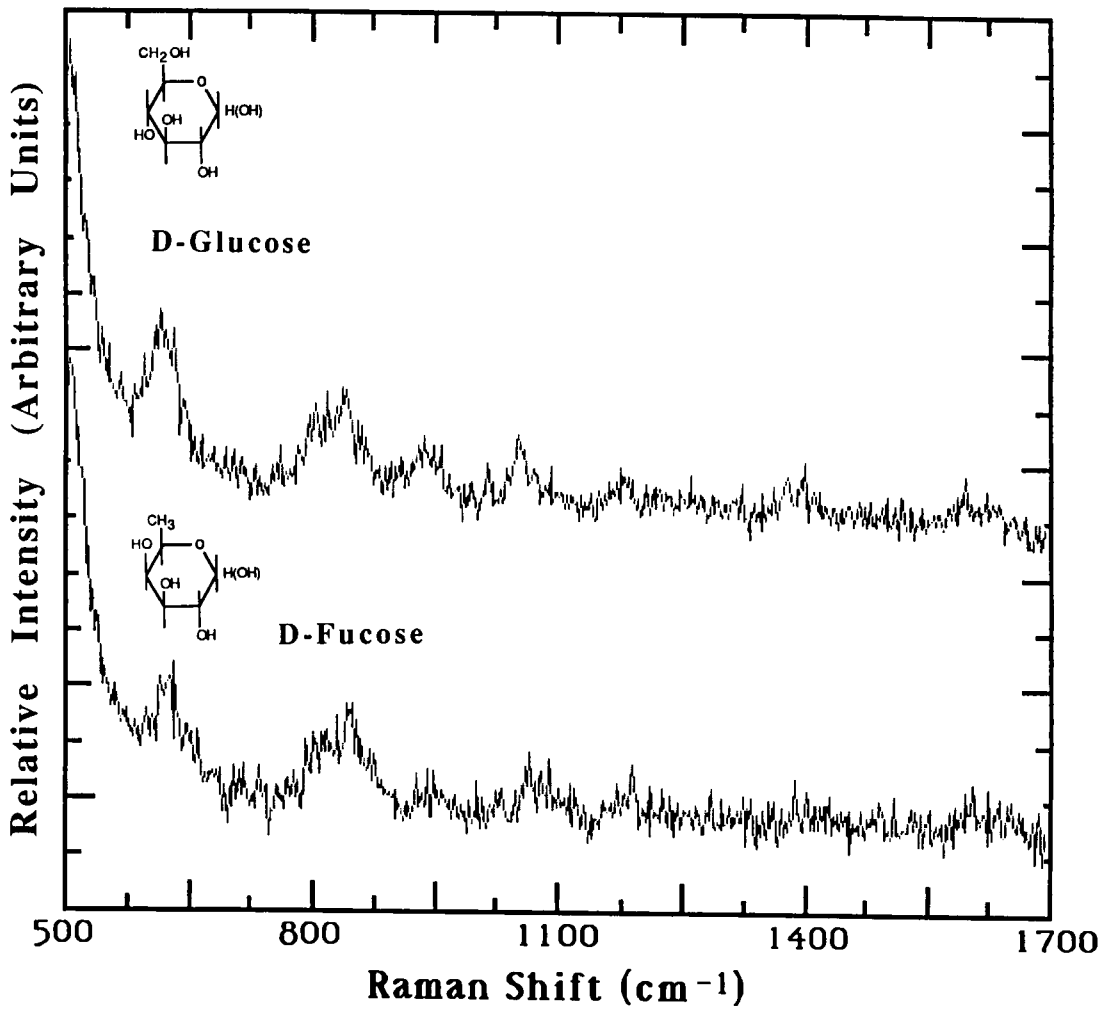

<FIGURE III-27> SERS Spectra of L-Ribose and D-Ribose Samples were 1.0-μL of 1x10$^{-4}$-m solutions in ethanol. Laser excitation was 70-mW (L-ribose) and 80-mW (D-ribose) at 647.1-nm.




<FIGURE III-28> SERS Spectra of D-Ribose and the Substrate Background Sample was 1.0-μL of a 1x10-4-M solution of D-ribose in ethanol. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.




**<FIGURE III-29> SERS Spectra of the Substrate Background and 2'-Deoxy-D-Ribose** Sample was 1.0-μL of a 2.083-mg/mL solution of 2'-deoxy-D-ribose in ethanol. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.




**<FIGURE III-30> SERS Spectra of D-Glucose and D-Fucose** Samples were 1.0-μL of 1x10$^{-4}$-M solutions in ethanol. Laser excitation was 75-mW at 647.1-nm.

III-18 and Figure III-19) yielded intense SERS spectra. It was possible that a chemical interaction between the phosphate and saline ions in the PBS and the silver surface of the substrate was responsible for the observed results. In any event, PBS was not routinely used as solvent because its use diminished the intensity of both background and molecule bands from 1000 $cm^{-1}$ to 1800 $cm^{-1}$. Scanning electron micrographs showed marked alteration of the substrate surface when a PBS solution had been applied. No SEM photographs are available of this phenomenon because the damage to the substrate surface exposed glass to the electron beam. The charging of the glass resulted in exceptionally poor SEM images of the substrate surface.

Absolute ethanol was used as solvent wherever chemical solubility allowed because the ethanol solutions spread more evenly and dried more quickly with insignificant damage to the SERS substrate. No significant SERS signal decrease was noticed when pure water was used as the solvent, although visual inspection and scanning electron micrographs have shown the water-treated substrate surface to be altered. Perhaps no spectral change was apparent because the silver layer was thick enough to survive slight disruption without loss of plasmon properties. A 1:1 ethanol:water mix applied to the substrate exhibited no surface damage, although the spot took a considerable amount of time to evaporate (20 minutes as compared with about 15 seconds for absolute ethanol) and did not spread as widely or as smoothly as an ethanol-based sample spot.

## *Summary*

Surface-enhanced Raman spectroscopy (SERS) detection and analysis were performed on several etheno adducts formed from the metabolism of vinyl chloride. Spectra of the etheno adducts 1,$N^6$-ethenoadenine, 1,$N^6$-ethenoadenosine, and 1,$N^6$-etheno-2'-deoxyadenosine were compared with unmodified adenine and 2'-deoxyadenosine. Spectra of 3,$N^4$-ethenocytosine and 3,$N^4$-ethenocytidine were compared to cytosine, cytidine, and 2'-deoxycytidine. Chemical group assignments were made for the SERS peaks of these adducts and the limit at which SERS could detect the adducts was assessed. SERS spectra could be obtained from one femtomole of 3,$N^4$-ethenocytosine and from approximately 10 picomoles of the other chemicals. In all cases, SERS could be used to differentiate the adducts from the unmodified bases, although molecularly similar 2'-deoxycytidine and cytidine could not be distinguished. These assignments were favorably comparable to previously published SERS analyses of nucleotides and nucleosides.

The results of this study may be of interest to researchers involved in the characterization of DNA adducts in DNA from individuals exposed to small molecules such as vinyl choride. While DNA adducts of large compounds, such as the polyaromatic hydrocarbons, can be analyzed by spectroscopic techniques such as fluorescence (31) and phosphorescence (32), it is difficult to use these techniques to analyze DNA adducts from less luminescent small molecules. While infrared (IR) spectroscopy could be used to differentiate between specific chemical groups, it is generally not sensitive enough for trace detection and analysis. In addition, the IR technique is limited by strong

influences from the water absorption band. The SERS technique, which combines the sensitivity of the surface enhancement effect with the spectral selectivity of Raman, may be an important biomonitoring tool for the sensitive detection of DNA adducts of small molecules.

This research has demonstrated that SERS may be an invaluable tool in the study of DNA adducts. The excellent limit of detection, for 3,$N^4$-ethenocytosine in particular, proves that SERS may rival other spectroscopic techniques such as fluorescence in its sensitivity. In addition, SERS may be used to selectively study multiple analytes in a complex solution. This may be especially important when looking at altered DNA. SERS may be used to differentiate between normal and altered bases, and is capable of detecting more than one substance in a mixture of bases (Figure III-12).

# CHAPTER 2

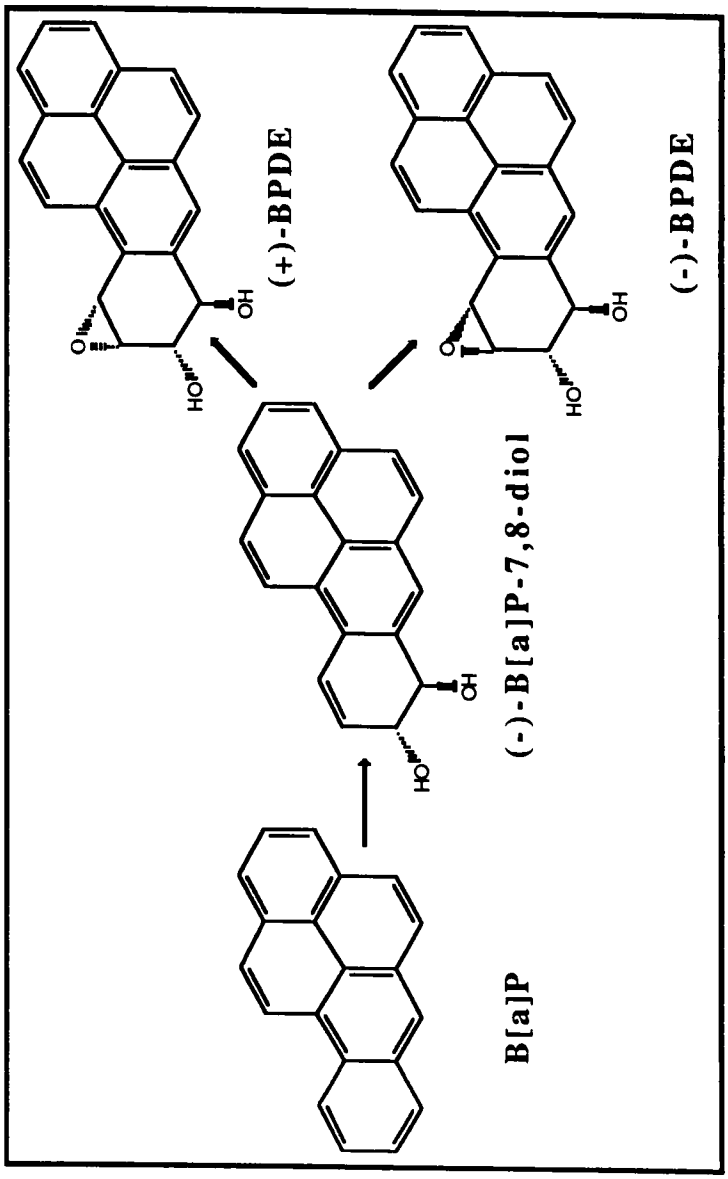
# BENZO[A]PYRENE STUDIES

## *Introduction*

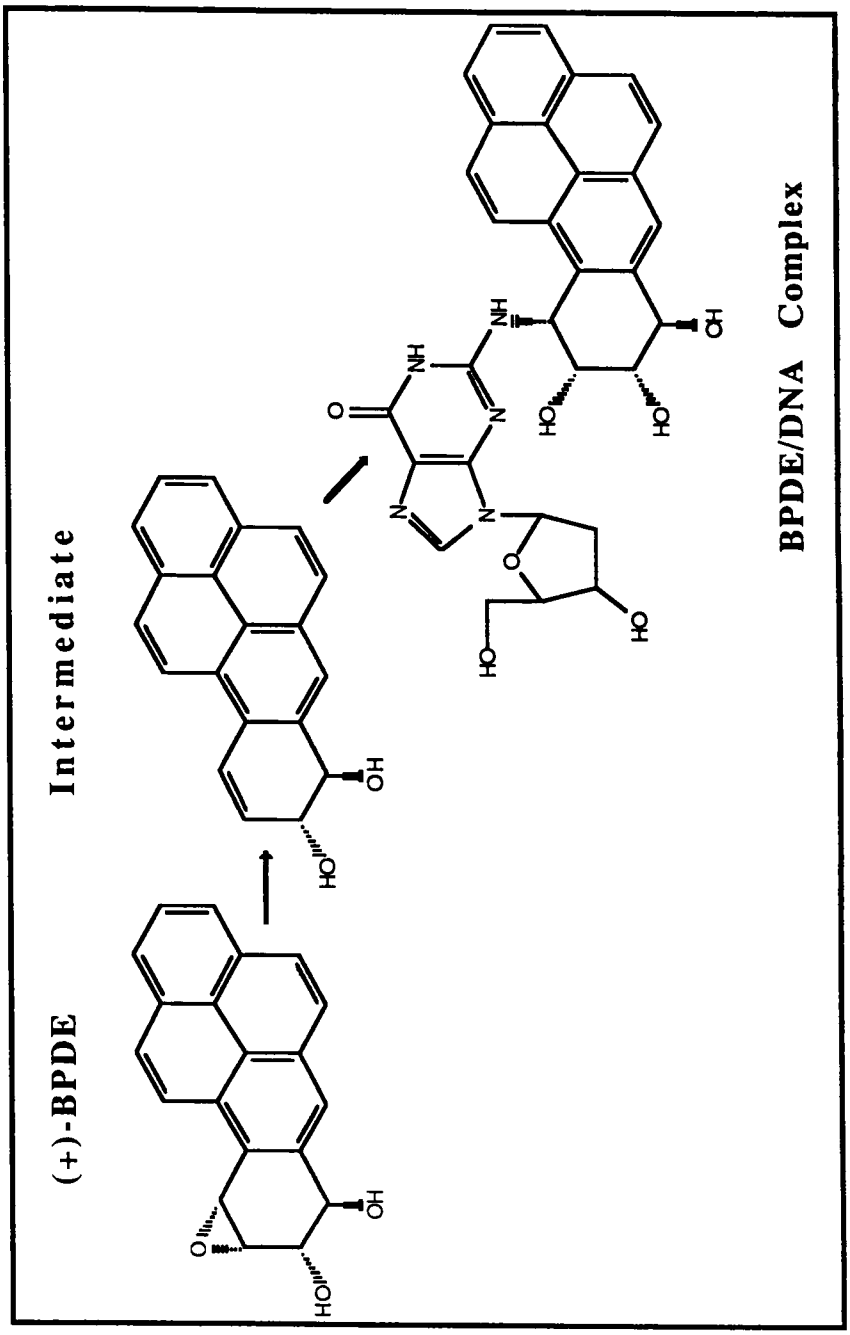
The measurement of human exposure to mutagenic and carcinogenic polynuclear aromatic (PNA) compounds (33) has been a concern of many scientists. A polynuclear aromatic compound (PAC) of especial interest to cancer researchers and toxicologists is benzo[a]pyrene (B[a]P), an ubiquitous byproduct of the combustion of wood, fuel, and food products (34) and a known animal carcinogen (35). Major industrial and workplace sources of B[a]P include chemical processes, coke-oven emissions, fossil fuel consumption, and diesel engine emissions (35-37). Unvented kerosene space heaters, cigarette smoke, cooking fumes, and fireplace smoke contribute to indoor exposure to B[a]P.

B[a]P is metabolically activated to electrophilic intermediates that can bind covalently to DNA. (See Figure III-31 and Figure III-32.) The major carcinogenic metabolic intermediate involved in DNA binding is the diol-epoxide of B[a]P, r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene (BPDE) (35,38). The resulting BPDE/DNA adducts are excreted in the urine and feces (39). BPDE/DNA is often acid hydrolyzed to form the highly fluorescent chemical species benzo[a]pyrene tetraol (BPT). Fluorescence measurement of BPT levels is a common means of providing evidence of an individual's exposure to B[a]P by indirectly determining the extent of DNA adduct formation.

Since the binding of B[a]P to DNA is the critical first step in its




<FIGURE III-31> Chemicals Structures of Benzo[a]Pyrene, a Chemical Intermediate, (+)-BPDE, and (-)-BPDE




<FIGURE III-32> Chemical Structures of (+)-BPDE, a Chemical Intermediate, and a BPDE/DNA Adduct

carcinogenic activity, it is important to develop sensitive analytical techniques that can detect the covalent linkage of BPDE to DNA. Several techniques, including mass spectrometry (40), synchronous fluorescence (41-43), liquid chromatography (44,45), fluorescence line-narrowing spectroscopy (46), $^{32}$P-post-labeling autoradiography (47), enzyme-linked immunoassay (48), laser-induced room temperature phosphorescence (32), and fiberoptic-based fluoro-immunosensors (49) are capable of detecting the carcinogen-DNA adduct. In these experiments SERS was demonstrated to be an effective tool for detection of benzo[a]pyrene epoxide. Additionally, SERS could be used to differentiate B[a]P, BPT, BPDE, DBAE (a related coal tar epoxide), and BPDE/DNA adducts.

## *Materials and Methods*

### Instrumentation

The SERS instrumentation was identical to the optical setup used for the etheno-adducts experiments. For specific details on the instrumentation, refer to Section I of this dissertation.

### Chemicals

Benzo[a]pyrene was obtained from Sigma Chemical Company (St. Louis, MO) and was used without further purification. Benzo[a]pyrene tetraol, BPDE, DBAE, and BPDE/DNA adducts were provided by Dr. Tuan Vo-Dinh and Dr. Mayo Uziel of Oak Ridge National Laboratory (Oak Ridge, TN). BPDE/DNA adducts and benzo[a]pyrene tetraol were prepared as follows (50): 50-μL of a $3x10^{-3}$- M solution of BPDE in methanol was added to 1-mL of aqueous 1.2-mg/mL DNA and

was incubated overnight at 37° C. The resulting solution was passed through a SepPak C-18 cartridge (Waters Corporation, Milford, MA) that was pre-equilibrated in water. The cartridge was washed with 5-mL water and the aqueous solutions were pooled to yield an adduct preparation containing 8.6x10-6-M of pyrenyl residues. This value was calculated from the absorbance at 343-nm and the extinction coefficient of 49,000 $cm^2M^{-1}$. The purified DNA adduct had no detectable free benzo[a]pyrene tetraol when it had been passed through a fresh SepPak cartidge. Acid hydrolysis of the BPDE/DNA adduct for 90-minutes at 90° C yielded an isomeric mixture of BPT's with the major component having the 7-r, 8-t, 9-t, 10-r (BPT-I) configuration. BPDE and DBAE were originally obtained from the National Cancer Institute, Bethesda, MD, and were identified as follows:

Benzo[a]pyrene-7,8-dihydroepoxide

NCI#L0622 MRI#568 Lot#CSL84-071-95 Batch#01

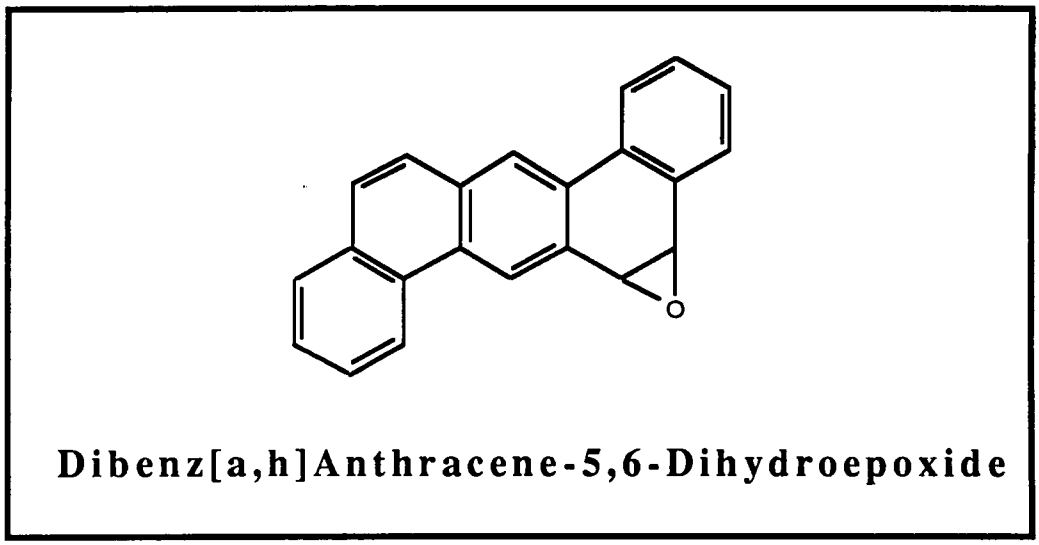
Dibenz[a,h]anthracene-5,6-dihydoepoxide

NCI#L0128 MRI#536 Lot#CR6-18-30 Batch#01

The structure of DBAE is shown in Figure III-33.

Procedures

Chemicals to be analyzed by SERS were 1.0-μL in volume and were spotted onto a SERS substrate. The samples were allowed to dry prior to exposure to the laser beam. Alumina-roughened SERS substrates were used for all analyses in this study. Laser excitation was either 647.1-nm (krypton-ion)




<FIGURE III-33> Chemical Structure of 1,2-Dibenz[a,h]anthracene-5,6-Dihydroepoxide

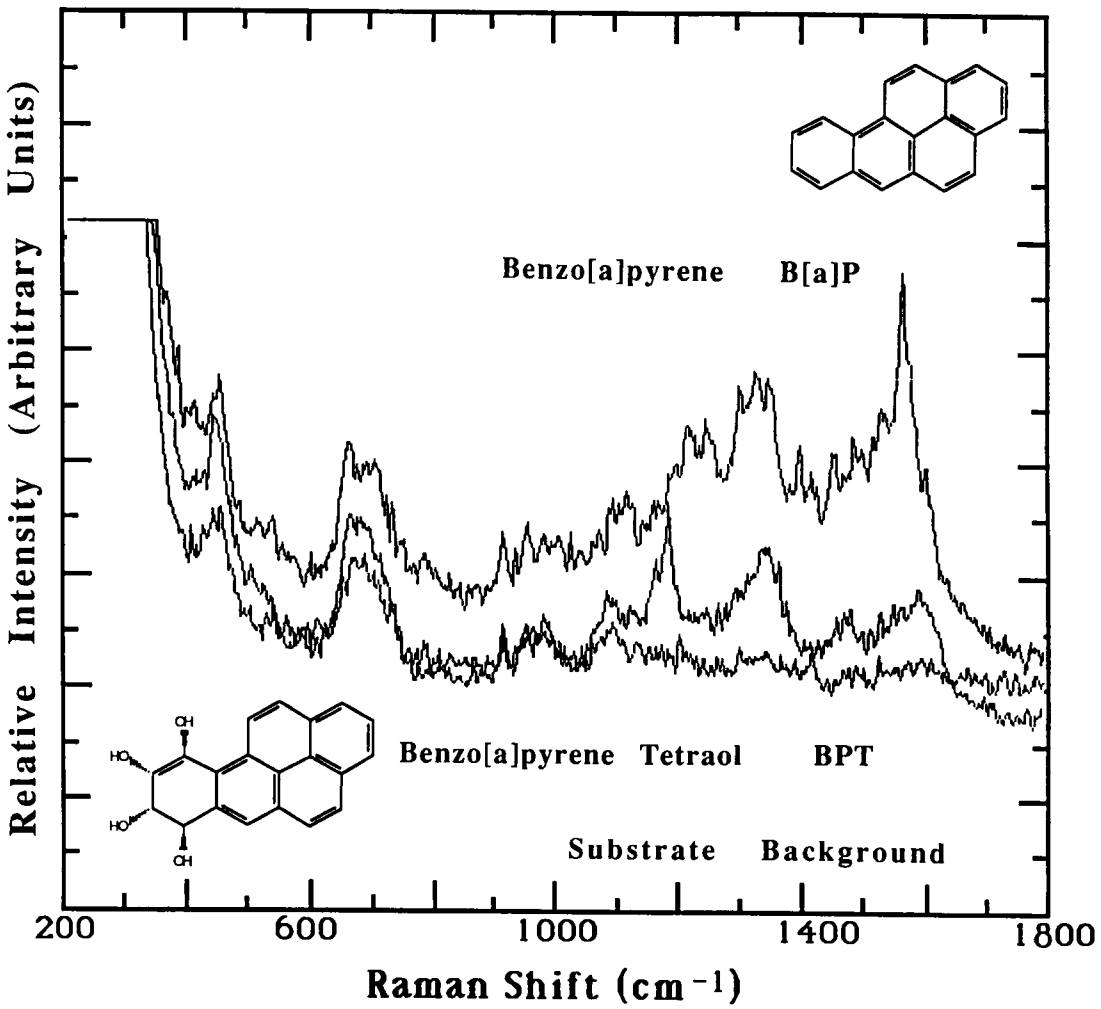
or 514.5-nm (argon-ion). Information on alumina content and laser wavelength is given in the legend of each spectrum.

*Results and Discussion*

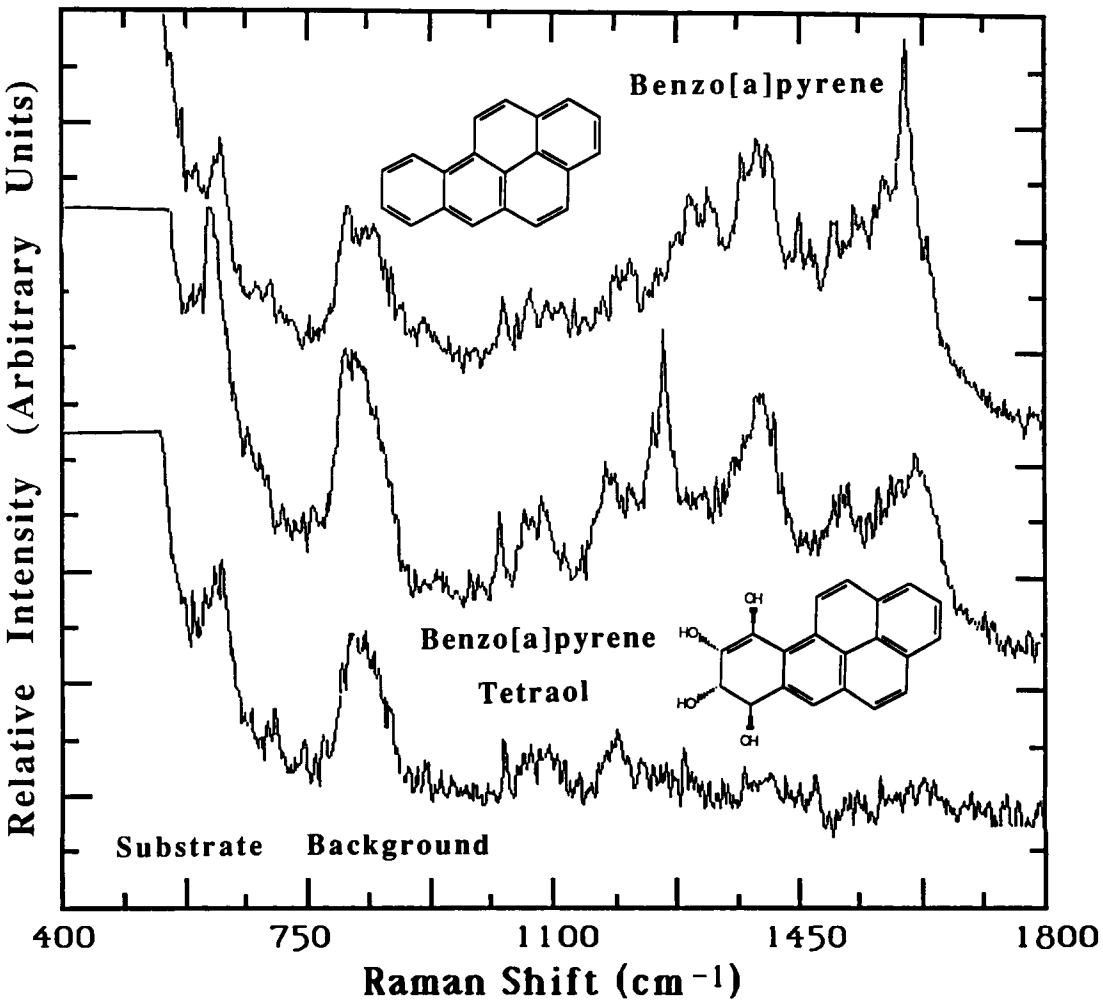
SERS spectra of benzo[a]pyrene, benzo[a]pyrene tetraol, and the substrate background are given in Figure III-34 (printed to scale and directly comparable). These spectra were separated to show detail in Figure III-35.

Spectra were also taken of benzo[a]pyrene and benzo[a]pyrene tetraol with laser excitation at 514.5-nm (argon-ion laser). Representative spectra of B[a]P and BPT are shown in Figure III-36. Spectra were also obtained from dibenz[a,h]anthracene epoxide (DBAE, a related carcinogen suspect substance derived from coal tar) and BPDE. Spectra of DBAE and BPDE are given in Figure III-37. DBAE and BPDE spectra were taken with laser excitation at 647.1-nm. Sample spectra are shown in Figure III-38 (solvent background), Figure III-39 (DBAE), and Figure III-40 (BPDE and a BPDE/DNA covalent complex). No SERS bands were detectable from the BPDE/DNA adduct shown in Figure III-40. Perhaps the BPDE/DNA adduct would have been detected had the sample been dissolved in water instead of in ethanol, which caused precipitation of the DNA. It is possible that the BPDE/DNA adduct would be a more efficient Raman scattering agent if adsorbed onto a different type of SERS substrate.

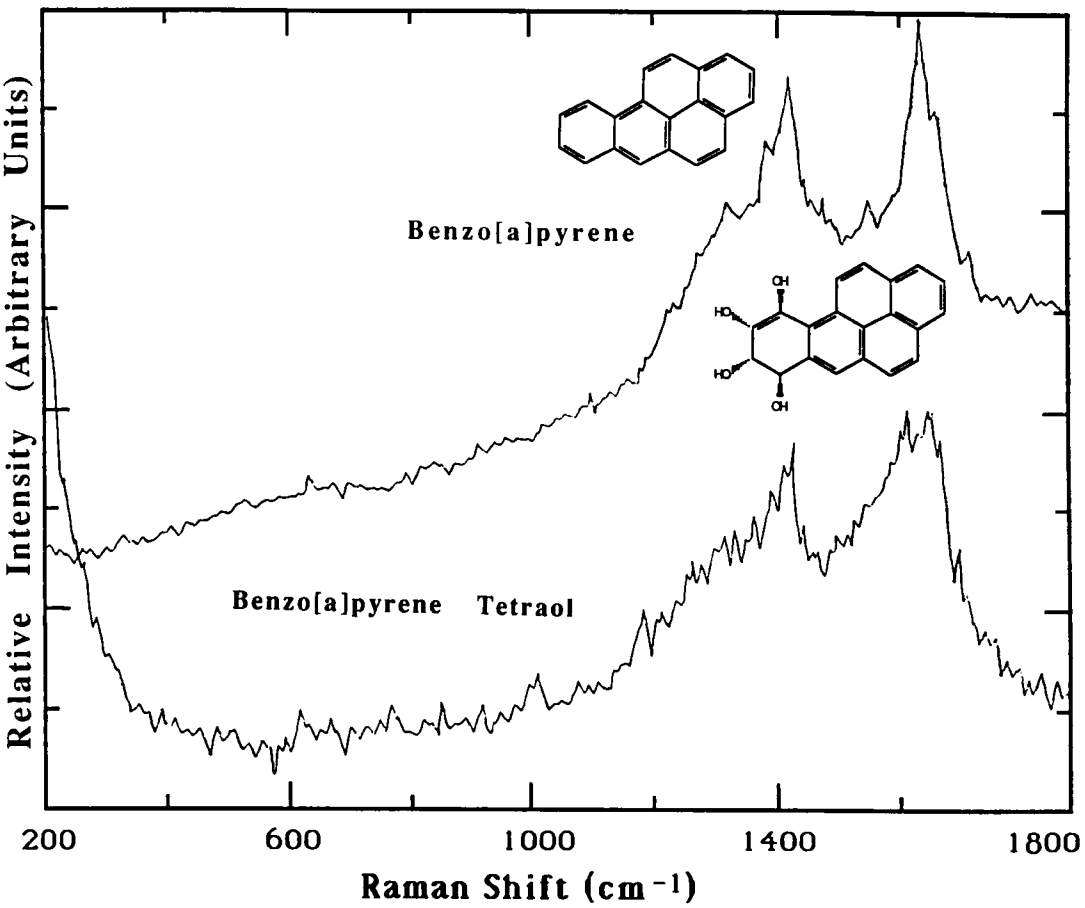
All pure samples observed in this study (B[a]P, BPT, BPDE, and DBAE) could be differentiated from each other. The molecules were routinely detected in 1.0-μL of a $1x10^{-5}$-M solution (picomole level). This detection limit is comparable to that seen with the etheno adenine and cytosine adducts.



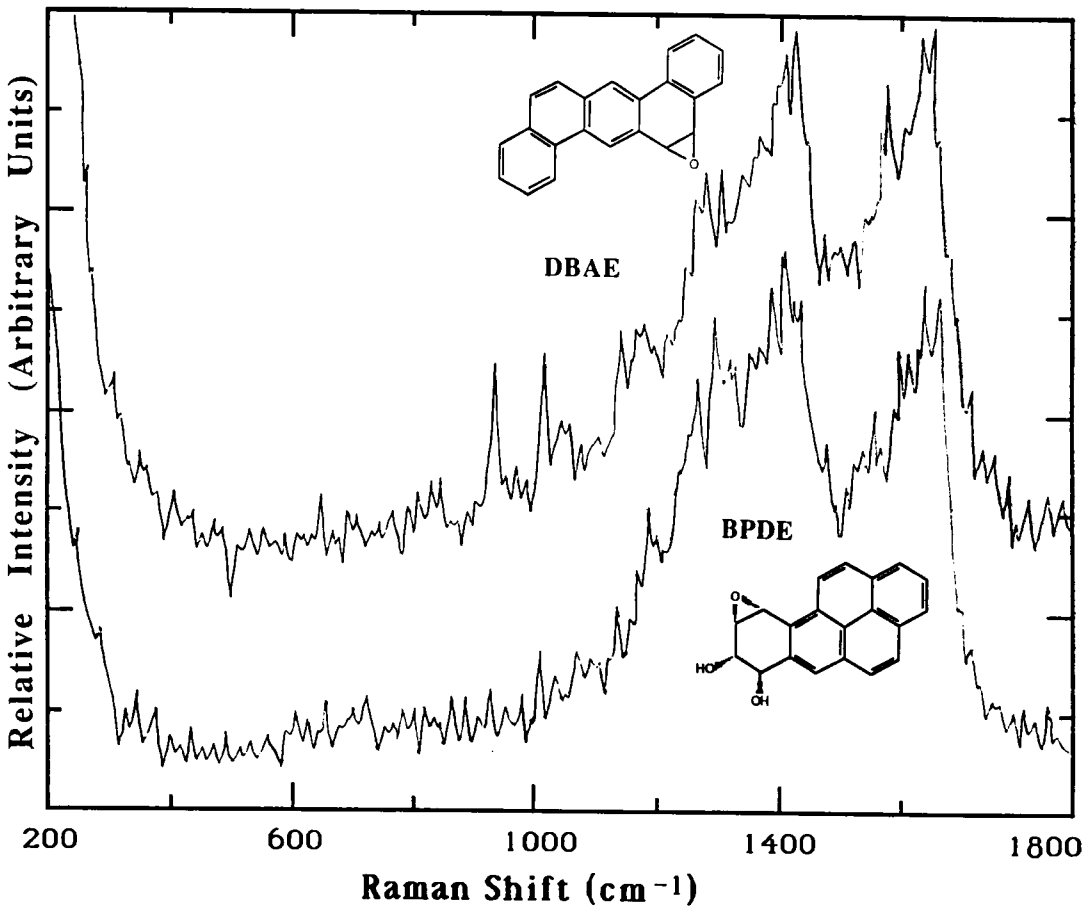

<FIGURE III-34> SERS Spectra of B[a]P, BPT, and the Substrate Background Samples were 1.0-μL of 1x10-4-M solutions in ethanol. Laser excitation was 80-mW at 647.1-nm. Spectra were printed to scale relative to each other.



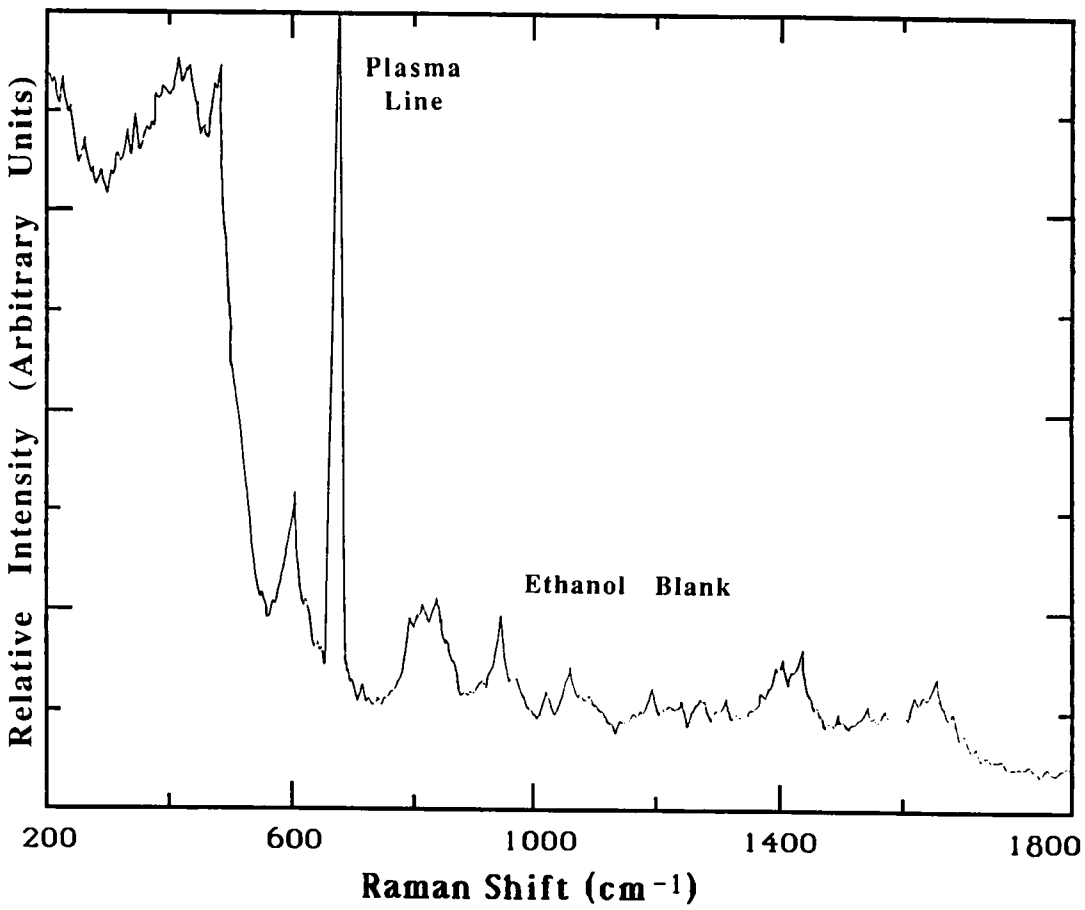

**<FIGURE III-35> SERS Spectra of Figure III-34 Enlarged to Show Detail** Spectra are of (Top to Bottom) B[a]P, BPT, and the 4.0% alumina-type substrate background. Spectra were printed to scale relative to each other.



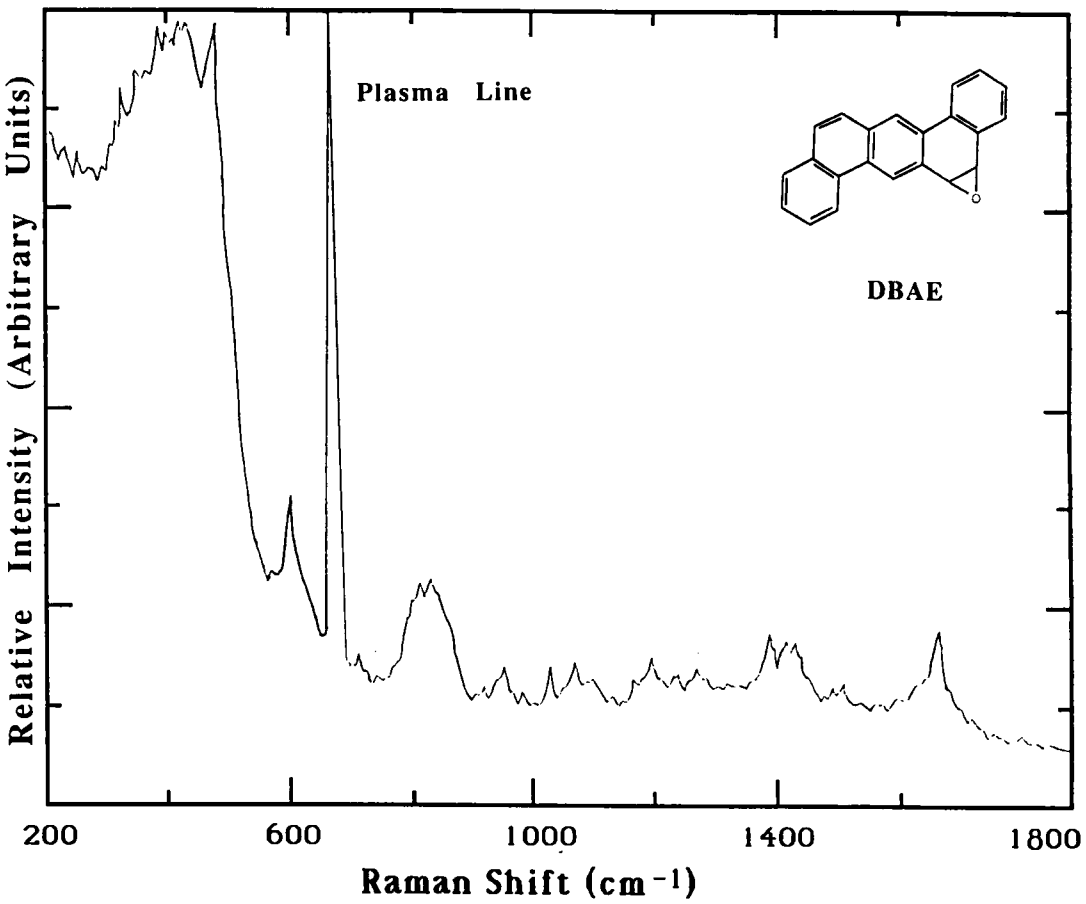

<FIGURE III-36> SERS Spectra of B[a]P and BPT Using Argon Laser Excitation Samples were 1.0-μL of 1x10$^{-4}$-M solutions in ethanol. Laser excitation was 50-mW at 514.5-nm using a direct excitation/collection optical setup. 5.0% alumina-type substrate background was shown in Figure III-9.



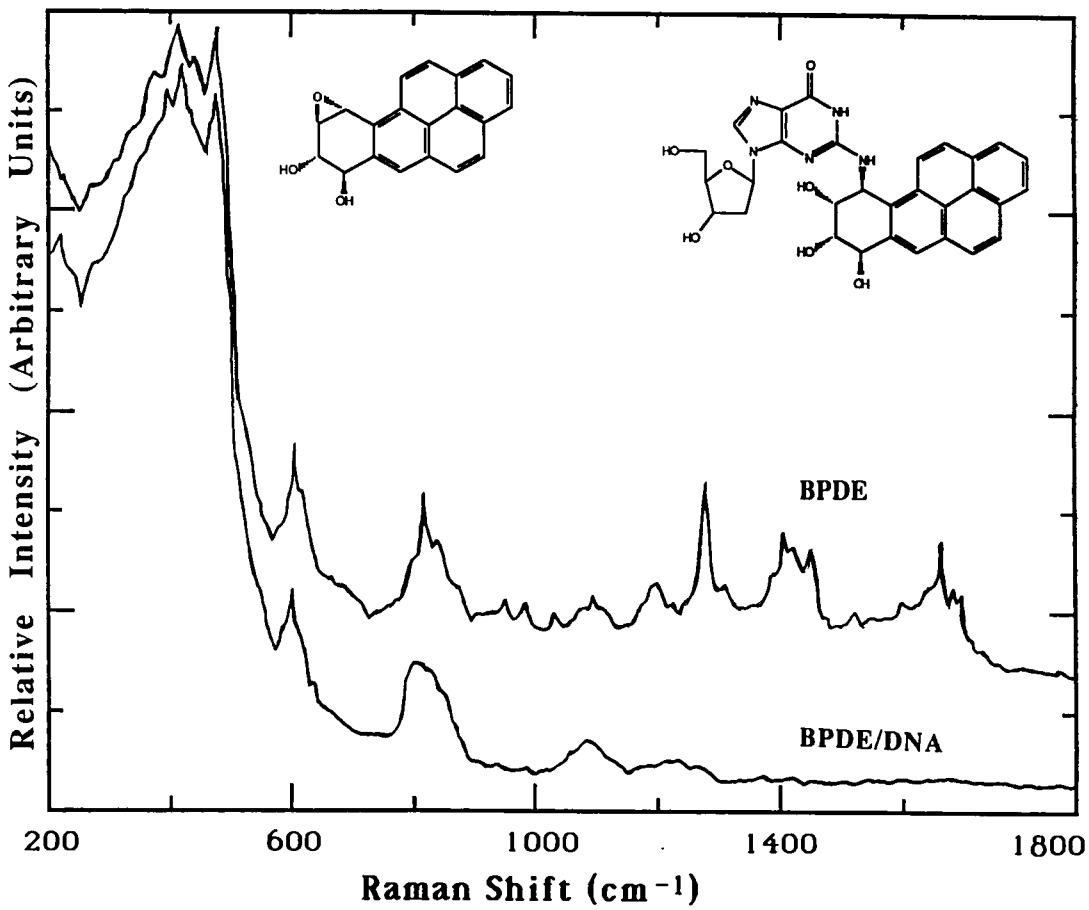

<FIGURE III-37> SERS Spectra of DBAE and BPDE Using Argon Laser Excitation Samples were 1.0-μL of 1x10⁻⁴-M solutions in ethanol. Laser excitation was 50-mW at 514.5-nm using a direct excitation/collection optical setup. Substrate background was shown in Figure III-9.




<FIGURE III-38> SERS Solvent Spectrum for Figure III-39 and Figure III-40 Sample was 1.0-μL of absolute ethanol. Laser excitation was 140-mW at 647.1-nm on a 5.0% alumina-type substrate.




<FIGURE III-39> SERS Spectrum of DBAE Sample was 1.0-μL of a $1x10^{-4}$-M solution in ethanol. Laser excitation was 140-mW at 647.1-nm on a 5.0% alumina-type substrate.




<FIGURE III-40> SERS Spectra of BPDE and a BPDE/DNA Adduct Spectra are of (Top) 1.0-μL of a $2.50 \times 10^{-5}$-M solution of BPDE in ethanol and (Bottom) a BPDE/DNA complex in ethanol (DNA precipitated). Laser excitation was 140-mW at 647.1-nm on a 5.0% alumina-type substrate.

## *Summary*

While SERS had been used earlier by Dr. Vo-Dinh's research group to detect benzo[a]pyrene and benzo[a]pyrene tetraol, the technique had not been applied to differentiation of these chemicals from the diol-epoxide of benzo[a]pyrene, BPDE. In this study, BPDE/DNA adducts exhibited no SERS bands. Chemicals which provided SERS spectra in this study were B[a]P, BPT, BPDE, and DBAE. These molecules produced well-defined SERS spectra under laser excitation wavelengths at both 647.1-nm and 514.5-nm using silvered, alumina-type SERS substrates.

SERS may be used to evaluate exposure to B[a]P by directly detecting B[a]P and BPDE. The SERS technique does not need to rely on an indirect measure of chemical exposure (e.g. BPT for fluorescence) to assess B[a]P contamination. In this study SERS demonstrated potential for the characterization of DNA adducts not only from small molecules such as vinyl chloride, but also from large molecular weight, ringed molecules such as benzo[a]pyrene.

# CHAPTER 3

# AFLATOXIN STUDIES

## *Introduction*

Aflatoxins are naturally occurring mold contaminants of several food sources, including nuts, grains, and rice (51,52). The aflatoxins are produced as metabolites of organisms including *Aspergillus flavus* and *Aspergillus parasiticus*. While aflatoxins have been proven to be extremely toxic, carcinogenic, and mutagenic in some animal species, their action in the human body is still unclear (53-55). Approximately thirty years ago, aflatoxin was recognized as a contaminant of peanuts and nut products (56-58). Several approaches have been devised to detect and limit aflatoxin levels in food sources, but the development of additional, improved techniques is advisable (59).

Fluorescence has been the major experimental technique used to detect aflatoxins because aflatoxins fluoresce with a reasonably high quantum yield in many different solvents (60,61). In some instances, however, the typical fluorescence from samples is an inconclusive indicator of aflatoxin contamination. For example, a bright greenish-yellow (BGY) fluorescence signal that probably signifies aflatoxin contamination can be obtained from the surfaces of corn (62-65), cotton (66-68), and pistachio nuts (69-71). The bright greenish-yellow color results from the interaction between kofic acid, a metabolite of *Aspergillus flavus*, and peroxidases residing in the plant tissue, rather than from aflatoxin itself (62,72,73). Peanuts contaminated by aflatoxins do not emit BGY fluorescence (74,75). In almonds, aflatoxin

contamination has been correlated with a violet-purple fluorescence, although the chemical responsible for this fluorescence was not identified (76,77). In experiments similar to the almond studies, peanuts exhibiting violet fluorescence under a near-ultraviolet excitation source, were found to contain 35 ppb aflatoxin (78). Clearly, different and improved techniques for assessing aflatoxin contamination need to be developed. In this study, SERS was used to detect aflatoxin $B_1$. No previous SERS detection or analysis of aflatoxin has been reported.

## *Materials and Methods*

### Instrumentation

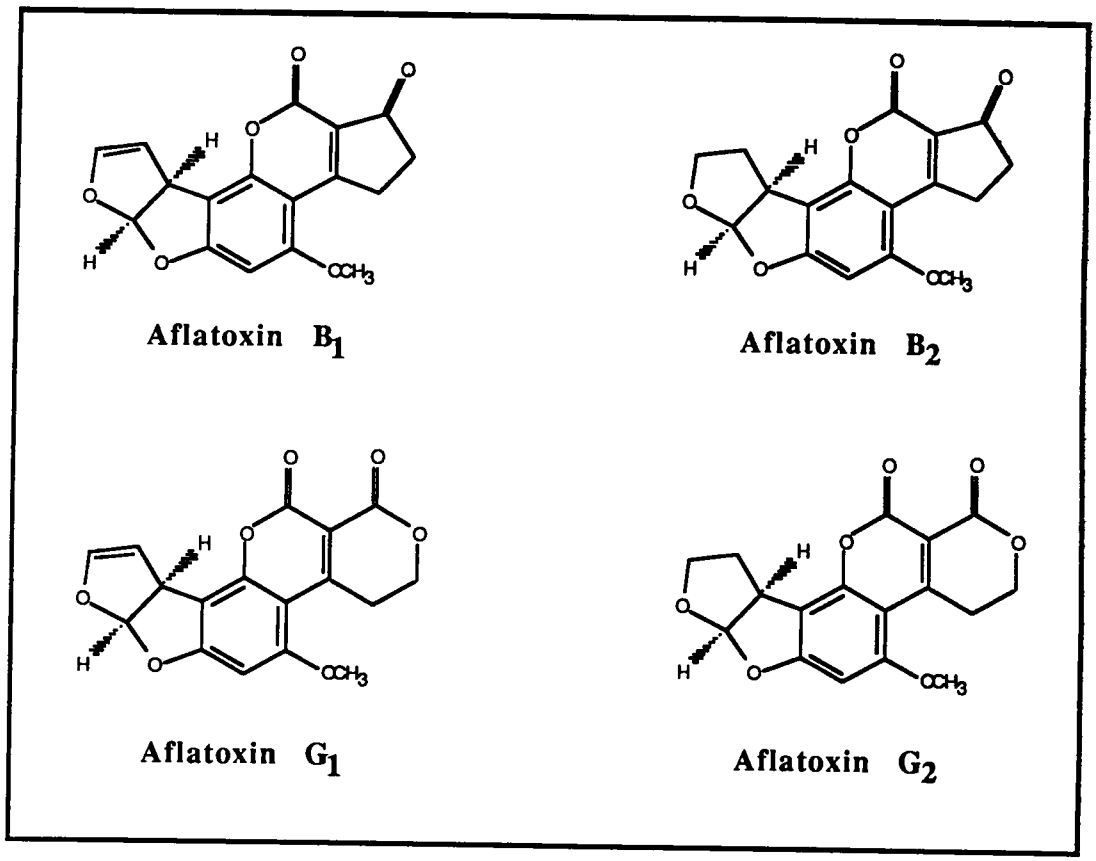
Instrumentation was similar to the optical setup described in Section I and diagrammed in Figure I-4 (p. 18).

### Chemicals

Aflatoxin $B_1$ was obtained from the Sigma Chemical Company (St. Louis, MO) and from the Fluka Chemical Corporation (Ronkonkoma, NY) and was used without further purification. Aflatoxins $B_2$, $G_1$, and $G_2$ were used as received from the Sigma Chemical Company (St. Louis, MO). The chemical structures of aflatoxin $B_1$, $B_2$, $G_1$, and $G_2$ are shown in Figure III-41.

### Procedures

The experimental procedures were identical to those used for the other studies in this section. Laser excitation power was usually 50-mW to 80-mW at



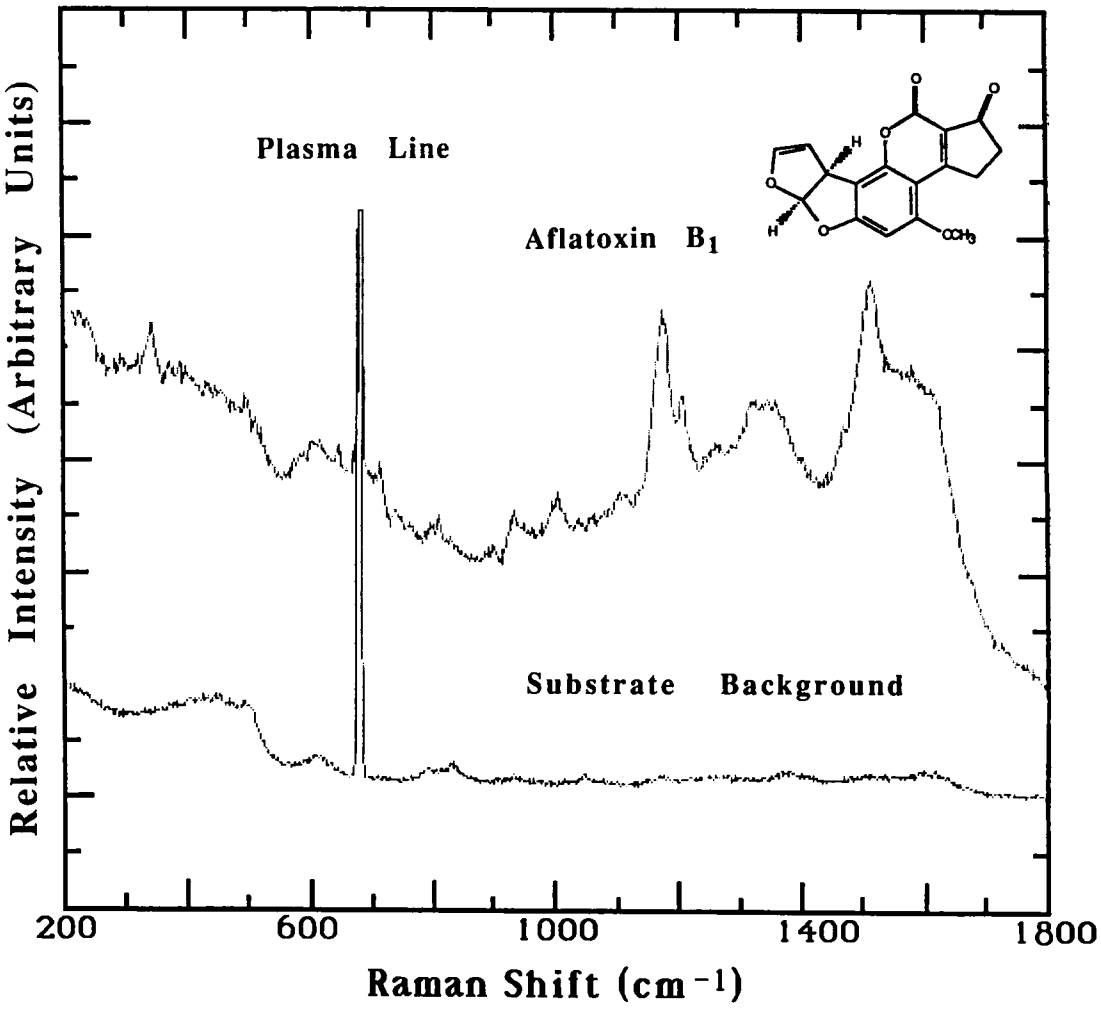

<FIGURE III-41> Chemical Structures of Aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$

647.1-nm. Aflatoxins $B_2$, $G_1$, and $G_2$ were examined using laser excitation at 616-nm from the dye head used in conjunction with the argon-ion laser. SERS substrates were alumina-roughened (4.0%) glass coated with 100-nm of thermally evaporated silver.

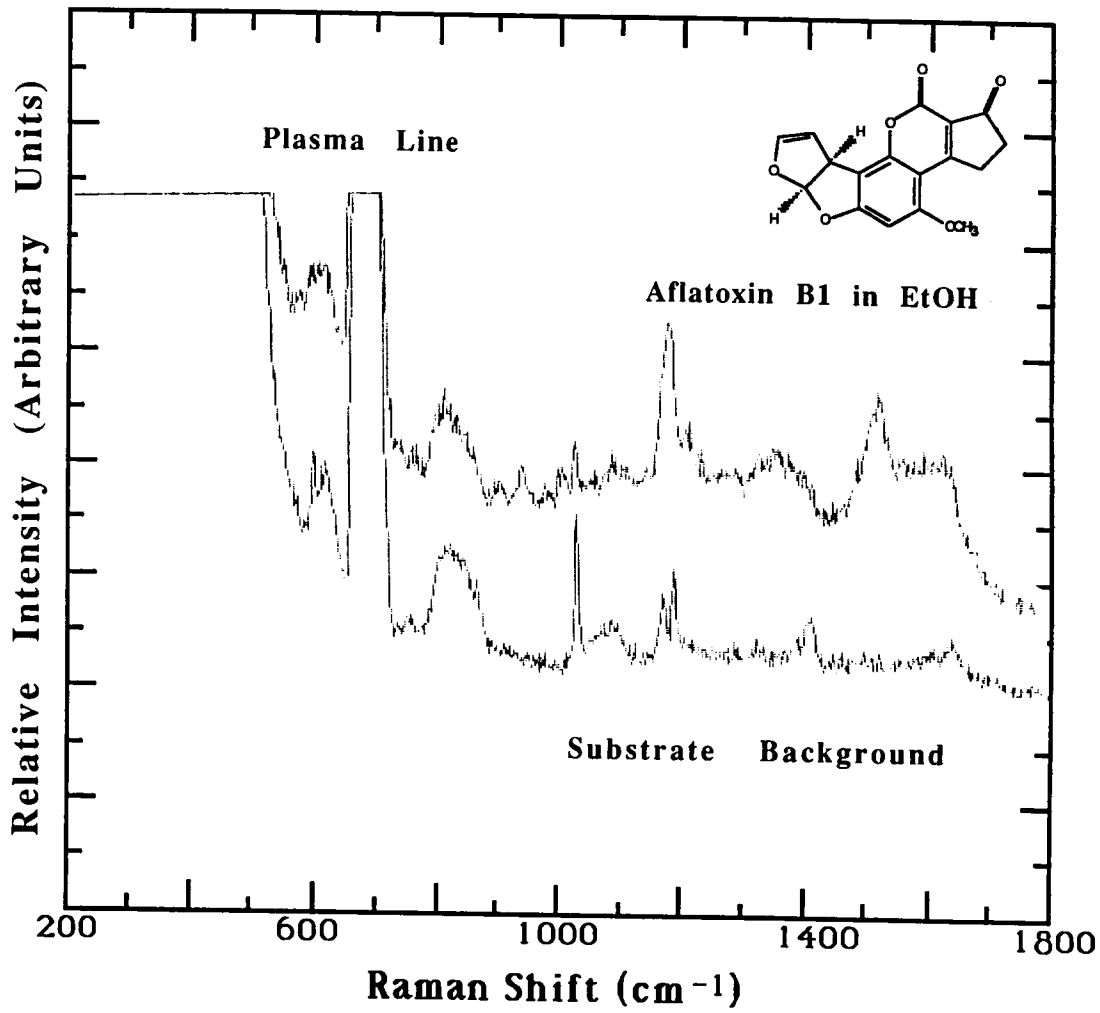
*Results and Discussion*

An excellent SERS spectrum of aflatoxin $B_1$ and the substrate background are shown in Figure III-42. The relationship between the background spectrum and the sample spectrum is an important consideration when reviewing the experimental results on aflatoxin. Figure III-43 also shows aflatoxin $B_1$ (same sample solution) and the background spectrum, but the background bands in this spectrum are more comparable in intensity to those of the samples than were the background peaks seen in Figure III-42. A tabular comparison of SERS background bands and aflatoxin $B_1$ bands is given in Table III-3.

Aflatoxins $B_2$, $G_1$, and $G_2$ were also examined using SERS. Spectra are presented in Figure III-44. The spectra revealed no intense SERS bands, although two broad bands were apparent for all three samples. The aflatoxins could be distinguished from the background spectrum because of the presence of these bands and because the aflatoxins were better scattering agents (higher spectral intensity) than was the substrate. The lack of intense Raman bands may have been a result of the low solubility of the aflatoxins in ethanol (crystals still remained in all three chemical solutions). The crystals of aflatoxin were also probably excellent scattering agents.




<FIGURE III-42> SERS Spectra of Aflatoxin $B_1$ and the Substrate Background Sample was 1.0-μL of a 2.14x10-4-M solution of aflatoxin in ethanol. Laser excitation was 55-mW at 647.1-nm. Spectra were printed to scale relative to each other.



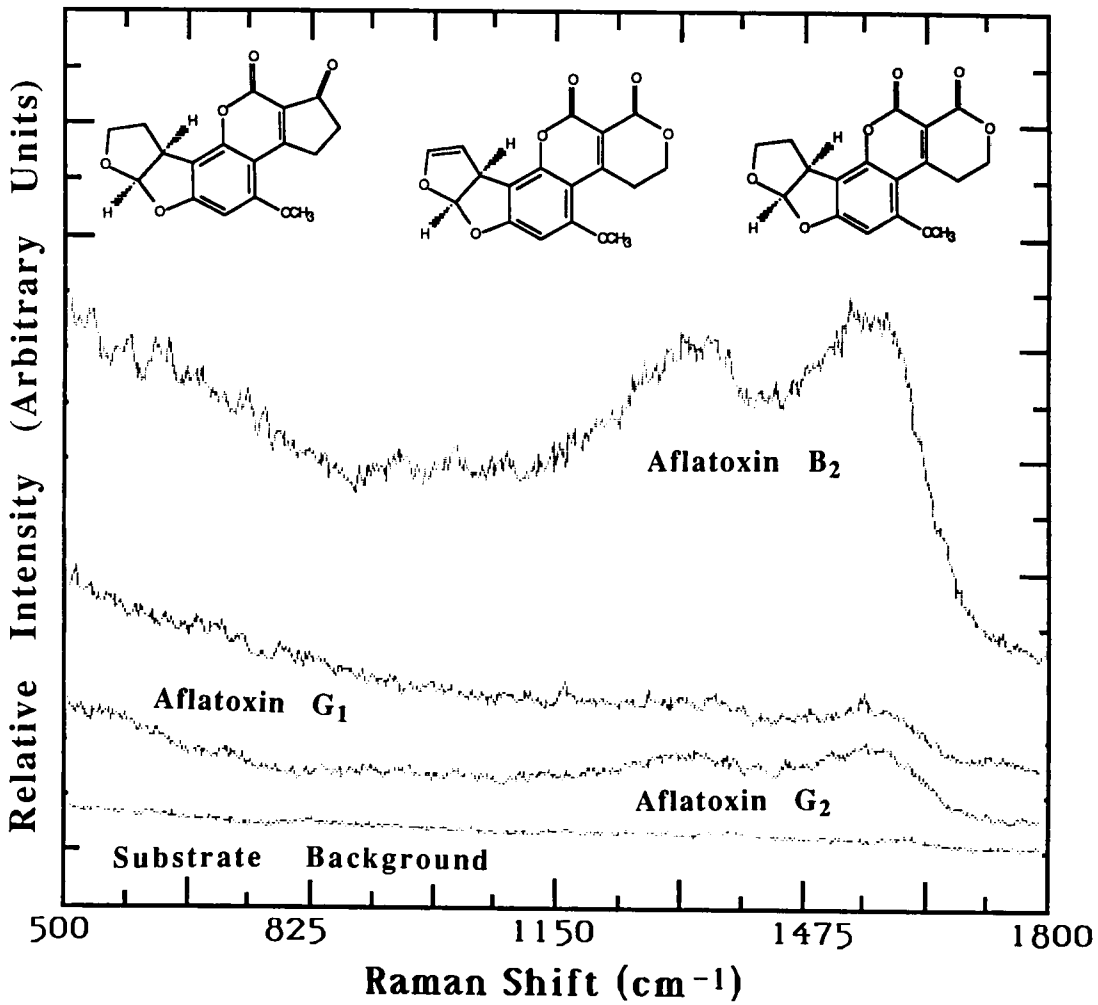

<FIGURE III-43> SERS Spectra of Aflatoxin $B_1$ and the Substrate Background Expanded to Show Detail Sample was 1.0-μL of a 2.14x10-4-M solution of aflatoxin $B_1$ in ethanol. Laser excitation was 70-mW at 647.1-nm. Spectra were printed to scale relative to each other.

TABLE III-3
Tentative Chemical Group Assignments for the Spectral Bands of Aflatoxin $B_1$

| Background | Aflatoxin $B_1$ | Assignment |
|---|---|---|
| 224 S | 240 S | Background SERS |
| 446 b | 440 b | Background SERS |
| 492 S,sh | 486 S,sh | Background SERS |
| 608 m | 602 m | Background SERS |
| 670 vS | 670 vS | Laser Plasma Line |
| 794 m,b | 796 m,b | Background SERS |
| | 918 m | Ring Vibration |
| 1050 w | 1060 w | Background SERS |
| | 1152 S | Aflatoxin $B_1$ |
| | 1186 m,sh | Aflatoxin $B_1$ |
| | 1244 w | Aflatoxin $B_1$ |
| | 1330 m,b | Aflatoxin $B_1$ |
| | 1494 S | In-Plane Ring Bending |
| | 1596 m,sh | Double-Bond Stretching |

*Abbreviations*

| | |
|---|---|
| S | strong |
| m | medium |
| w | weak |
| sh | shoulder |
| b | broad |

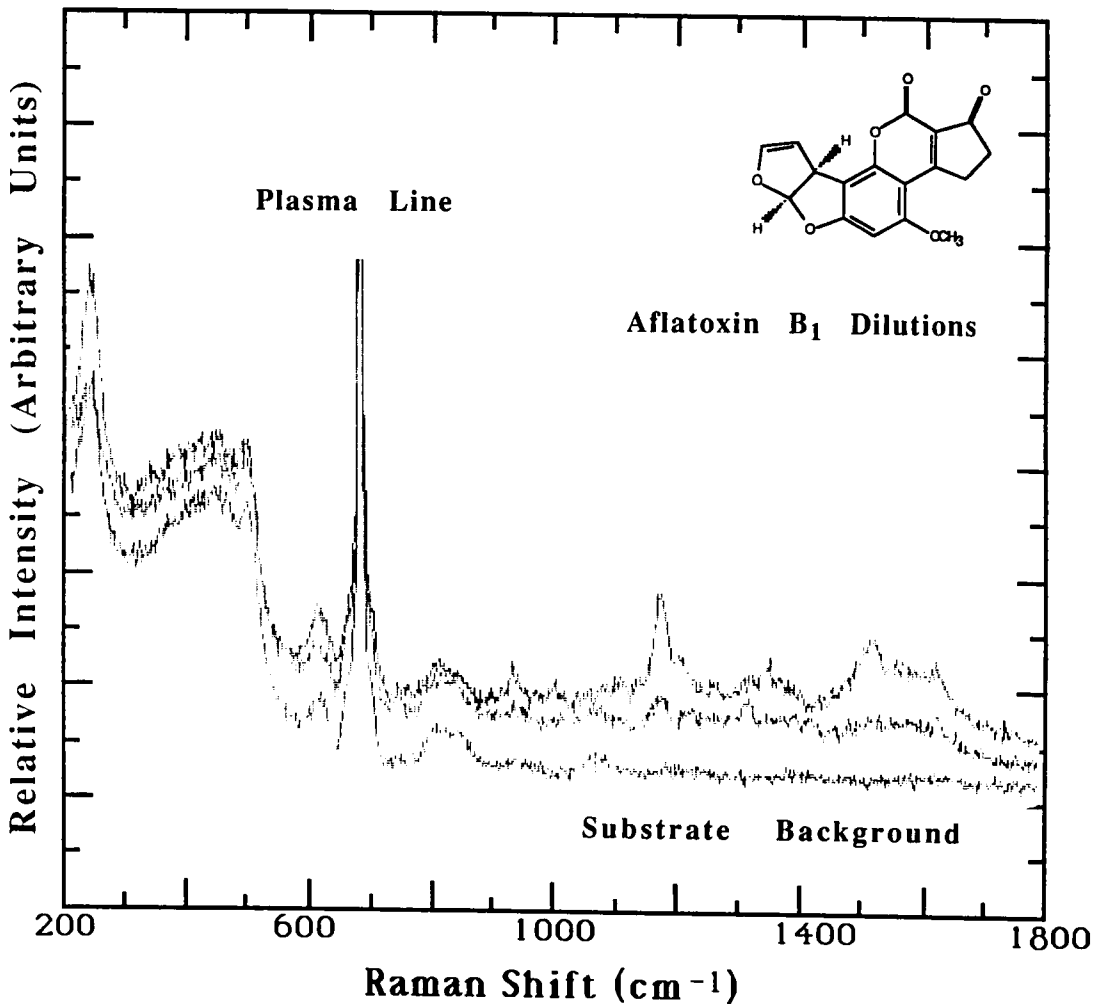



<FIGURE III-44> SERS Spectra of Aflatoxins $B_2$, $G_1$, and $G_2$ and the Substrate Background Spectra are of (Top to Bottom) 1.0-μL of a $1.59 \times 10^{-2}$-M solution of aflatoxin $B_2$ in EtOH, 1.0-μL of a $1.52 \times 10^{-3}$-M solution of aflatoxin $G_1$ in EtOH, 1.0-μL of a $1.51 \times 10^{-2}$-M solution of aflatoxin $G_2$ in EtOH and the 4.0% alumina-type substrate background. Aflatoxin samples were not fully dissolved at these concentrations. Laser excitation was 140-mW at 616-nm ($Ar^+$ laser with dye head) using a direct excitation/collection optical setup. Spectra were printed to scale relative to each other.

Aflatoxin $B_1$ bands were not discernable at the extremely low concentrations at which the etheno adducts could be detected. Spectra from a sample limit-of-detection study are shown in Figure III-45. Aflatoxin $B_1$ was marginally detectable in a 1.0-μL drop of a $8.92 \times 10^{-5}$-M solution (picomole level). Low chemical solubility prevented samples from eliciting spectra from solutions more concentrated than $2 \times 10^{-4}$-M.

*Summary*

These spectra represent the first published SERS detection or analysis of an aflatoxin. Broad SERS bands were observed for aflatoxins $B_2$, $G_1$, and $G_2$. SERS spectra of aflatoxin $B_1$ demonstrate that SERS may be developed as a powerful technique for assessing the health risk from aflatoxin contamination. At this juncture, SERS may not be used to detect aflatoxin $B_1$ at the extremely low level of concentration at which it may be found in animal tissue. As with the other molecules examined by SERS, the laser beam did not impinge on the entire sample spot, so the amount of aflatoxin detectable may be less than the picomole level. Modification of the optical setup and the incorporation of a more sensitive substrate may overcome current sample detection limitations.




<FIGURE III-45> SERS Limit-of-Detection Study of Aflatoxin $B_1$ Spectra are of (Top) 1.0-μL of a $2.14 \times 10^{-4}$-M solution of aflatoxin $B_1$ in ethanol, (Center) 1.0-μL of a $8.9197 \times 10^{-5}$-M solution of aflatoxin $B_1$ in ethanol, and (Bottom) substrate background. Laser excitation was 70-mW at 647.1-nm. Spectra were printed to scale relative to each other.

# CHAPTER 4

# CONCLUSIONS

This section focused on complementary studies of some of the genotoxins which may be detected using SERS. The data presented in this section contributed original structural/vibrational information for these molecules. There was scientific value for these studies not only from the principal detection of these samples by SERS, but also from the comparisons which were made with unaltered precursors of these genotoxins. For example, SERS spectra of purines and pyrmidines adenine, cytosine, thymine, and guanine were contrasted with spectra of 2'-deoxyadenosine, cytidine, and 2'-deoxycytidine and with spectra of etheno adducts 1,$N^6$-ethenoadenine, 1,$N^6$-ethenoadenosine, 1,$N^6$-etheno-2'-deoxyadenosine, 3,$N^4$-ethenocytosine, and 3,$N^4$-ethenocytidine. Also, SERS spectra of benzo[a]pyrene and benzo[a]pyrene tetraol were compared with spectra of BPDE, the most significant carcinogenic intermediate in B[a]P binding to DNA. The SERS spectrum of BPDE was compared with that of a BPDE/DNA adduct and with that of a related epoxide, DBAE. SERS was used to address the need for new methods of chemical detection. To that effect, the first aflatoxin (types $B_1$, $B_2$, $G_1$, and $G_2$) SERS spectra were recorded.

The quantity of molecules detectable by SERS rivaled that of other spectroscopic techniques. Ten picomoles to 100 picomoles could be detected for all analytes that exhibited SERS bands in this study. 3,$N^4$-ethenocytosine could be detected at the femtomole level.

In this study, SERS was shown to be an effective means of detecting and

characterizing biologically-important molecules. The samples examined and discussed in this section represent only a small fraction of potentially SERS-active genotoxins. It must be noted that not every molecule reveals a SERS spectrum and that some molecules only yield SERS spectra under specific experimental conditions. This limitation affects any spectroscopic technique. SERS is a valuable analytical technique not only because of its sensitivity and chemical group specificity, but also because the experimental parameters may be readily manipulated in a manner not possible for more standardized techniques. Thus with SERS one technique may be used to provide a wealth of structural information about a single sample.

# SECTION III

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## REFERENCES


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# PART IV: APPLICATIONS OF SERS TO BIOLOGICAL PROBLEMS

# CHAPTER 1

# A SERS BIOSENSOR

## *Introduction*

One aspect of this research was to address the feasibility of utilizing SERS for *in vivo* scattering analysis of biologically-important substances. The major professor for this dissertation, Dr. Tuan Vo-Dinh, was among the spectroscopists to have successfully integrated SERS with fiberoptics technology. His group devised a sensitive SERS fiberoptic sensor and a fiberoptic remote sensor for *in situ* SERS scattering analysis (1). The development of a SERS *bio*sensor was justified by the many positive aspects it could contribute to biochemical and clinical analyses. The first of these capabilities would be the high chemical selectivity available with SERS. A wide range of chemicals including many small, ringed compounds may be detected using SERS. Excellent sample detectability was not possible for dilute samples at the time this research was begun, but was accomplished as experimental parameters were optimized (see Section II). With the proper substrate, SERS could be done under low laser excitation powers and at a variety of wavelengths, so damage to sample molecules would be minimized. Organic molecules would not need to be subjected to high energy wavelengths and intense excitation power that could result in alteration of the specimen and thermal damage to surrounding tissue. Because SERS could be done with thin fibers, a biosensor could function using extremely minute sample volumes (nanoliter to microliter) -- it was hoped that SERS measurements could be performed in otherwise inaccessible body fluids, such as the

interstitial fluids of soft tissues. SERS could be used to provide simultaneous sampling in which a single analyte could be examined in a variety of sites (thus the possibility for providing valuable metabolic information). Because of the unique spectral signatures of similar chemicals, rapid, multi-analyte measurements could be performed simultaneously with a single sensor.

### *Biosensor Designs*

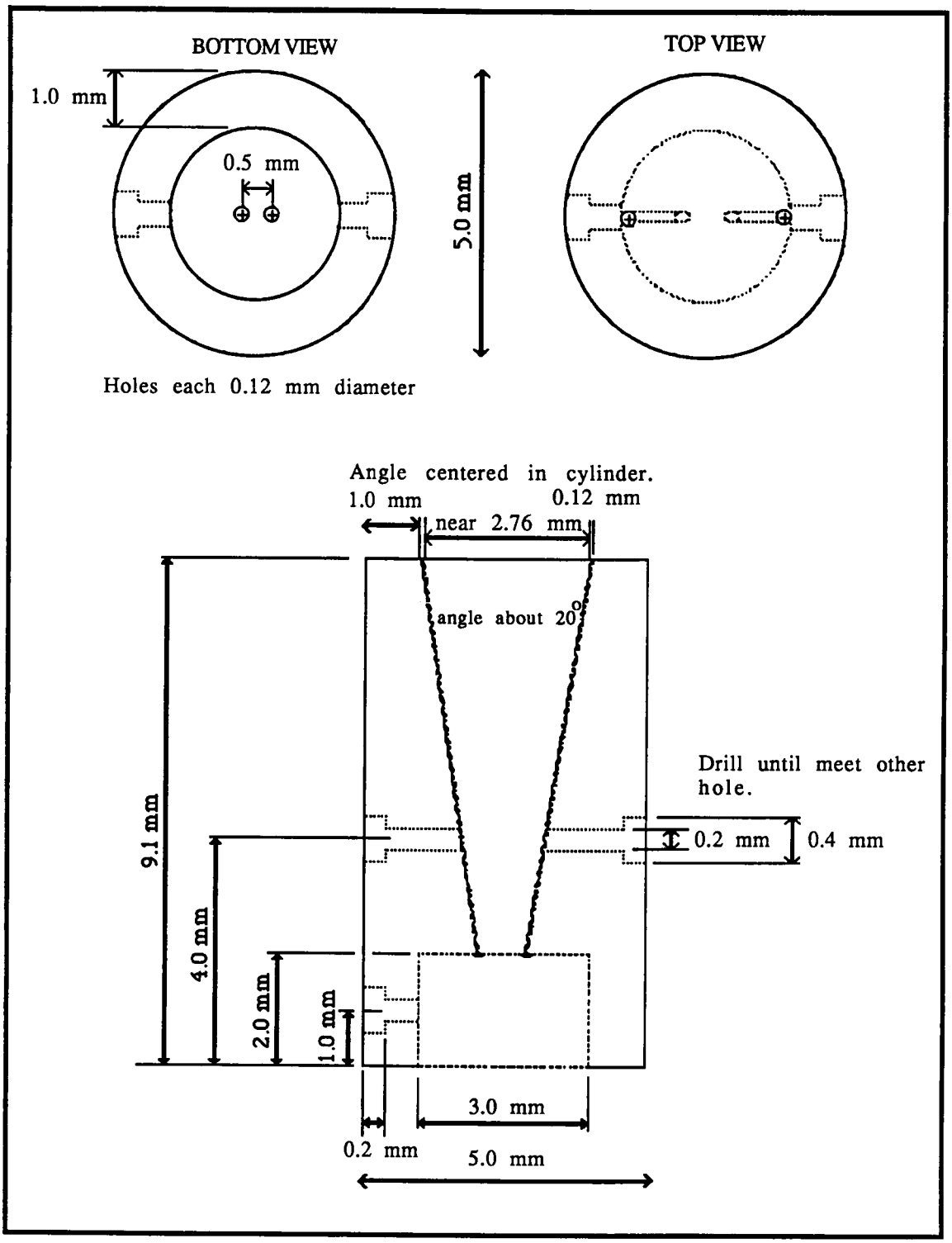
Several possible designs for the proposed biosensor were explored. The principal requirements for the biosensor were small size, to minimize tissue damage and facilitate *in vivo* examination, and high detectability, so that molecules in dilute, complex mixtures could be studied. A major obstacle in the biosensor application was discovering a suitable biologically-important chemical system that could be detected with SERS. This problem was solved through educated guesswork (many small, ringed molecules are SERS-active) and trial-and-error analysis of chemicals. Selection of a chemical system was complicated by the fact that SERS sensitivity varied for each proposed molecule to be detected. Another difficulty was that SERS was incapable of detecting very dilute chemical samples. As described earlier, an extensive amount of time was spent optimizing the experimental parameters associated with SERS to bring the limit-of-detection of the samples to a range practical for the study of biological contaminants.

Remote sensing of samples was easy to achieve, since many spectra taken in this study used from 0.5-m to 3.0-m each of excitation and collection optical fiber with little signal loss. Optimal biosensor size could be determined fairly readily when the effectiveness of SERS with fiberoptics was

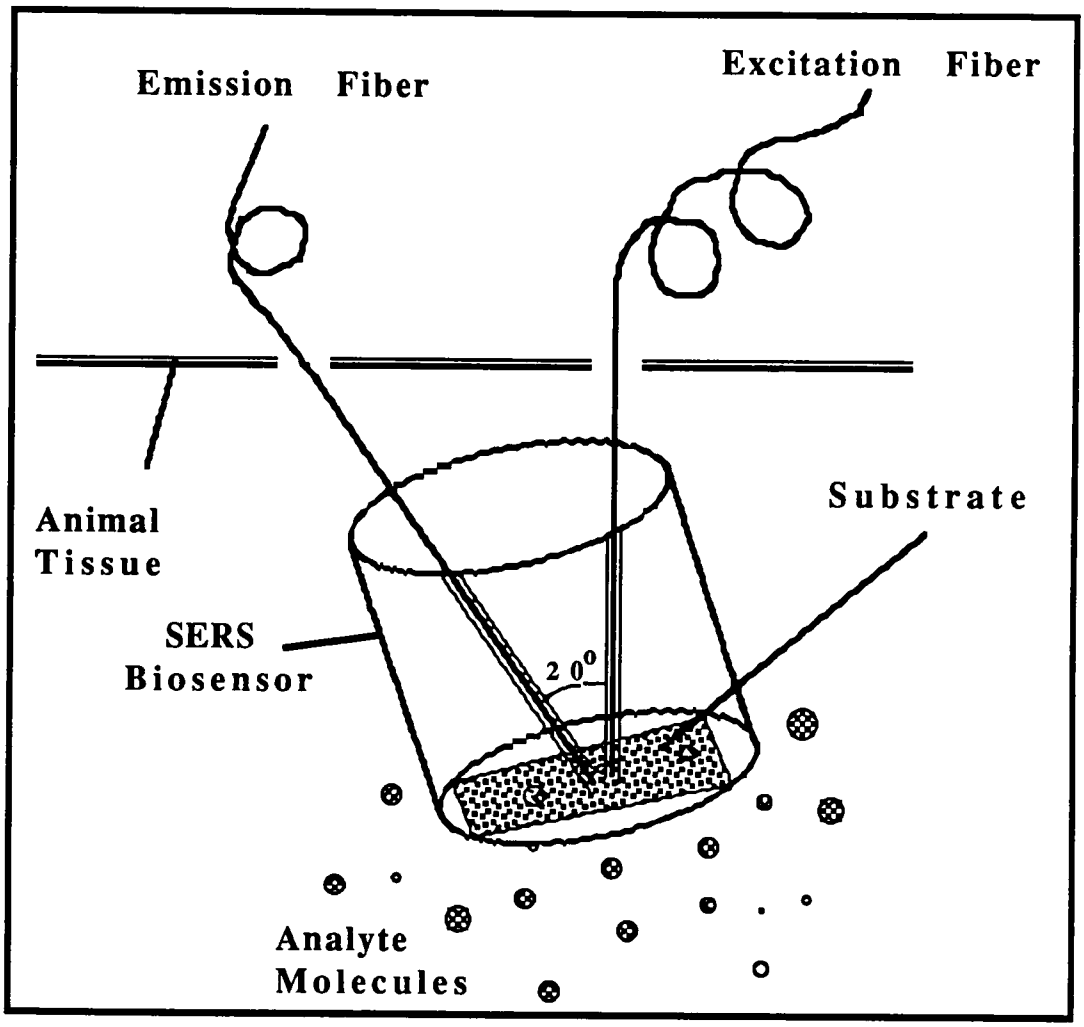
demonstrated. All-silica fibers were available at 100-μm in diameter and could be used to detect a sample with only a twenty degree angle of fiber separation. Two viable biosensor designs are shown in Figure IV-1/Figure IV-2 and Figure IV-3/Figure IV-4. One adaptation that could be incorporated into these schematics would be the use of optical fiber bundles to collect scattered light, either encircling the excitation fiber to minimize biosensor size, or opposed to the excitation fiber to maximize efficiency. Additional possible modifications include evaporation or chemical deposition of silver directly onto the fibers as a SERS substrate, containment of a silver hydrosol within a semi-permeable membrane for sample detection, or polymer coating of a SERS substrate to protect the sample against metal contamination or to increase technique sensitivity.

### *Limit-of-Detection Studies*

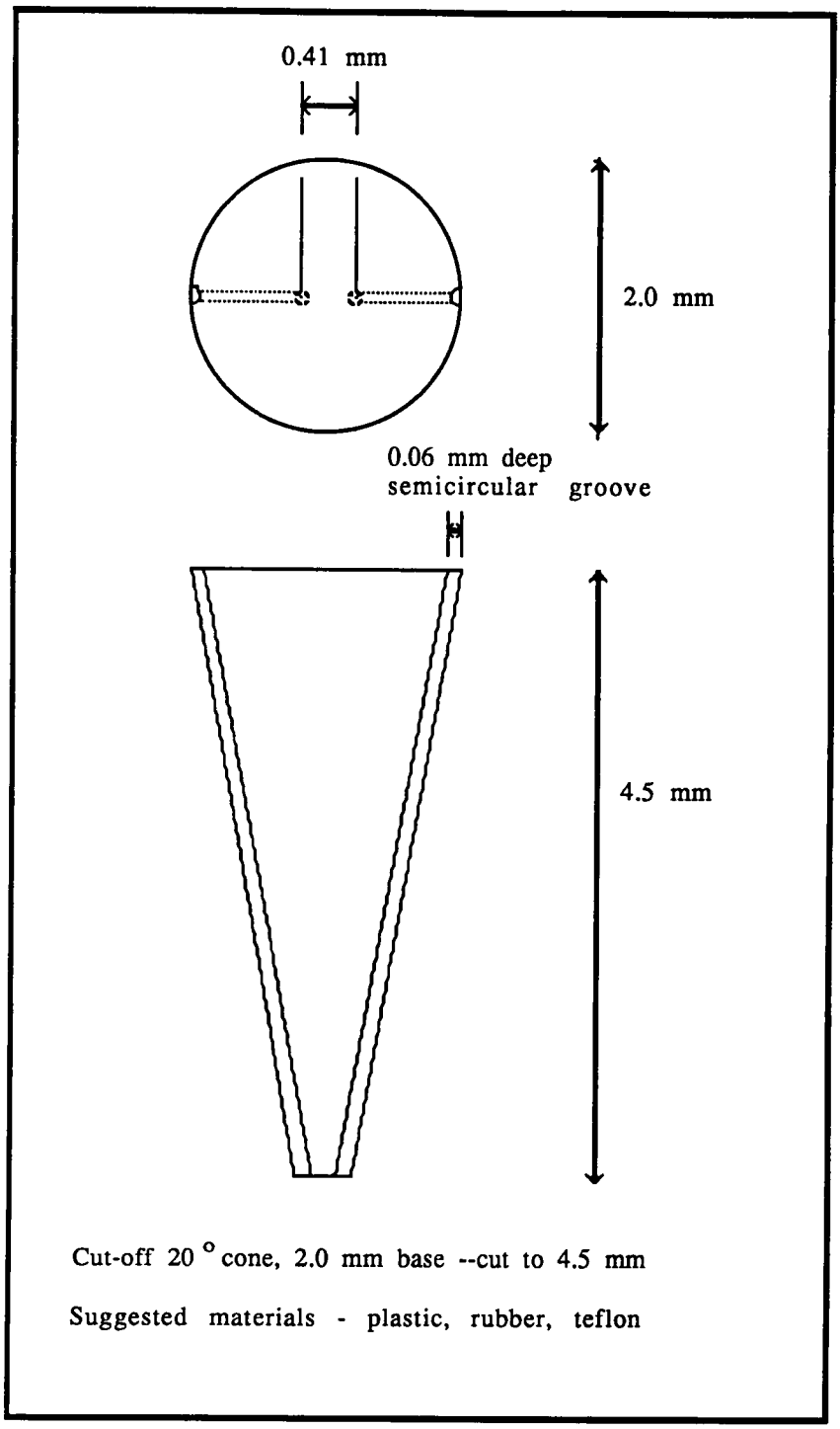
When a SERS sample spectrum that differed from a substrate/solvent background spectrum was discovered, a type of limit-of-detection study was performed to determine the best sample concentration and the minimum sample concentration for SERS detection. This was an experimentally determined value equal to the lowest sample concentration (from a set of serial dilutions) that could be distinguished from the background noise. It must be noted that this value did not necessarily correspond to the IUPAC convention for limit-of-detection because many of the substances in these experiments could be detected at the same order of magnitude as the background noise. Sample limit-of-detection spectra were presented in the preceding section (Section III) on SERS detection of genotoxins to allow the reader to make an



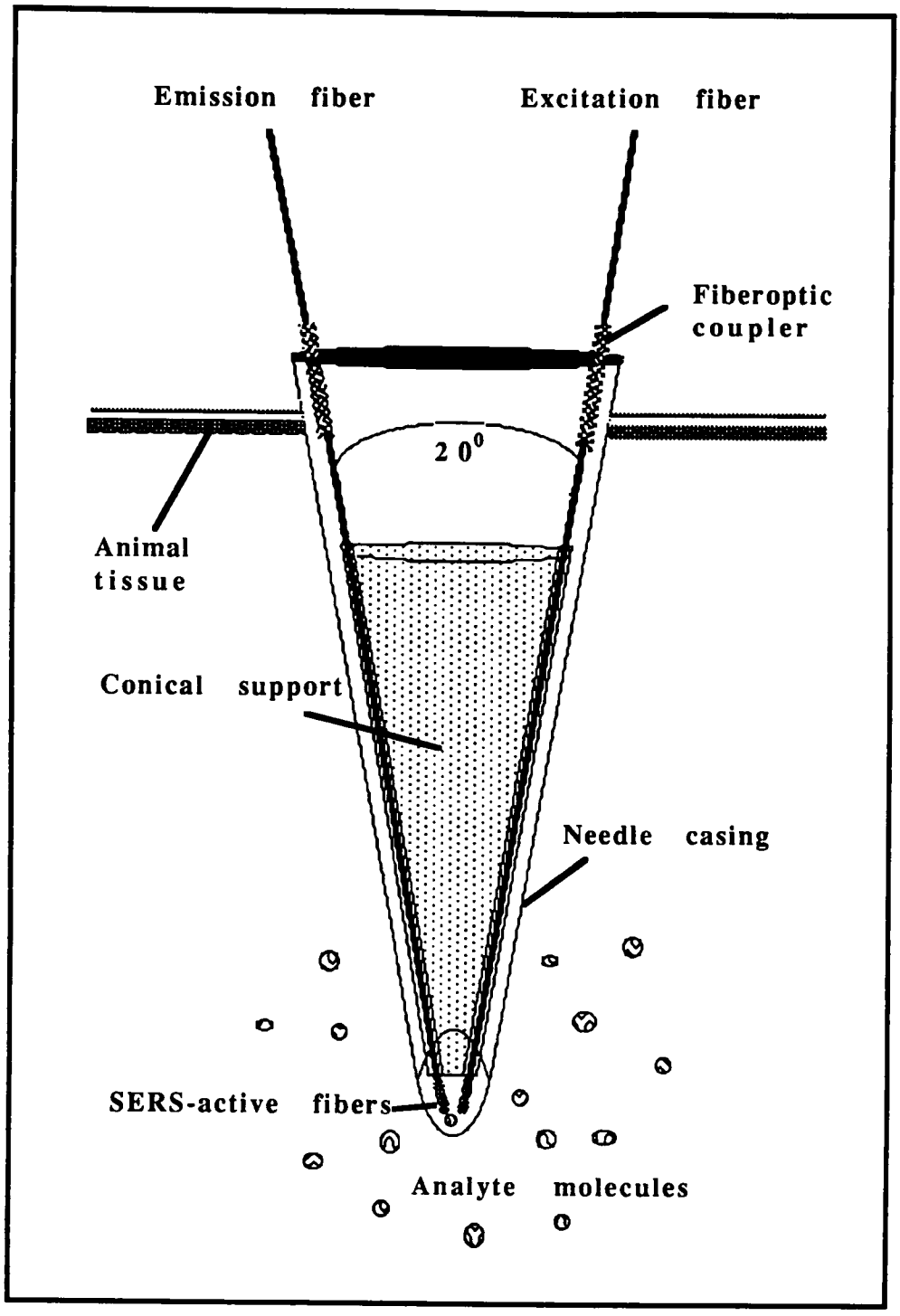

**<FIGURE IV-1> Blueprint of a SERS Biosensor Utilizing a Silvered, Microparticle-Roughened Substrate**




<FIGURE IV-2> Diagram of an Application of the SERS Biosensor of Figure IV-1




**<FIGURE IV-3> Blueprint of a SERS Biosensor with Silvered Optical Fibers**




<FIGURE IV-4> Diagram of an Application of the SERS Biosensor of Figure IV-3 This type of biosensor may be encased in a hypodermic needle.

individual decision about the level at which a chemical could be detected. The limit-of-detection values could be used to help predict whether or not a chemical could be expected to be detected and could be used to roughly quantify the sample being examined.

### *Summary*

The problems encountered with the biosensor proposal originated from several sources. First, when any optical fiber was used for the collection of scattered light some percentage of signal was inevitably lost. Some loss was due to inefficient fiber collection of strongly scattered light (such as in a liquid sample solution). This problem could be partially remedied through using a fiber array (bundle) surrounding the sample to collect light from many angles. Use of a fiberoptic bundle would worsen a second source of signal loss -- scattering inside the collection fiber. Loss of photons within and through the sides of the fiber may be substantially reduced by limiting fiber length. Optical fiber manufacturers list the expected amount of signal loss for a given wavelength and fiber length, so this loss can be predicted. In the experiments discussed in this dissertation, all fibers were relatively short (about one meter) and negligible signal loss occurred. Remote sensing through a biosensor with a fairly long fiber would only exacerbate the problem of detecting extremely dilute samples in very complex matrices. As will be discussed later in this section, the excellent chemical sensitivity obtained in this study through optimizing experimental parameters is, however, barely sufficient for detection of biologically-important molecules in fresh tissues. In addition, a signal loss factor is encountered when fiber

diameter or fiber length is increased using the experimental setup described here. Application of the biosensor to *in vivo* animal studies is not practical unless a means of protecting the animal against silver exposure (metal poisoning) is incorporated into the biosensor design. Use of the biosensor in living systems is not advisable at this time with the substrates and instrumentation employed here.

# CHAPTER 2

# AGAROSE GEL AS A COATING FOR SERS SUBSTRATES

## *Introduction and Rationale*

Among the problems to be overcome in adapting SERS to biological analysis was the toxicity of the silver on the silvered alumina-coated substrates and the difficulty of using SERS to detect bioindicators of chemical exposure *in vivo* because of the large dilution factor involved. An experiment using agarose gel as a coating for SERS substrates was performed to determine if the toxicity of the substrates could be reduced without seriously compromising the sensitivity of the substrates for use with SERS.

Extensive studies have been performed at ORNL and other facilities concerning the effects of polymer coatings on SERS substrate effectiveness. Agarose gels were especially interesting substrate coating candidates because agarose hydroxyl groups can be activated for chemical binding. Gels may also be impregnated with electroactive species for indirect binding. Samples may be embedded in an agarose matrix, yet due to the porous nature of agarose gels, still retain much of their binding activity. Agarose is readily available, non-toxic, and inexpensive.

Agarose proved to be an effective coating for SERS substrates because SERS spectral intensity was not diminished by the overcoat on the substrate. In fact, the SERS spectra appear to have been enhanced by the agarose coating. It should be noted, however, that the coating substantially diminished the lifetime of the substrates and took considerably more preparation time than did the usual silvered, alumina-type substrates. Terephthalic acid and

benzoic acid were selected as molecules for this study because both chemicals have been well-characterized by SERS (2-6) and because they yield very intense, well-defined SERS spectra under these experimental conditions.

## Agarose

Agarose is a naturally occurring polysaccharide widely used in immunoelectrophoresis, electrode formation, and affinity chromatography. Agarose gels result from thermally reversible multiple hydrogen bonding of double stranded helices of galactose chains. This process leads to the formation of polymeric bundles separated by aqueous channels. Agarose gel matrices are produced by cooling hot agarose solutions. Increasing the agarose concentration in a gel decreases the average pore diameter in the matrix. Typically, this diameter is tens of nanometers (7).

## *Materials and Methods*

## Instrumentation

Once again, the instrumentation was based on an integration of SERS with fiber optics (Figure I-4, p. 18). Similar setups have previously been described as effective for SERS (8-10). The monochromator slit widths were set at 500-nm. A model Innova 70 krypton-ion laser (Coherent Inc. Laser Products Division, Palo Alto, CA) 647.1-nm line served as the excitation source for the samples. Optical fiber diameter was 600-μm (without the soft plastic cladding) with a typical fiber length of 0.75-m to 1.00-m.

## Chemicals

Terephthalic acid (Pfaltz and Bauer, Waterburg, CT), benzoic acid (Aldrich, Milwaukee, WI) and electrophoresis-grade agarose (Aldrich) were purchased at the highest available quality and were used as received. Spectroscopic grade absolute ethanol (Warner-Graham Company, Cockeysville, MD) and HPLC-grade deionized water (Millipore Corporation, Bedford, MA) were used to prepare solutions.

## Procedures

Silvered, microparticle-coated substrates were prepared as follows: Approximately 10-μL of a 5.0% sonicated aqueous suspension of alumina were spin-coated (2000-rpm to 3000-rpm, 20-s) onto a precleaned 20x10x1-mm piece of microscope slide. Next, a 100-nm layer of silver was evaporated onto the alumina-coated plates under a pressure of approximately $2x10^{-6}$ Torr. These substrates were coated with agarose within 24 hours of preparation.

Aqueous agarose gels ranging in concentration from 0.0% (pure water) to 5.0% agarose (w/v) were melted and heated to 85° C. The agarose was kept in a hot water bath until the instant of spin-coating. Three drops of an agarose solution were spin-coated (2000-rpm, 10-s) onto a prepared substrate. The agarose drop volume varied with concentration; the more concentrated solutions were more viscuous and cooled more rapidly, consequently a greater total volume was delivered to the substrate surface. The average volume of melted agarose that was spin-coated onto the glass was 300-μL. Spin-coating was selected as the method of gel application because it resulted in a uniform covering and was easily controlled (rate and duration of spin) to produce an optimal surface. Agarose-covered plates were stored until use in a high

humidity chamber to prevent dessication and subsequent irreparable alteration of the agarose matrices. Agarose-covered plates were usually used within two hours of preparation.

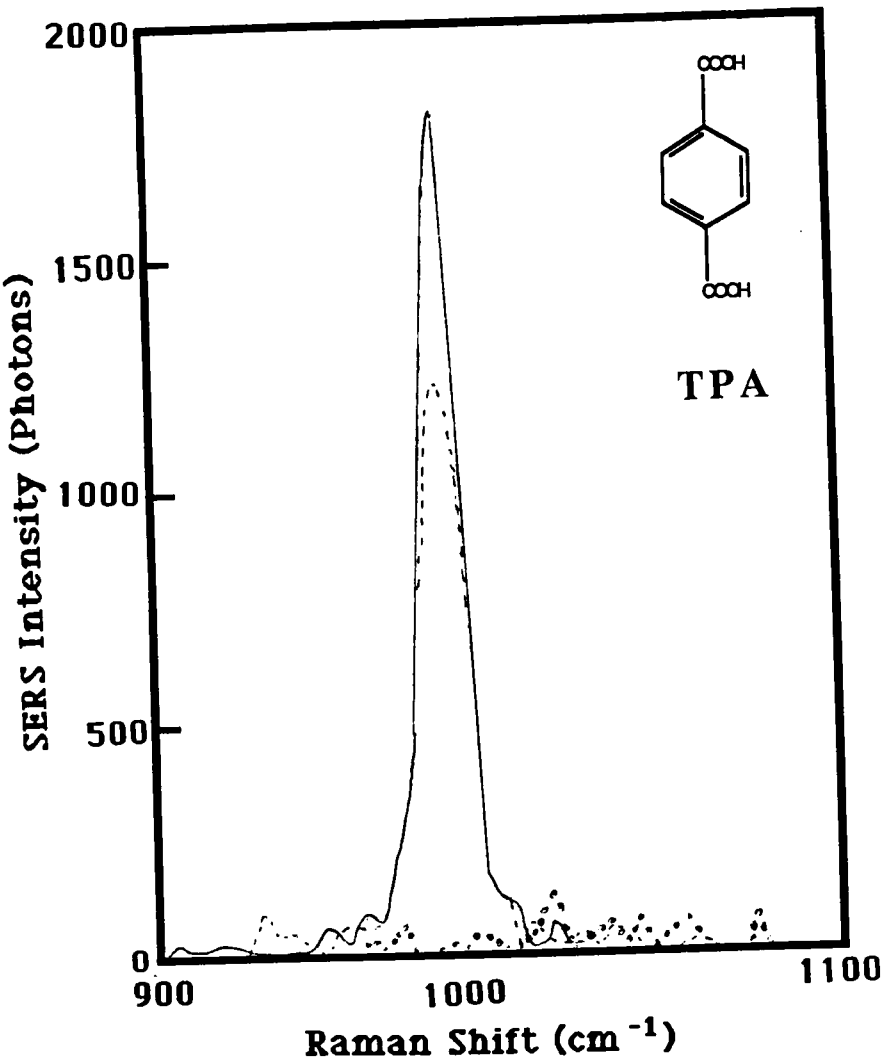
The SERS experiment consisted of scanning a series of negative and positive control plates and plates to which a 1.0-μL drop of 0.2-M terephthalic acid (TPA) in ethanol had been applied. Sample excitation was 100-mW at 647.1-nm by an optical fiber through the glass underside of each plate. Scattered light was collected with another fiber positioned directly above a dried analyte spot. Spectra of a major TPA SERS band (1590 $cm^{-1}$) were recorded for each agarose-coated substrate to determine what effect, if any, the agarose coating had on the resulting sample SERS spectra.

The TPA experiment was verified by repeating the SERS experiment with 3.0-μL of 0.1-M benzoic acid in ethanol. This experiment differed from the TPA study only in that the optical setup was direct excitation/collection instead of fiberoptic excitation/collection. Excitation power delivered to the sample of benzoic acid was still 100-mW at 647.1-nm. TPA and benzoic acid data were analyzed to discern the effect of the agarose coating on the SERS substrate and signal.

## *Results and Discussion*

### Terephthalic Acid

Typical SERS spectra of TPA are depicted in Figure IV-5. The 1590 $cm^{-1}$ scattering off TPA yields a well-resolved, sharp peak. Tabular (Table IV-1) and graphic (Figure IV-6) representations of the data illustrate a definite effect of agarose-coating on the effectiveness of this type of silvered microparticle-

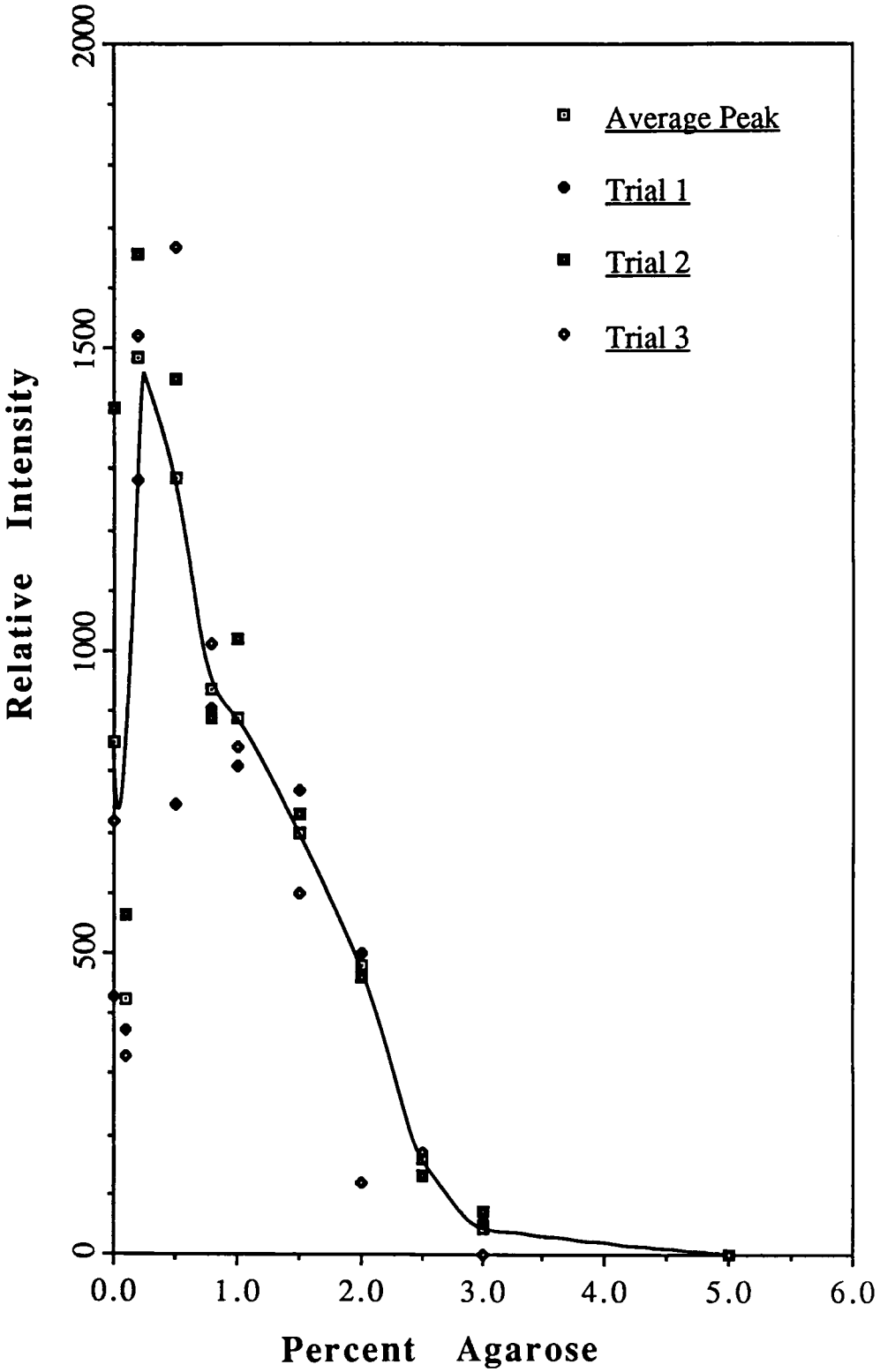



**<FIGURE IV-5> Sample SERS Spectra of Terephthalic Acid on Agarose-Coated Substrates** Spectra are of (__) 1.0-μL of a 0.2-M solution of TPA in ethanol on a 0.5% agarose-coated substrate, (- - -) SERS substrate spin-coated with water (0.0% agarose), and (. . .) 1.0-μL of a 0.2-M solution of TPA in ethanol on a 5.0% agarose-coated substrate.

**TABLE IV-1**
**Sample SERS Peak Intensities from 3.0-μL of 0.2-M Terephthalic Acid on Agarose-Coated Substrates**

| Substrate | Average SERS Peak (Photons) |
|---|---|
| Humid Blank | 0 |
| Wet Blank | 0 |
| 0.1% Agarose, No TPA | 0 |
| 0.2% Agarose, No TPA | 0 |
| 0.5% Agarose, No TPA | 0 |
| 0.8% Agarose, No TPA | 0 |
| 1.0% Agarose, No TPA | 0 |
| 1.5% Agarose, No TPA | 0 |
| 2.0% Agarose, No TPA | 0 |
| 2.5% Agarose, No TPA | 0 |
| 3.0% Agarose, No TPA | 0 |
| 5.0% Agarose, No TPA | 0 |
| Humid Substrate, 0.2 M TPA | 1000 |
| Wet Substrate, 0.2 M TPA | 720 |
| 0.1% Agarose, 0.2 M TPA | 330 |
| 0.2% Agarose, 0.2 M TPA | 1520 |
| 0.5% Agarose, 0.2 M TPA | 1665 |
| 0.8% Agarose, 0.2 M TPA | 1010 |
| 1.0% Agarose, 0.2 M TPA | 840 |
| 1.5% Agarose, 0.2 M TPA | 600 |
| 2.0% Agarose, 0.2 M TPA | 120 |
| 2.5% Agarose, 0.2 M TPA | 17 |
| 3.0% Agarose, 0.2 M TPA | 0 |
| 5.0% Agarose, 0.2 M TPA | 0 |

*Note* All SERS substrates consisted of 100-nm silver on spin-coated 5.0% alumina on glass. Trial sets used to form average intensity values took place on different days with freshly prepared substrates.




**<FIGURE IV-6> Interpolated graph of TPA on Agarose-Coated Substrates**

coated substrate. Neither negative control plates nor agarose-coated plates exhibited SERS bands upon laser excitation.

A 0.0% (water spin-coated onto an unaltered substrate) agarose coating was found to reduce the TPA SERS signal to about 75% of its original value. Phenomena have been observed which may account for the signal reduction: SERS substrates rinsed in water or ethanol have visibly disrupted surfaces. A similar abrasive effect may also take place when water is spin-coated onto a silvered, microparticle-roughened substrate. Since the effectiveness of the substrate is dependent upon its surface characteristics, it is likely that modification of the surface affected substrate performance.

A 0.1% agarose gel coating reduced scattering detected from the TPA sample to one-third of that detected from TPA adsorbed onto an untreated substrate. The 0.1% solution exhibited more charateristics of a viscuous liquid than of a gel matrix, so the "washing" effect may again have played a role in signal reduction. Also, agarose is known to bind well to metals. The agarose in the solution may have bonded to the evaporated silver, disturbing and abrading the silver layer during the spin-coating process.

While an extremely dilute agarose solution (0.1% agarose) appeared to diminish the usefulness of a substrate, intermediate concentrations were found to actually *enhance* the TPA SERS signal. This enhancement maximized at 0.5% agarose resulting in a 66.5% increase in detectable SERS scattering over that recorded for an untreated substrate. The spectral band returned to its usual intensity by 0.8% agarose gel composition, declining to no average detectable signal at 3.0% agarose.

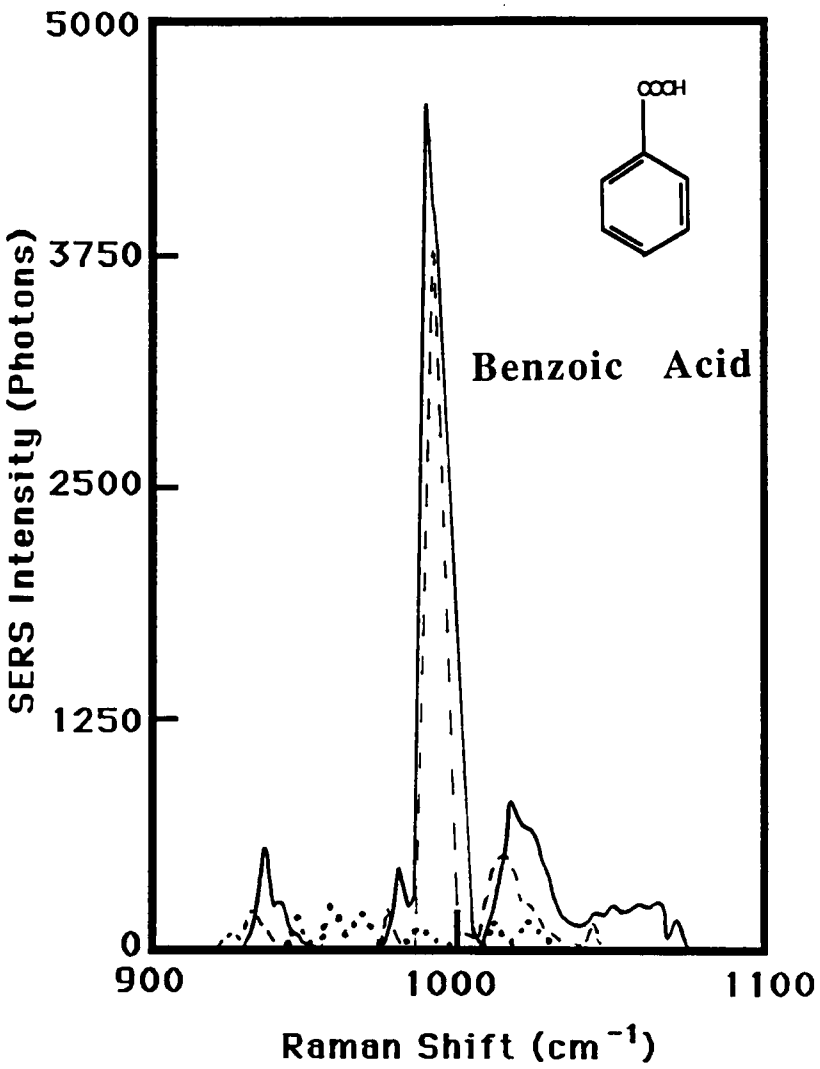
Benzoic Acid

The results of the TPA experiment were tested using the 990 $cm^{-1}$ peak of benzoic acid. All new agarose solutions and substrates were prepared. Sample benzoic acid spectra are given in Figure IV-7. Analysis of the data (Table IV-2 and Figure IV-8) supported the general trend seen with TPA. One difference between the results of the two experiments was with the effects of a 0.1% agarose coating. In the benzoic acid experiment, the SERS signal was virtually unaffected rather than diminished as in the TPA experiment. This may have been because the 0.1% solution in the benzoic acid experiment formed a gel matrix. The 0.1% agarose solution remained liquid in the TPA experiment. At 0.2% agarose, a 35.8% increase in benzoic acid SERS signal over that from an untreated substrate was observed. As in the TPA study, 3.0% agarose completely obstructed any SERS peaks.

Agarose coating thickness was too thin to measure with conventional microscopy and could not be measured with electron microscopy because the dessication of the gels under vacuum would have altered their structure. At low and intermediate coating concentrations, the substrates appeared slightly darker than usual, as though damp. The substrates at low agarose concentration coatings appeared dull; at intermediate concentrations they began to appear glazed. At high agarose concentration, the agarose coating was visible on the substrates as a glassy, wet covering.

## *Summary*

No demonstrable theory has been proposed for the signal gain observed with dilute agarose gel coating of the substrates. The gradual inhibition of




**<FIGURE IV-7> Sample SERS Spectra of Benzoic Acid on Agarose-Coated Substrates** Spectra are of 1.0-μL of a 0.1-M solution of benzoic acid in ethanol on a (__) 0.2% agarose-coated substrate, (- - -) 0.8% agarose-coated substrate, and (. . .) 3.0% agarose-coated substrate.

**TABLE IV-2**
**Sample SERS Peak Intensities from 3.0-μL of 0.1-M Benzoic Acid on Agarose-Coated Substrates**

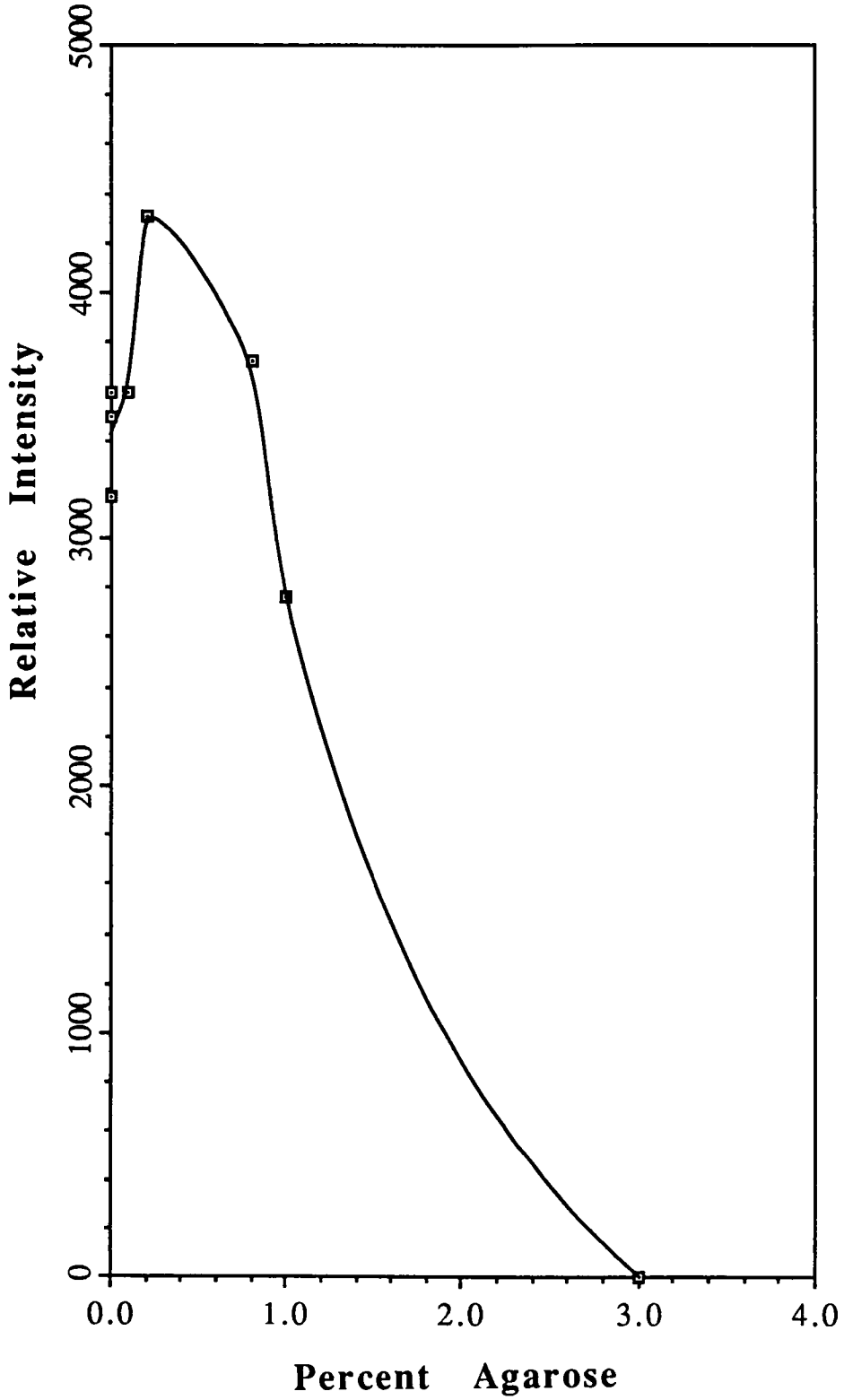
| Substrate | Peak Intensity (Photons) | Average Peak (Photons) | Plotted Peak Value |
|---|---|---|---|
| Trial Set 1 | | | |
| Dry Blank | 1206.15 | | |
| Dry Blank | 2722.30 | | |
| Dry Blank | 5339.00 | 3089.15 | |
| Trial Set 2 | | | |
| Dry Blank | 3798.45 | | |
| Dry Blank | 3022.30 | | |
| Dry Blank | 2950.95 | 3257.23 | 3173.2 |
| Trial Set 1 | | | |
| Humid Blank | 4209.70 | | |
| Humid Blank | 3262.20 | | |
| Humid Blank | 3022.30 | 3498.70 | 3498.7 |
| Trial Set 1 | | | |
| Wet Blank | 2567.30 | | |
| Wet Blank | 2937.20 | | |
| Wet Blank | 5264.60 | 3589.70 | 3589.7 |
| Trial Set 1 | | | |
| 0.1% Agarose | 2777.30 | | |
| 0.1% Agarose | 4442.20 | | |
| 0.1% Agarose | 4309.60 | 3843.60 | |
| Trial Set 2 | | | |
| 0.1% Agarose | 3452.20 | | |
| 0.1% Agarose | 3088.45 | | |
| 0.1% Agarose | 3485.95 | 3342.20 | 3592.6 |
| Trial Set 1 | | | |
| 0.2% Agarose | 2934.80 | | |
| 0.2% Agarose | 4214.60 | | |
| 0.2% Agarose | 4382.20 | 3843.87 | |
| Trial Set 2 | | | |
| 0.2% Agarose | 5772.00 | | |
| 0.2% Agarose | 4492.10 | | |
| 0.2% Agarose | 4057.20 | 4773.77 | 4308.8 |
| Trial Set 1 | | | |
| 0.8% Agarose | 3715.95 | | |
| 0.8% Agarose | 2903.55 | | |
| 0.8% Agarose | 3859.70 | 3493.07 | |

**TABLE IV-2 (cont.)**
**Sample SERS Peak Intensities from 3.0-μL of 0.1-M Benzoic Acid on Agarose-Coated Substrates**

| Substrate | Peak Intensity (Photons) | Average Peak (Photons) | Plotted Peak Value |
|---|---|---|---|
| Trial Set 2 | | | |
| 0.8% Agarose | 3959.70 | | |
| 0.8% Agarose | 4074.70 | | |
| 0.8% Agarose | 3844.70 | 3959.70 | 3726.4 |
| Trial Set 1 | | | |
| 1.0% Agarose | 2712.30 | | |
| 1.0% Agarose | 2787.30 | | |
| 1.0% Agarose | 2789.80 | 2763.13 | |
| Trial Set 2 | | | |
| 1.0% Agarose | 0 | | |
| 1.0% Agarose | 214.90 | Left Out | |
| 1.0% Agarose | 0 | Of Average[1] | 2763.1 |
| Trial Set 1 | | | |
| 3.0% Agarose | 0 | | |
| 3.0% Agarose | 0 | | |
| 3.0% Agarose | 0 | 0 | |
| Trial Set 2 | | | |
| 3.0% Agarose | 0 | | |
| 3.0% Agarose | 0 | 0 | 0.0 |

*Note* All SERS substrates consisted of 100-nm silver on spin-coated 5.0% alumina on glass. Trial sets 1 and 2 took place on different days with freshly prepared substrates. Each value within a trial set was obtained from a separate substrate.

[1] Data was inconsistent with that of other experiments (not shown) so this trial was omitted from the plot of the data.




<FIGURE IV-8> Interpolated Graph of Benzoic Acid on Agarose-Coated SERS Substrates

detected sample scattering as the agarose concentration increased may have been due to a lack of free electrons for the surface plasmon. In other words, at low and intermediate agarose concentrations the large pores in the matrix may have permitted some electron movement. As the agarose percentage increased, the average pore size in the matrix decreased and the analyte may have been unable to interact with the electrons from the silver. Also, as agarose concentration was increased more agarose molecules were available to bind the silver directly. Again, fewer electrons would have been able to resonate and to interact with the sample molecules. High agarose concentrations formed a visible barrier on the substrate. One factor in reduced SERS signal may have been increased scattering of light as it attempted to penetrate the thick agarose coatings.

One of the great advantages the silvered, alumina-coated substrates had over some other types of substrates (e.g. silver islands) was their long shelf life (approximately one year when stored in a sealed container). This advantage was compromised by the agarose coating. Agarose-treated substrates were only effective for perhaps three days before a visual yellowing appeared. Substrates which had turned yellow were incapable of yielding any SERS signal except background peaks from the optical fibers. (These background peaks could be detected when no substrate was in place in the optical setup.) The yellow color probably indicated oxidation of the silver or bacterial growth on the substrates. High humidity was requisite for sustaining hydrated agarose matrices, but in general was not conducive to long substrate shelf life.

In conclusion, despite the signal inhibition at very low and very high agarose concentrations and the short life span of substrates treated in this

fashion, this coating holds much promise for future SERS studies. The most remarkable aspect of the results was the detection of a signal enhancement observed using intermediate agarose percentages. While interesting in itself, a substantial increase in SERS substrate effectiveness could prove important in expanding the limit of detection of samples using the SERS technique. Only limited extrapolations may be made for the effect of the agarose coating on the SERS spectra of molecules chemically dissimilar to TPA and benzoic acid. Analysis of a wide range of samples using agarose and other polymer-coated substrates will determine the extent of the enhancement coatings can have on SERS and any dependence the enhancement may have on the type of analyte used. Since agarose is composed of non-toxic galactose residues, it could be used as an effective coating in *in vivo* procedures attempting to detect and analyze low levels of SERS-responsive chemicals. It remains to be determined whether the effectiveness of an agarose coating would be adversely affected by interaction with proteins and other substances in a biological matrix.

# CHAPTER 3

# PLASMA AND SERUM EXPERIMENTS

## *Introduction*

One step in assessing the effectiveness of SERS for the study of biological samples was to determine if chemicals previously found to possess excellent SERS limits of detection could be detected in body fluids. The first fluids tested were serum and plasma. Samples were not initially doped into whole blood because SERS scattering was purportedly easier to detect in a partially purified matrix.

## *Materials and Methods*

### Instrumentation

Both "direct" and "fiberoptic" optical configurations were tested in this study. Unless otherwise noted, spectra were taken with a fiberoptic SERS setup as shown in Figure I-4 (p. 18).

### Chemicals

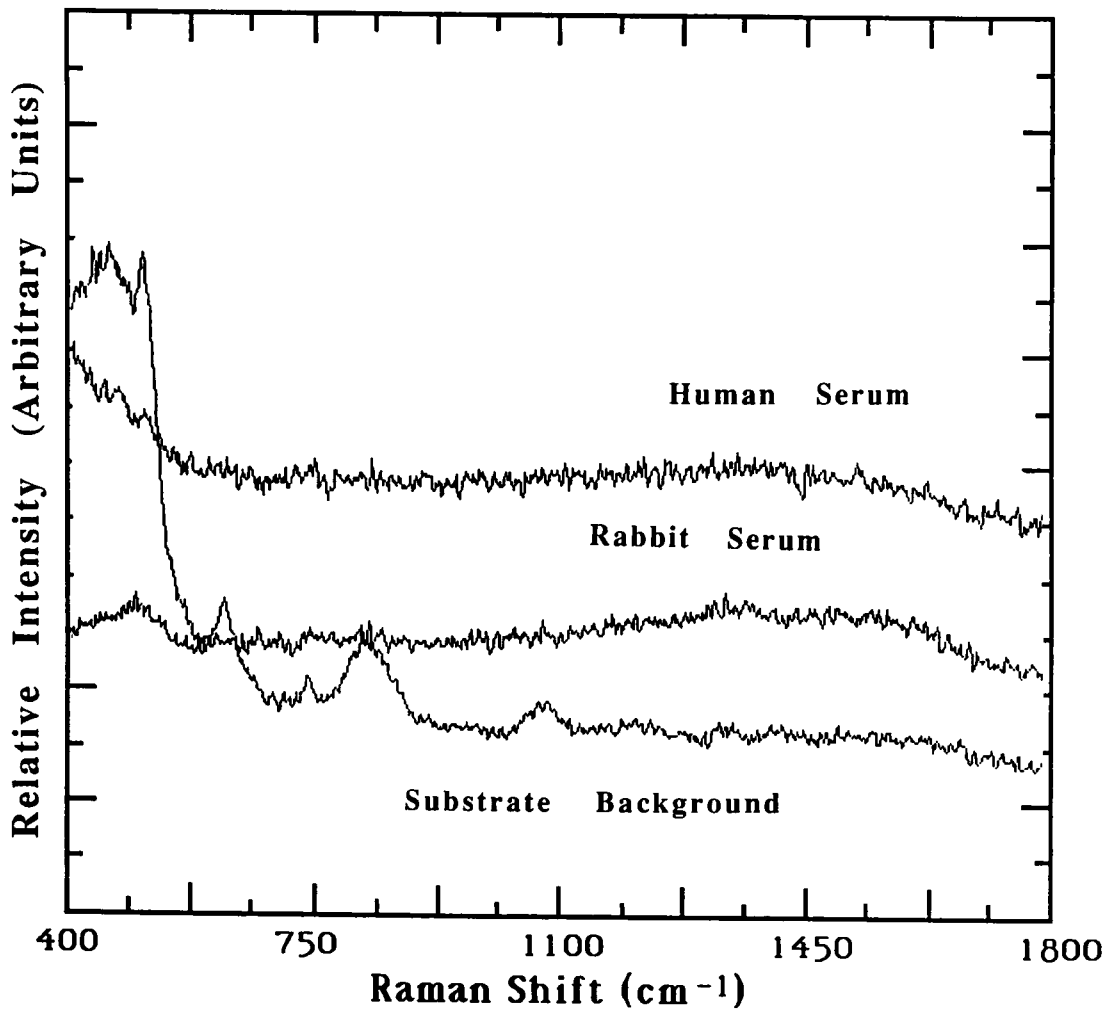
Plasma and sera were from the Sigma Chemical Company (St. Louis, MO) and were stored as 10-mL aliquots at -20°C until use. While in other studies samples were preferentially dissolved and diluted in ethanol, samples involving plasma or serum were dissolved and diluted in water or in PBS to prevent precipitation of plasma/serum proteins and to avoid alteration of fluid pH levels.

Procedures

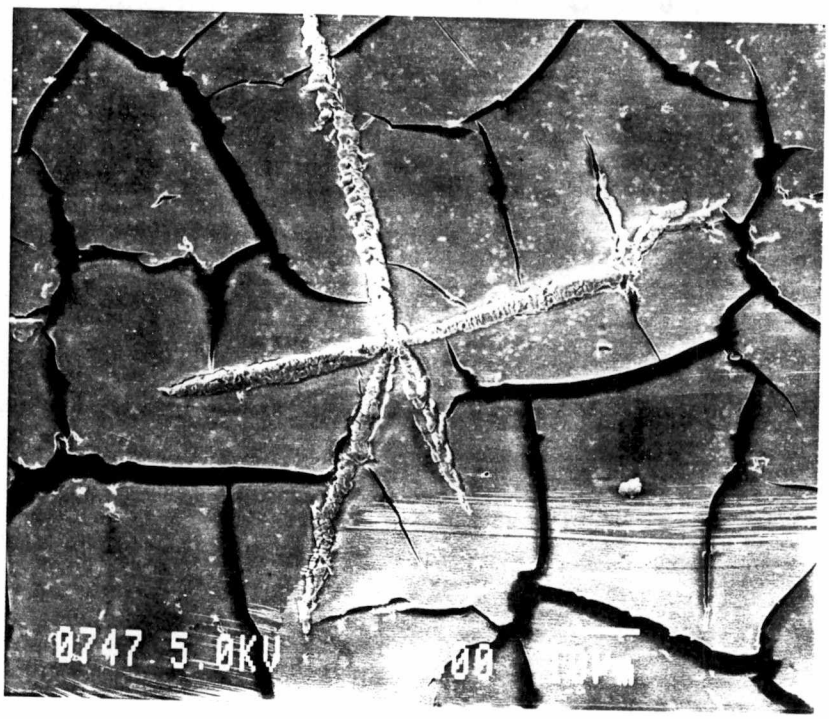
SERS substrates were comprised of glass spin-coated with a sonicated 4.0% or 5.0% aqueous suspension of agglomerate-free alumina (0.1-μm diameter). 100.0-nm of silver was then thermally evaporated onto the roughened glass under a pressure of $1x10^{-6}$-torr at a rate of 0.1-nm/s to 0.2-nm/s. Substrates were stored in a dessicated, sealed container until use.

*Results and Discussion*

Neither human or mouse plasma nor human male type AB, mouse, or porcine serum exhibited any SERS bands under optimized experimental conditions. The application of plasma or serum onto a SERS substrate was found to mask even the background Raman bands. This effect may be clearly seen in the human and rabbit sera spectra of Figure IV-9. Scanning electron micrographs (e.g. human male serum on an alumina-type substrate, Figure IV-10) revealed that serum glazed the substrate surface. (This phenomenon could not be studied in detail with the SEM as the serum spot was highly unstable in the electron beam of the microscope -- the crystal prominent in Figure IV-10 burned away within a matter of seconds.) No SERS spectra were obtained from porcine serum because a 1.0-μL drop applied to a substrate irreversibly damaged the substrate surface, visibly pulling away the silver and microparticles from the glass support. All samples of molecules (B[a]P and ethenocytosine) in serum or plasma examined with SERS appeared to the naked eye as gelatinous, glazed coatings on the substrate surface. Because the coating of the substrate surface was so marked, it is likely that much of the SERS scattering from the molecules was trapped or absorbed within the glazed




**<FIGURE IV-9> SERS Spectra of Human and Rabbit Sera and the Substrate Background** Spectra are of (From Left, Top to Bottom)) substrate background, 1.0-μL of human serum, and 1.0-μL of rabbit serum. Laser excitation was 90-mW at 647.1-nm. Spectra were printed to scale relative to each other.



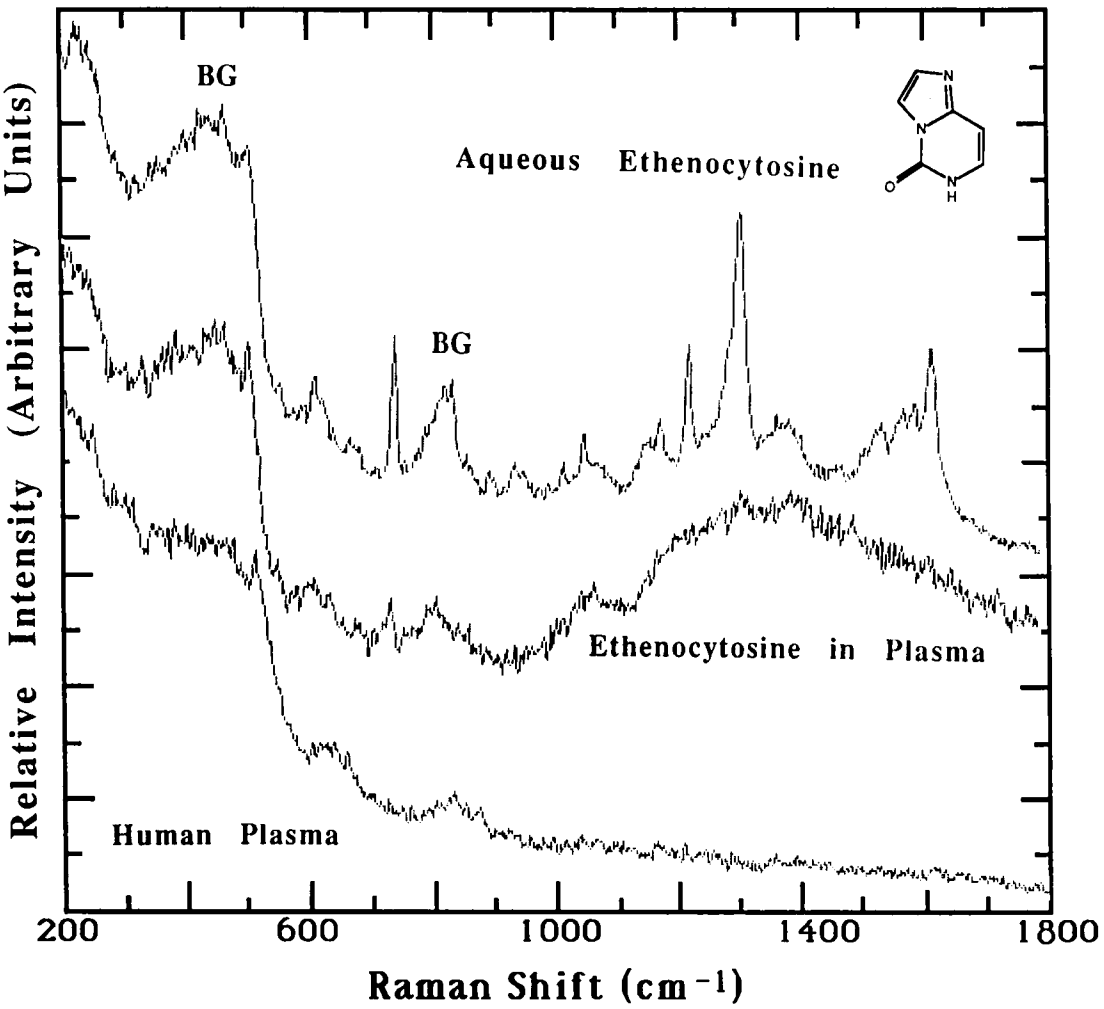

**<FIGURE IV-10> Scanning Electron Micrograph of Human Male Serum on a SERS Substrate** Substrate composition was 100-nm silver evaporated onto glass roughened with a 4.0% alumina suspension. Note that substrate surface is not visible beneath the serum glaze.

gel or was so scattered that it was not accumulated by the collection fiber. The glutinous semblence of the sample spots also suggests a sort of "damping" of molecular vibrations through interactions (including binding) of the sample molecules to the serum or plasma proteins.

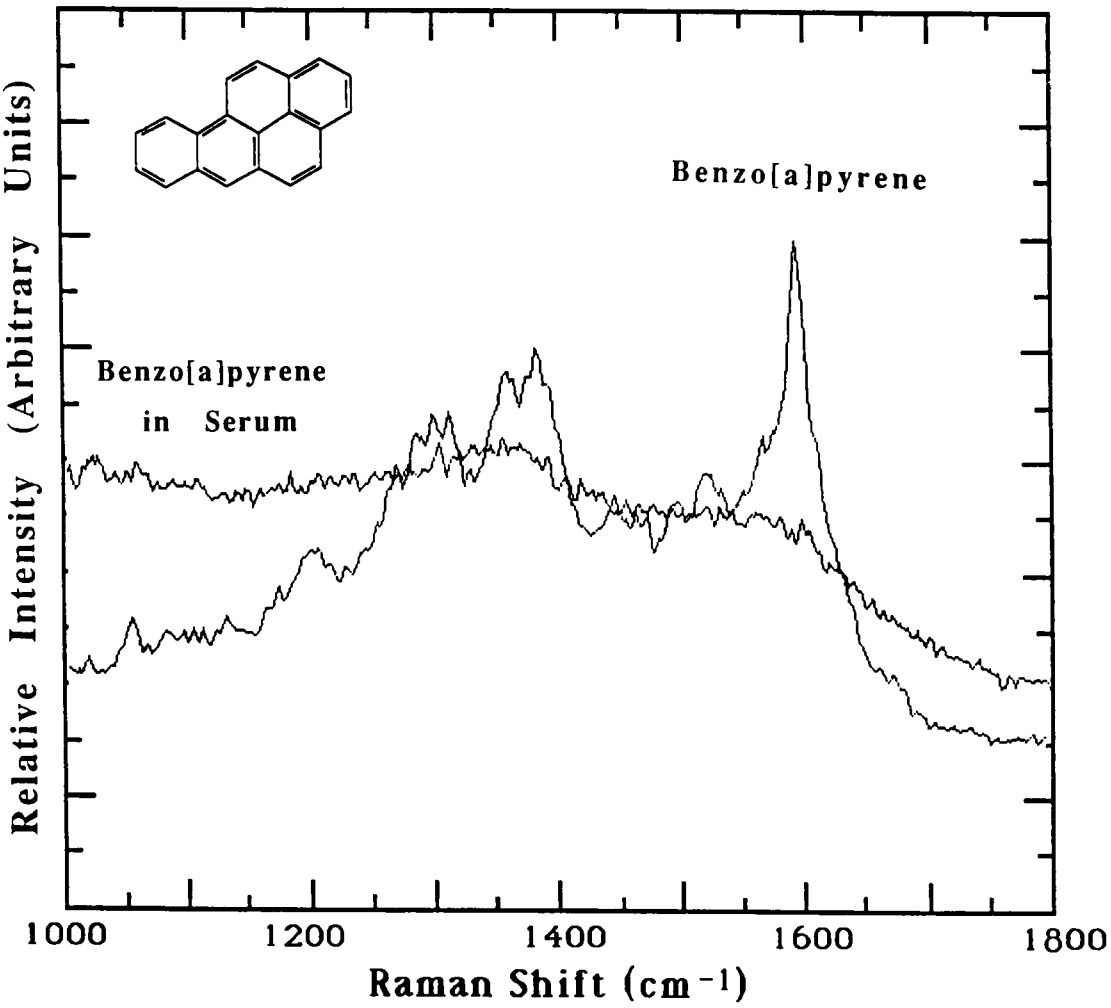
When plasma was doped with a SERS-active chemical normally detectable even at low concentrations, (e.g. 3,$N^4$-ethenocytosine, Figure IV-11) the general shape of the expected SERS spectrum could be discerned, but no detailed vibrational structure was evident. In this experiment, chemical concentrations were well within previously defined parameters for SERS detection. The conclusion reached from analysis of the spectra was that the sample molecules probably interacted with and bound to proteins or other constituents of the plasma, even in the diluted samples.

Similar behavior was found when plasma was replaced with serum. These results are demonstrated by the spectra shown in Figure IV-12 (benzo[a]pyrene). Figure IV-13 directly compares SERS spectra of 3,$N^4$-ethenocytosine and benzo[a]pyrene with their "solvent" background. In Figure IV-13 very broad bands which may correspond to sample SERS peaks were seen in the 1300 $cm^{-1}$ to 1700 $cm^{-1}$ region for both ethenocytosine and B[a]P. These bands were too indistinct to be used to discriminate between ethenocytosine and B[a]P. The choice of solvents had no noticeable effect on the results of these experiments (ethenocytosine was in water, benzo[a]pyrene was in ethanol). The spectra from ethenocytosine and benzo[a]pyrene in both plasma and sera were too undefined to provide any structural information about these molecules.

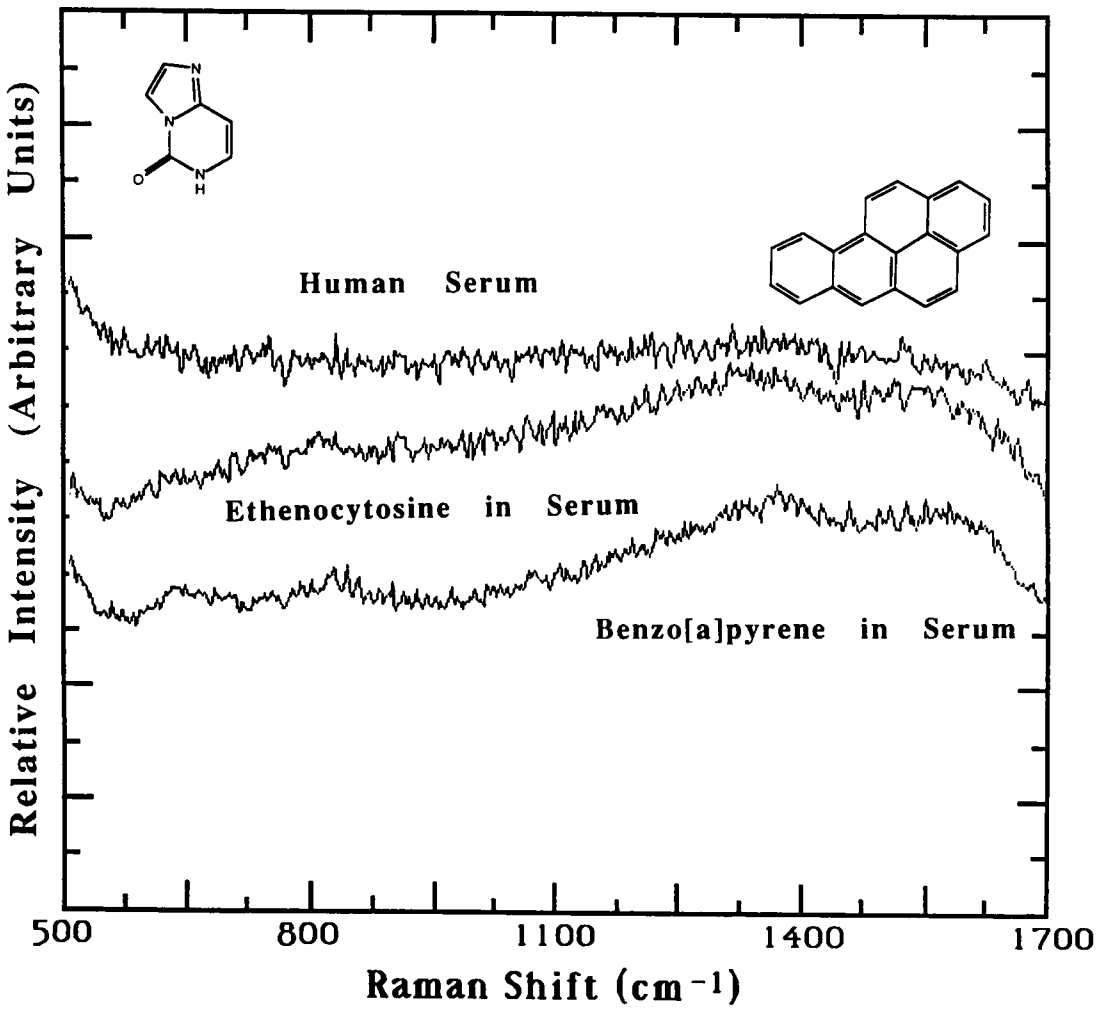
No intense sample SERS bands were seen even when water was the predominant component of an analyte solution. This point is illustrated by




<FIGURE IV-11> SERS Spectra of 3,$N^4$-Ethenocytosine in Human Plasma Spectra are of (Top) 1.0-μL of a 1.93-mg/mL solution of 3,$N^4$-ethenocytosine in water, (Center) 1.0-μL of a 1:1 (v/v) solution of a 1.93-mg/mL solution of ethenocytosine in water:human plasma, (Bottom) 1.0-μL of human plasma. Laser excitation was 70-mW at 647.1-nm.




**<FIGURE IV-12> SERS Spectra of Benzo[a]pyrene in Human Serum** Spectra are of 1.0-μL of a 1x10$^{-4}$-M solution of B[a]P in ethanol and 1.0-μL of a 1:1 (v/v) solution of 1x10$^{-4}$-M B[a]P in EtOH:human serum. Laser excitation was 90-mW at 647.1-nm. Spectra were printed to scale relative to each other.

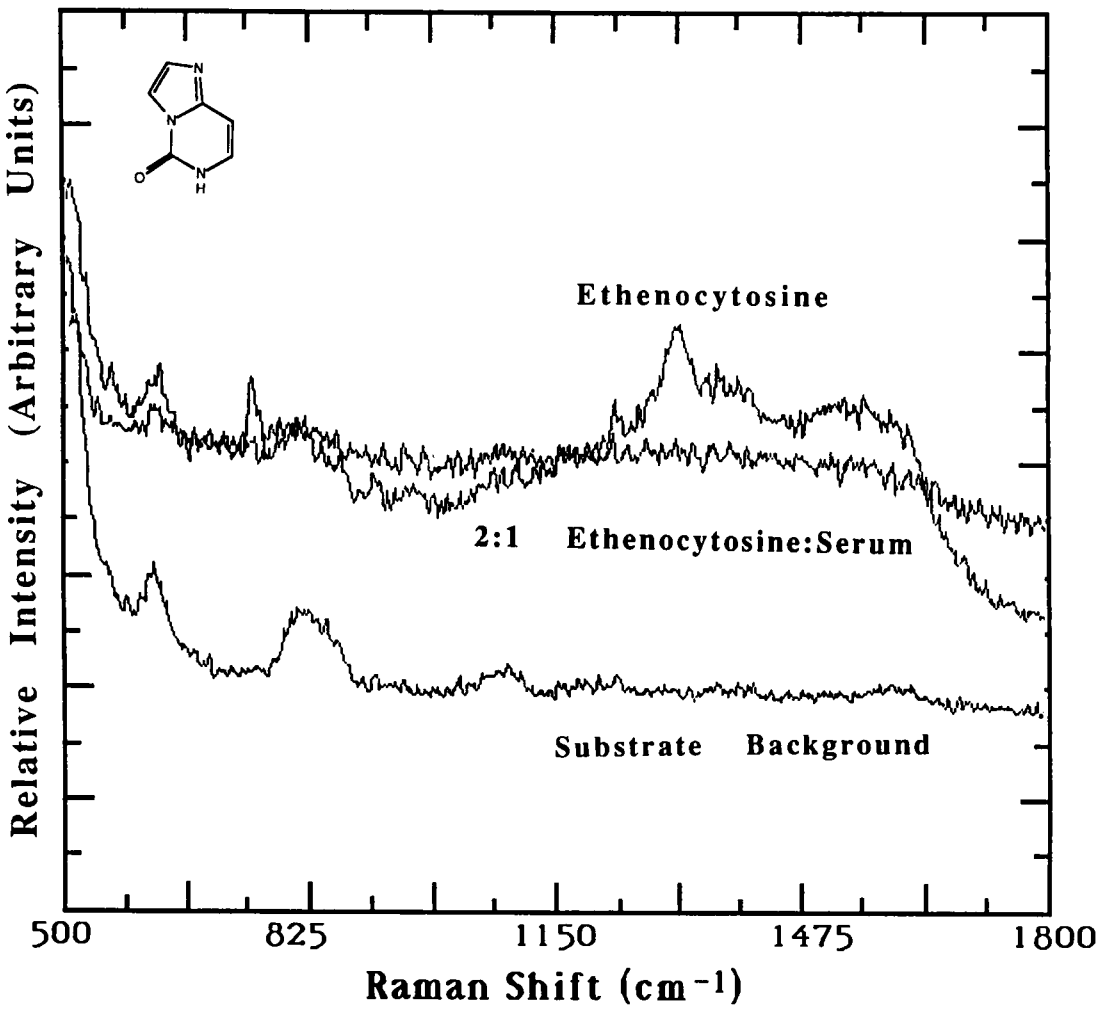



<FIGURE IV-13> SERS Spectra of 3,$N^4$-Ethenocytosine and Benzo[a]pyrene in Human Serum Spectra are of (Top) 1.0-µL of human serum, (Center) 1.0-µL of a 1:1 (v/v) solution of 1.93-mg/mL ethenocytosine in water:serum, and (Bottom) 1.0-µL of a 1:1 (v/v) solution of $1x10^{-4}$-M B[a]P in ethanol:serum. Laser excitation was 90-mW at 647.1-nm. Spectra were printed to scale relative to each other.

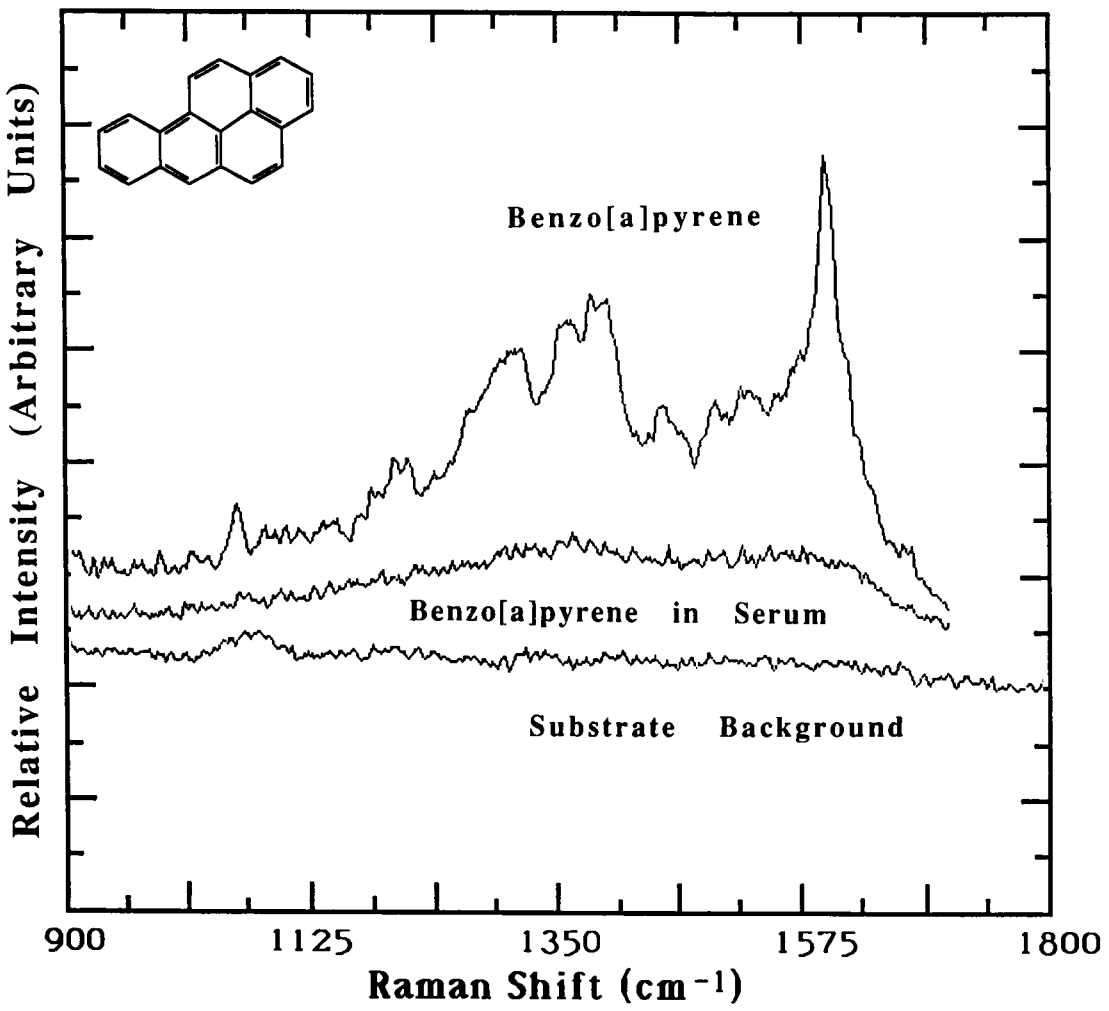
Figure IV-14 (ethenocytosine) and Figure IV-15 (B[a]P). No SERS bands could be discerned even when the ratio of analyte molecules and water to serum was very high (Figure IV-16, ethenocytosine was detected in vanishingly small amounts in a pure solution). A possible conclusion is that even a small quantity of plasma or serum proteins or other constituents was sufficient to bind the sample molecules or otherwise hinder the excitation of the vibrational levels in these molecules. Any close interaction between the B[a]P or ethenocytosine and a larger, complex molecule would lessen the degrees of vibrational freedom available to that molecule, hence masking the sample SERS spectrum that would be observed in a pure solution.

*Summary*

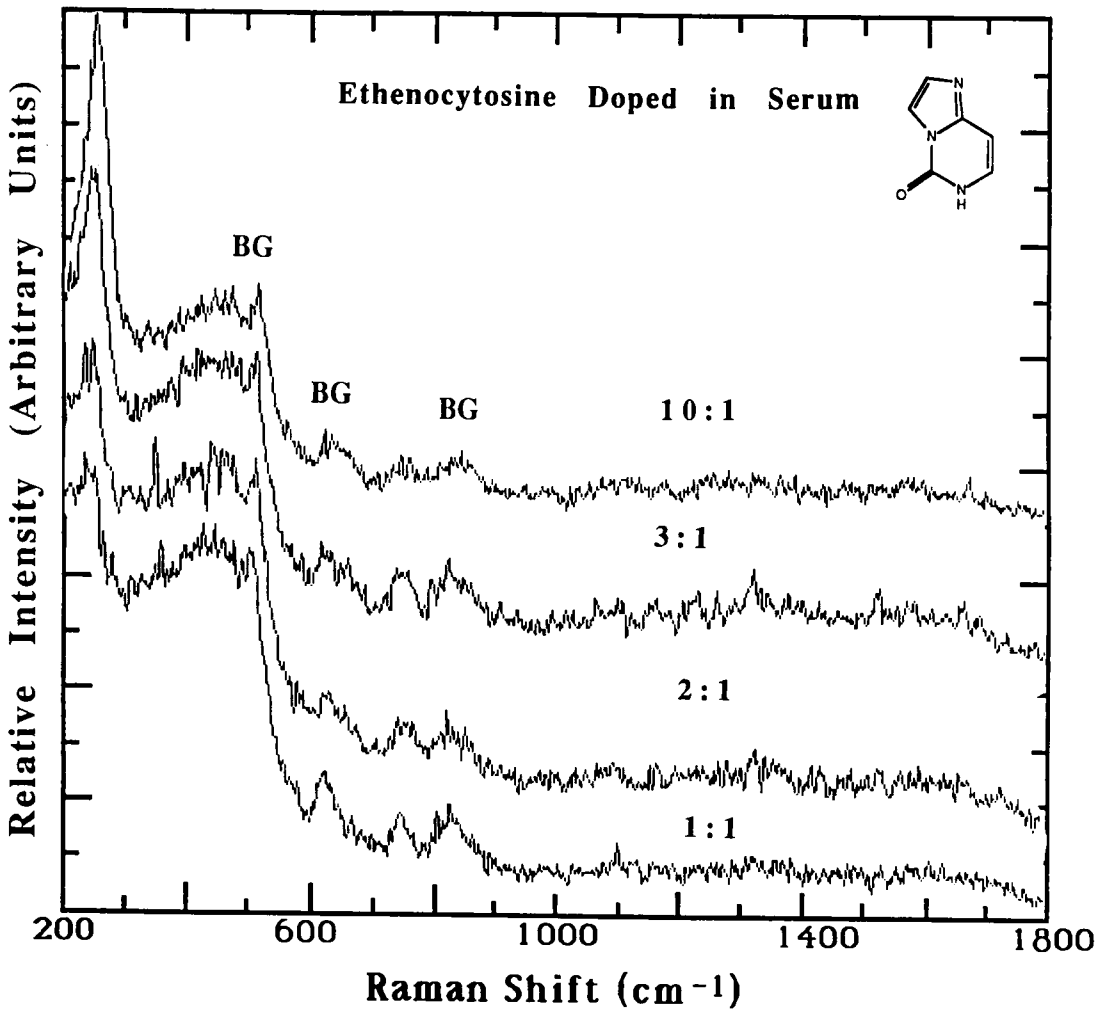
Before contemplating tests of the biosensor *in vivo*, it was deemed important to see how SERS performed in body fluids. Fluids tested were human plasma, mouse plasma, human male type AB serum, mouse serum, and porcine serum. At best, SERS spectra exhibited broad bands where chemical peaks were expected to occur. These broad bands could not be assigned to any specfic chemical group and supplied no useful vibrational information about the sample molecules (benzo[a]pyrene and 3,$N^4$-ethenocytosine). Even dilution of the body fluid with a concentrated SERS-active sample failed to alleviate the spectral resolution problem. SERS was found to be an unsatisfactory technique for detection of the molecules used in these experiments when doped into the aforementioned blood components.




**<FIGURE IV-14> SERS Spectra of Human Serum Diluted with Aqueous 3,N4-Ethenocytosine** Spectra are of (From Right, Top to Bottom) 1.0-µL of a 2:1 (v/v) solution of 1.93-mg/mL ethenocytosine in water: human serum, 1.0-µL of a 1.93-mg/mL solution of ethenocytosine in water, and the substrate background. Laser excitation was 90-mW at 647.1-nm. Spectra were printed to scale relative to each other.




<FIGURE IV-15> SERS Spectra of Human Serum Diluted with Benzo[a]pyrene in Ethanol Spectra are of (Top to Bottom) 1.0-μL of a 2:1 (v/v) solution of $1x10^{-4}$-M B[a]P in EtOH:human serum, 1.0-μL of a $1x10^{-4}$-M solution of B[a]P in EtOH, and the substrate background. Laser excitation was 90-mW at 647.1-m. Spectra were printed to scale relative to each other.




**<FIGURE IV-16> SERS Spectra of Human Serum Highly Diluted with Aqueous 3,N⁴-Ethenocytosine** Spectra are of 1.0-μL of a (Top to Bottom) 10:1, 3:1, 2:1, and 1:1 (v/v) solution of 1.93-mg/mL 3,N⁴-ethenocytosine in water:human serum. Laser excitation was 50-mW at 647.1-nm.

# CHAPTER 4

# URINE FROM HUMANS EXPOSED TO AFLATOXIN

## *Introduction*

In addition to testing SERS samples in plasma and serum, a study was also performed to determine if SERS-active chemicals could be detected in human urine. This experiment differed from the plasma/serum study in that the urine was not doped with a SERS-active chemical in the laboratory, but was obtained from humans that had been exposed to aflatoxin. The aflatoxin content of the samples was evaluated with ELISA (Dr. Regina Santella's laboratory, Columbia University, NY).

## *Materials and Methods*

### Instrumentation

A fiberoptic setup as in Figure I-4 (p. 18) was used. Laser excitation was 647.1-nm on silvered alumina-roughened (4.0%) substrates.

### Chemicals

Samples for this study were generously supplied by Dr. Regina Santella from the Division of Environmental Sciences at Columbia University in New York. Samples were extracts of urine from humans (Taiwan) exposed to aflatoxin. Urine was passed through a SepPak column (Waters Corporation, Milford, MA). The retained material was eluted with methanol and dissolved in phosphate-buffered saline. All sample processing occurred prior to shipment

to ORNL; specific information on sample preparation is unknown. A list of all samples examined in this study is given in Table IV-3.

Procedures

All substrates were prepared at once, so that spectra would be directly comparable with each other. Samples were 1.0-μL spots, which spread evenly across the substrate surface (a characteristic of PBS as a solvent) and were allowed to dry before exposure to the laser beam.

During the SERS examination only numerical assignments were given to the samples. At the behest of Dr. Santella, no knowledge of sample composition was initially available to prevent any bias in the experimental results. Since the location of spectral bands could not be predicted, prior to sample analysis the optical setup was aligned on a dilute aqueous solution of 3,$N^4$-ethenocytosine. Samples were later identified as shown in Table IV-3. Spectral bands for aflatoxin should correspond to those found in Section III, since no change was made between optical setup, excitation wavelength, or substrate between the initial detection studies of aflatoxin and the experiment with human urine extracts.

## *Results and Discussion*

Each sample in this study was examined with the SERS technique a minimum of three times on different days with new preparations of alumina-type SERS substrates. As with most studies presented in this dissertation, the quantity of data produced was enormous and only representative spectra will be displayed. So that spectra may be directly compared as much as possible,

TABLE IV-3
Identification of Human Urine Extracts

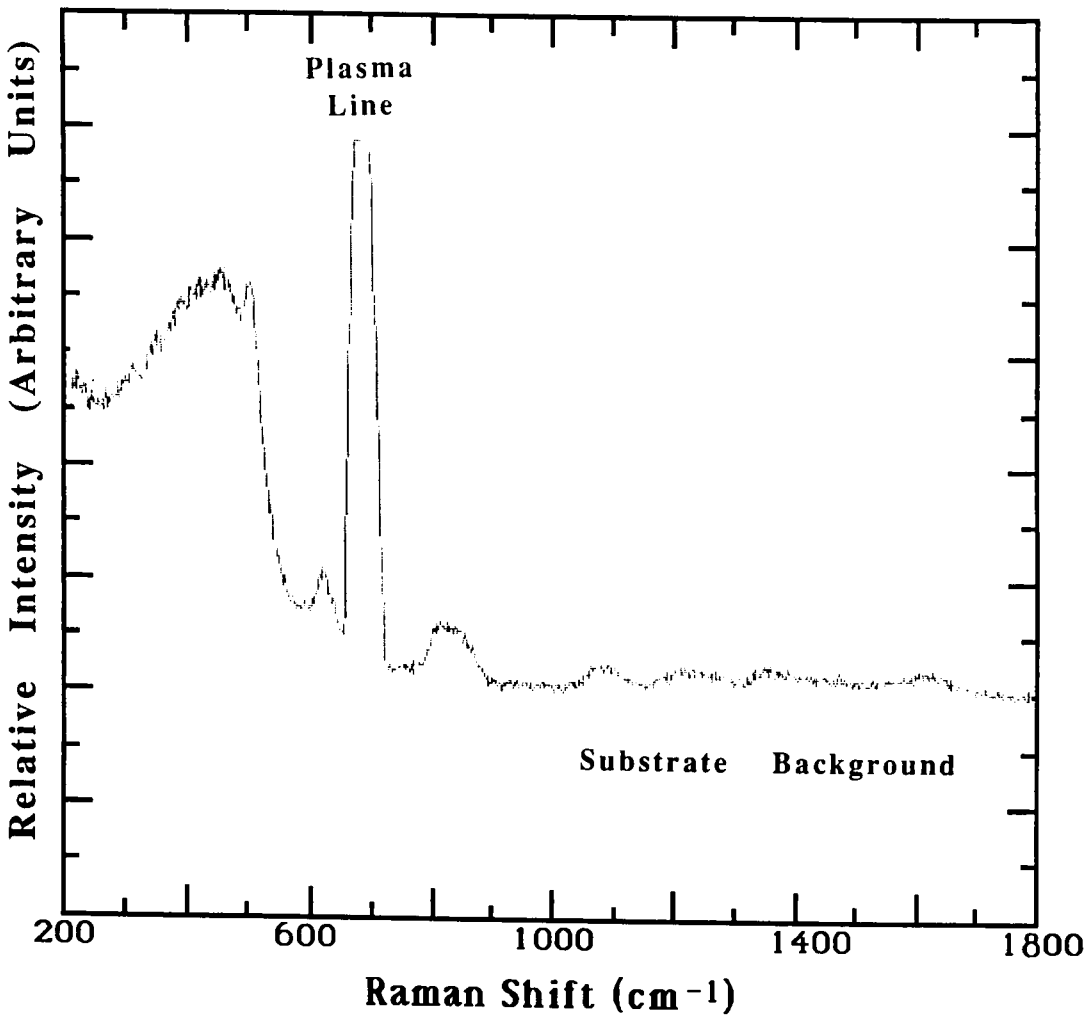
| Sample Number | | Contents (fm of Aflatoxin/μL) | |
|---|---|---|---|
| 1. | - 0028071 - 102810 | 0.316 | Human Urine Extract |
| 2. | - 0041066 - 102820 | 0.404 | Human Urine Extract |
| 3. | - 0074071 - 300520 | 0.085 | Human Urine Extract |
| 4. | - 0057066 - 300710 | 0.049 | Human Urine Extract |
| 5. | PBS | 0 | Negative Control |
| 6. | 250 fm of aflatoxin/50μL | 5.0 | Positive Control |
| 7. | - 0063071 - 301310 | 0.325 | Human Urine Extract |
| 8. | - 0076071 - 301020 | 0.732 | Human Urine Extract |
| 9. | 100 fm of aflatoxin/50 μL | 2.0 | Positive Control |
| 10. | PBS | 0 | Negative Control |
| 11. | - 0070066 - 302010 | 0.224 | Human Urine Extract |
| 12. | - 0080071 - 302230 | 0.041 | Human Urine Extract |
| 13. | - 0073066 - 302310 | 0.056 | Human Urine Extract |
| 14. | - 0073071 - 302310 | 0.056 | Human Urine Extract |

Note: All samples were then diluted with 1.0-mL PBS.

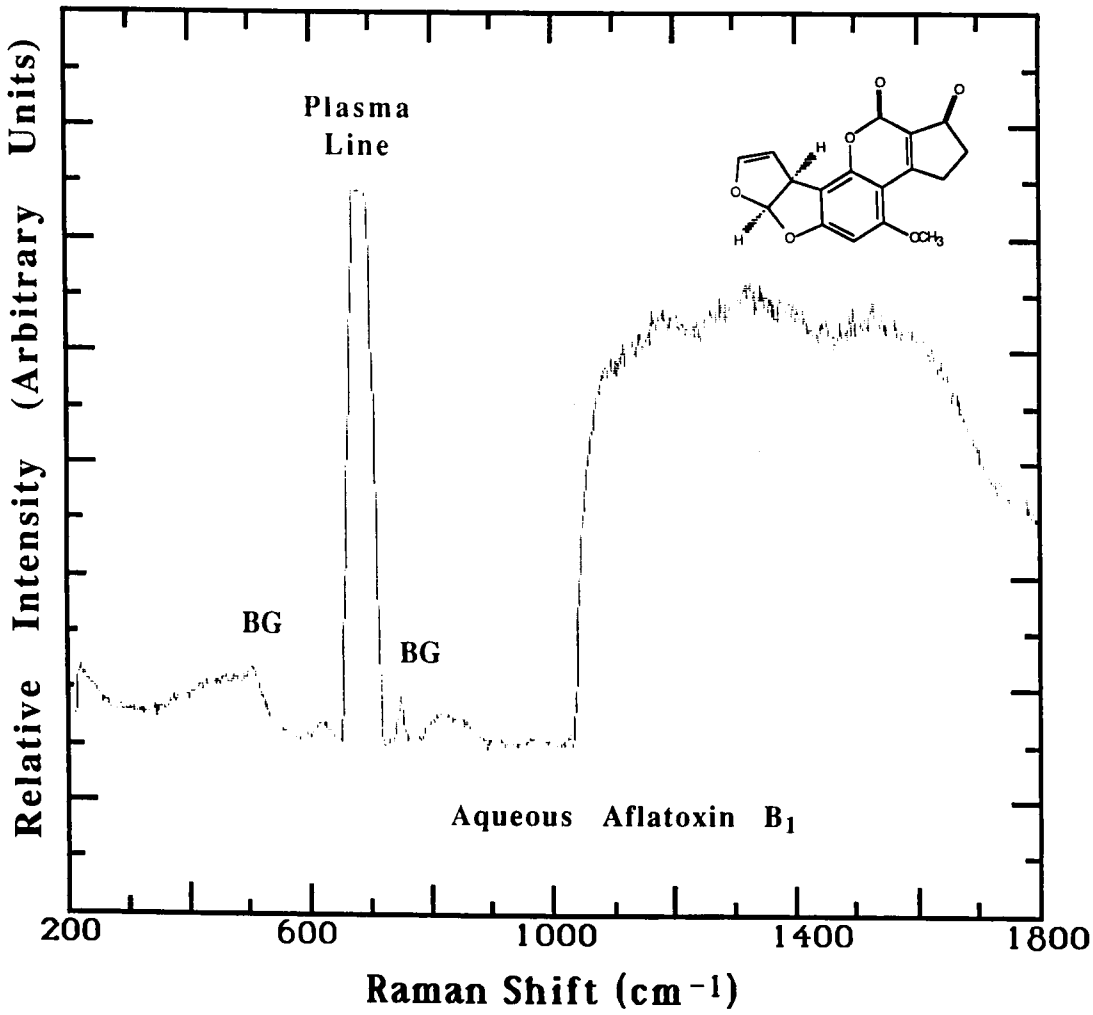
most data shown here were taken on the same set of substrates on the same day.

A typical substrate background spectrum for this study is shown in Figure IV-17. A SERS spectrum of aflatoxin $B_1$ (aqueous solution) taken under current experimental conditions is given in Figure IV-18. Note that this spectrum exhibits bands with different relative intensities than were seen for the spectra of aflatoxin $B_1$ in Section III. One explanation for this variation is that the spectra for Section III and Section IV were taken at different times and with alumina produced in separate batches by the manufacturer. (Baikowski makes the agglomerate-free aluminas for polishing optical fibers; it was adapted for SERS. Different preparations of alumina conforming to their specifications for fiber polishing have variations that are noticeable when the alumina is used for SERS.) Another explanation for the differences observed between this aflatoxin spectrum and the spectra of Section III is that the solvents used for sample preparation of the two spectral sets were different. Aflatoxin is only slightly soluble in water and the aflatoxin solution used to produce Figure IV-18 still contained some aflatoxin $B_1$ crystals. The spectrum of aflatoxin $B_1$ in Figure IV-18 resembles the spectra of aflatoxins $B_2$, $G_1$, and $G_2$ in Section III (all concentrated enough to have been incompletely dissolved, although prepared in ethanol). The solvent used to produce the spectra of aflatoxin $B_1$ in Section III was absolute ethanol and the aflatoxin was completely dissolved. Comparison of all available spectra indicates it is the insolubility of components of a sample that result in broad, ill-defined SERS bands.

A SERS spectrum of a negative control (sample #5, phosphate-buffered




**<FIGURE IV-17> SERS Spectrum of the Substrate Background for the Urine Extract Study** Laser excitation was 50-mW at 647.1-nm. This is the exact substrate used to produce many spectra in this study.

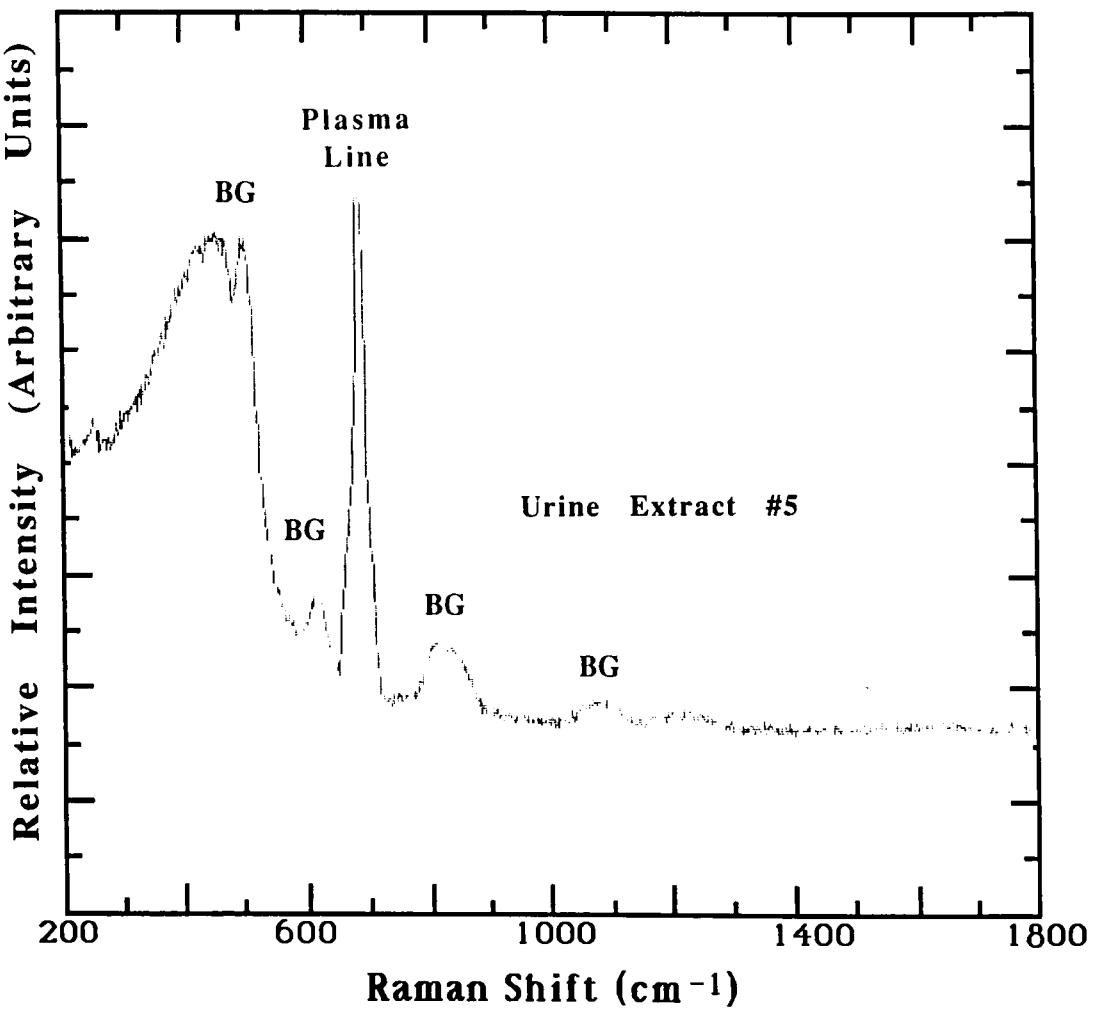



**<FIGURE IV-18> SERS Spectrum of Aflatoxin $B_1$ in Water** Sample was 1.0-μL of a 0.16-mg/mL solution of aflatoxin in water. Some crystals remained. Laser excitation was 50-mW at 647.1-nm.

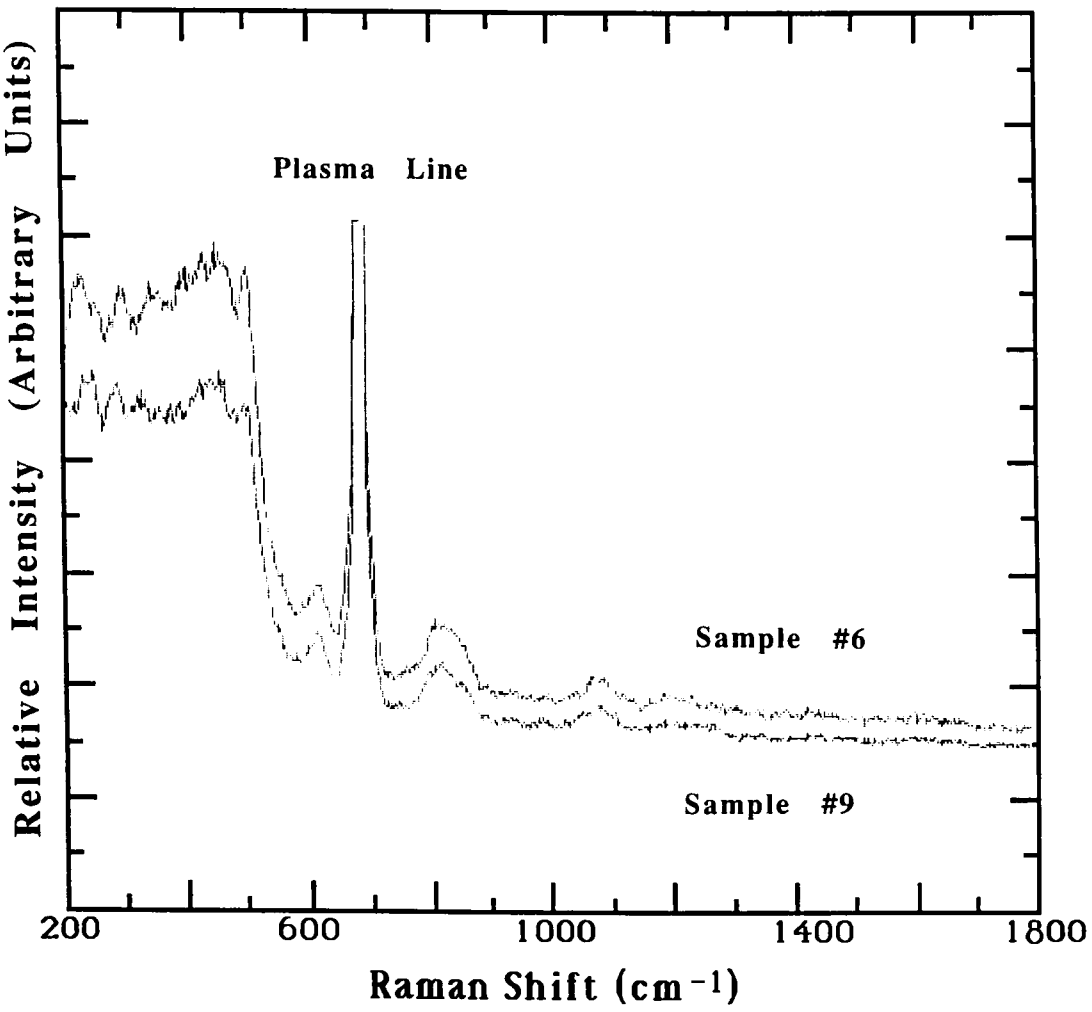
saline) is shown in Figure IV-19. As has been shown to be typical of PBS, this spectrum contained somewhat less detail in the 1100 $cm^{-1}$ to 1800 $cm^{-1}$ Raman shifted region than did the background spectrum of Figure IV-17. Spectra of the two positive controls (samples #6 and #9) are shown in Figure IV-20. Spectra of the more concentrated positive control (sample #6, 5.0-fm aflatoxin/μL compared with the substrate background) are given in Figure IV-21. The spectra for the negative and positive controls were reproducible from day-to-day. The positive control (sample #6) did exhibit more spectral structure than did the solvent blank (PBS), but in the author's opinion the spectral features of the positive control were not clear enough for definitive assignment to aflatoxin. A direct spectral comparison for aflatoxin $B_1$, a positive control (sample #9), and a negative control (sample #10) is shown for the reader's benefit in Figure IV-22.

SERS spectra for sample #2 (0.404-fm aflatoxin/μL sample) are shown in Figure IV-23 and Figure IV-24. Note that the two spectra of the same sample are markedly different. This inability to reproduce the spectral signature was true for all urine extracts examined in this study. This effect is unfortunately fairly common when SERS is used to examine extremely complex samples. Further purification of the samples might help to improve reproducibility.

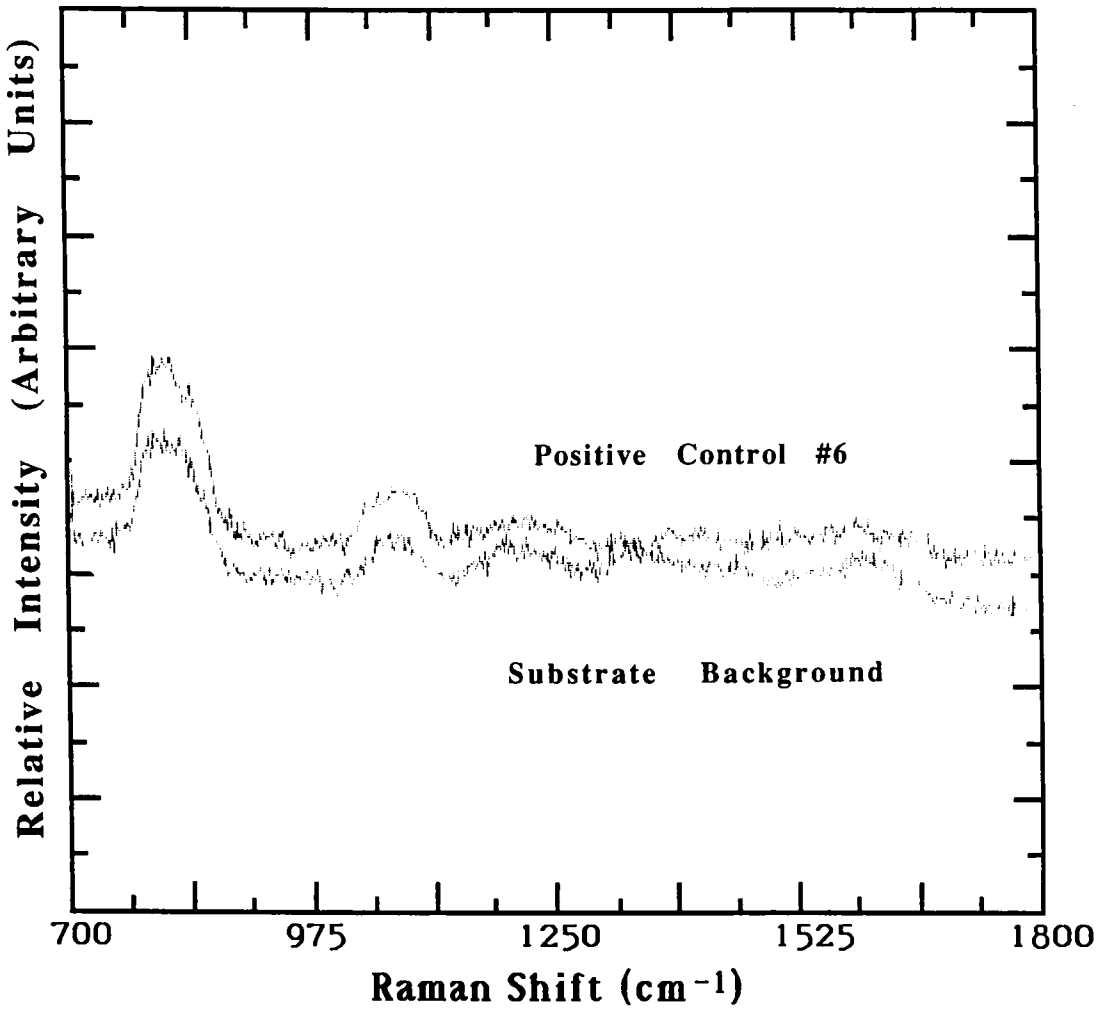
Spectra from sample #8 (0.732-fm/μL, the urine extract containing the highest concentration of aflatoxin) and from sample #11 (0.224-fm of aflatoxin/μL, an intermediate concentration sample) are shown in Figure IV-25 and Figure IV-26, respectively. A direct spectral comparison of a urine extract (sample #8), a positive control (sample #6), and the substrate background is given in Figure IV-27.



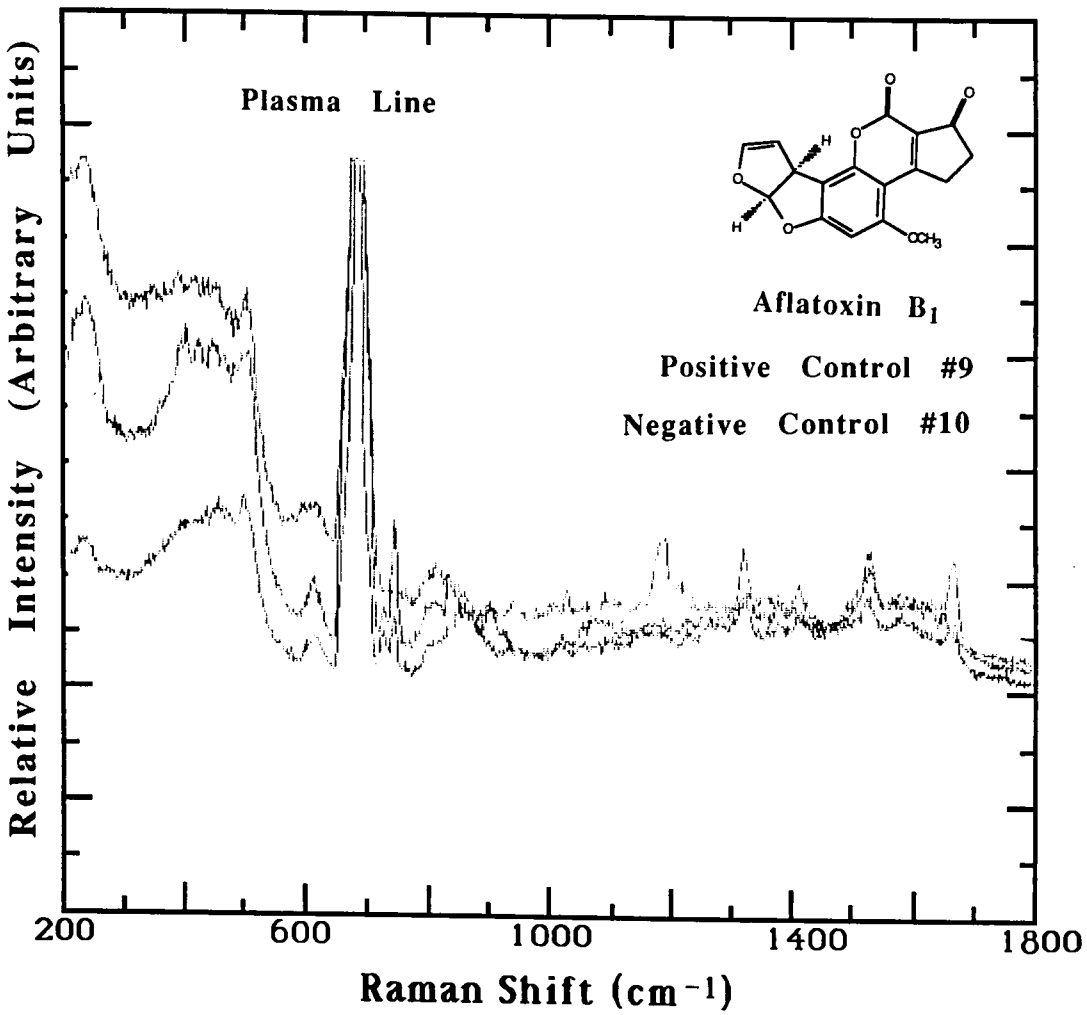

**<FIGURE IV-19> SERS Spectrum of Human Urine Extract #5** Sample was 1.0-μL of phosphate-buffered saline, a negative control. Laser excitation was 70-mW at 647.1-nm.



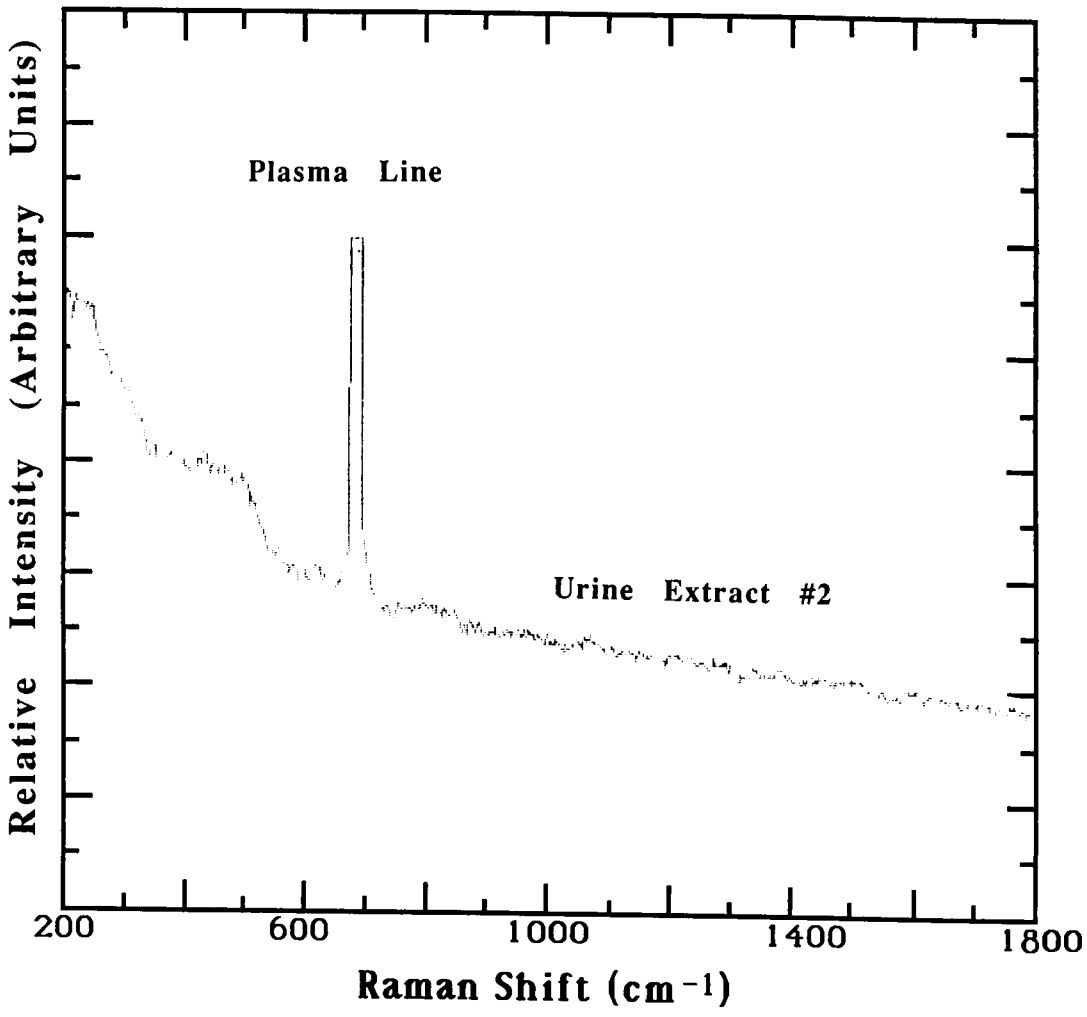

**<FIGURE IV-20> SERS Spectra of Aflatoxin Positive Control Samples #6 and #9** Spectra are of (Top) 1.0-μL of sample #6 (5.0-fm aflatoxin/μL) and (Bottom) 1.0-μL of sample #9 (2.0-fm aflatoxin/μL). Laser excitation was 70-mW at 647.1-nm. Spectra were printed to scale relative to each other.



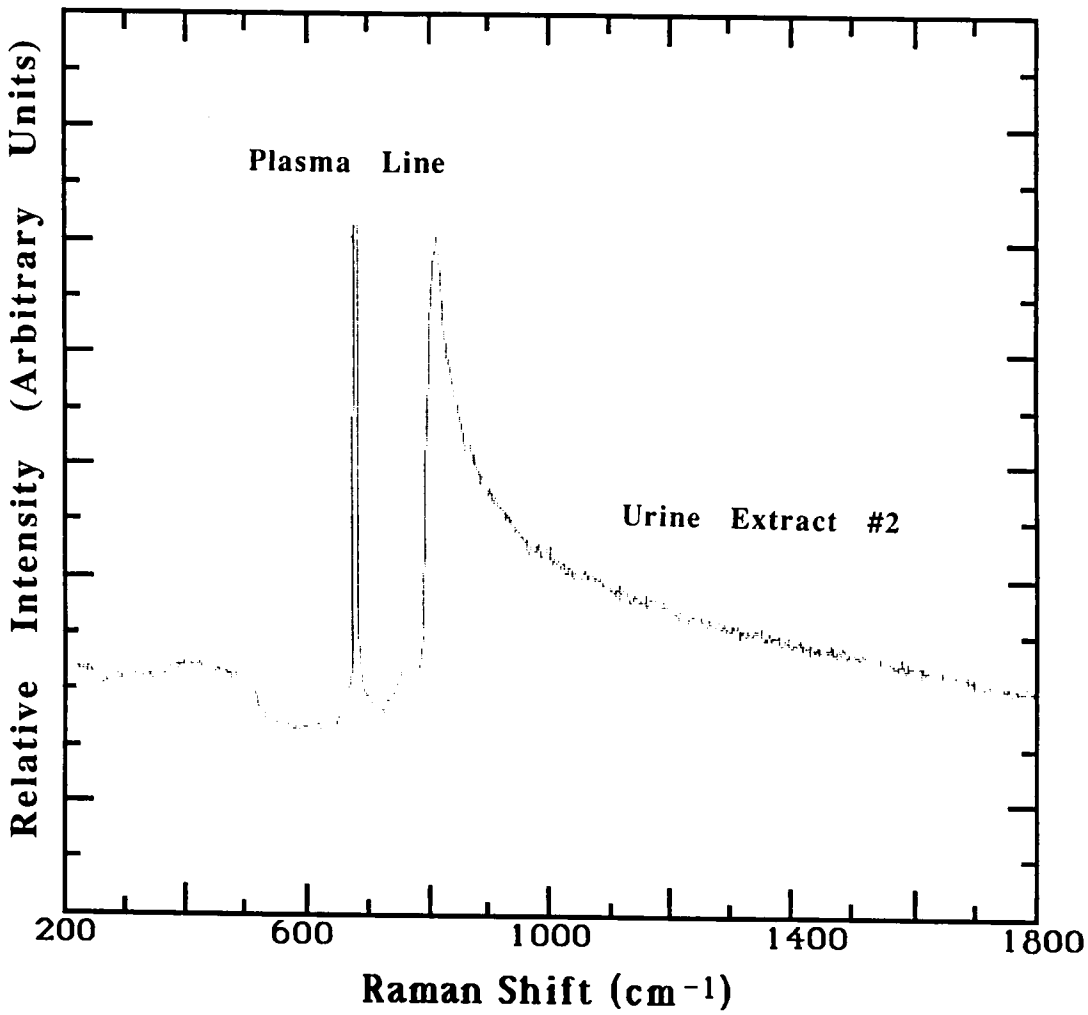

**<FIGURE IV-21> SERS Spectra of Aflatoxin Positive Control #6 and the Substrate Background** Spectra are of (Top) 1.0-μL of sample #6 (5.0-fm aflatoxin/μL) and (Bottom) substrate background. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.



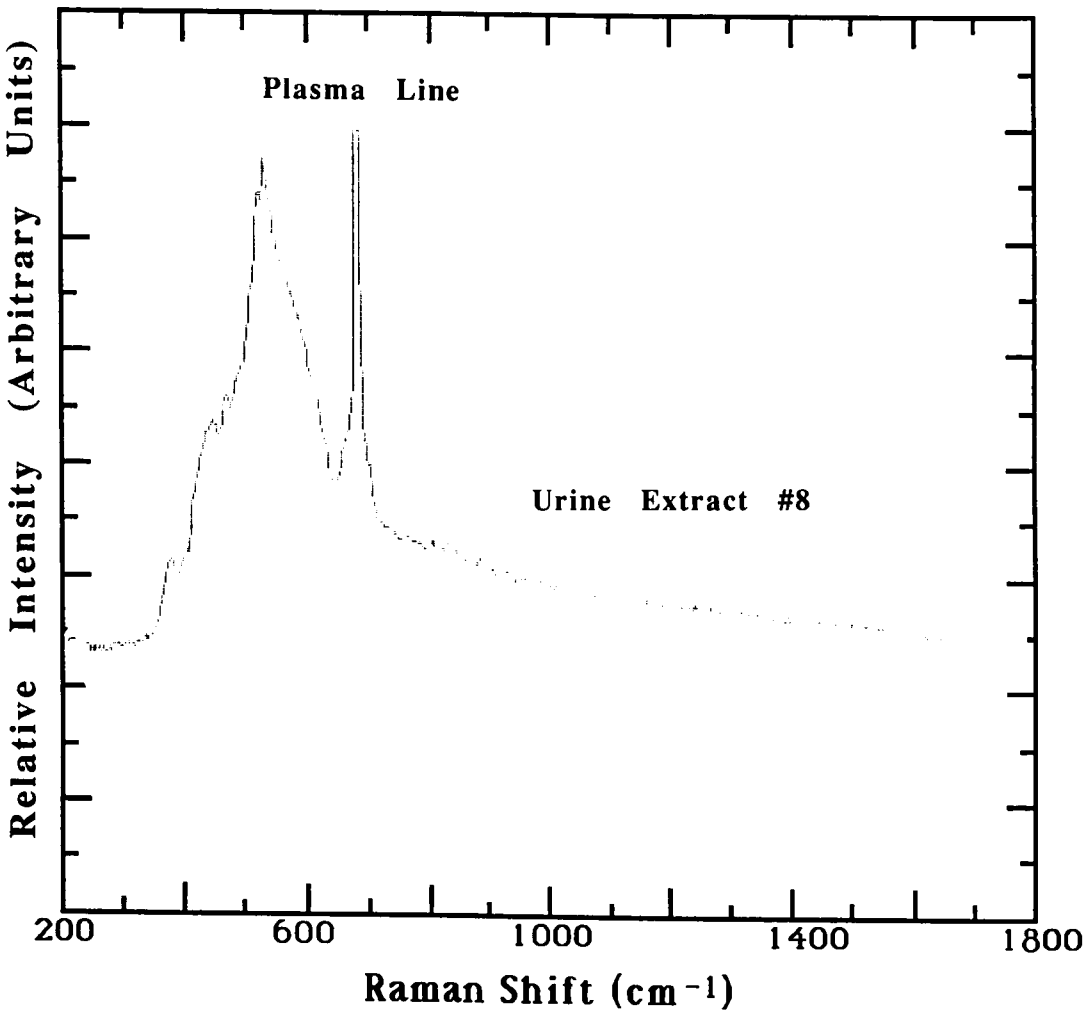

<FIGURE IV-22> SERS Spectra Comparing Aflatoxin $B_1$, Positive Control #9, and Negative Control #10 Spectra are of (Top) 1.0-μL of a $2.14 \times 10^{-4}$-M solution of aflatoxin $B_1$ in ethanol, (Center) 1.0-μL of sample #9 (2.0-fm aflatoxin/μL), and (Bottom) 1.0-μL of sample #10 (PBS). Laser excitation was 70-mW at 647.1-nm. Note the laser plasma line at 670 $cm^{-1}$. Spectra were printed to scale relative to each other.



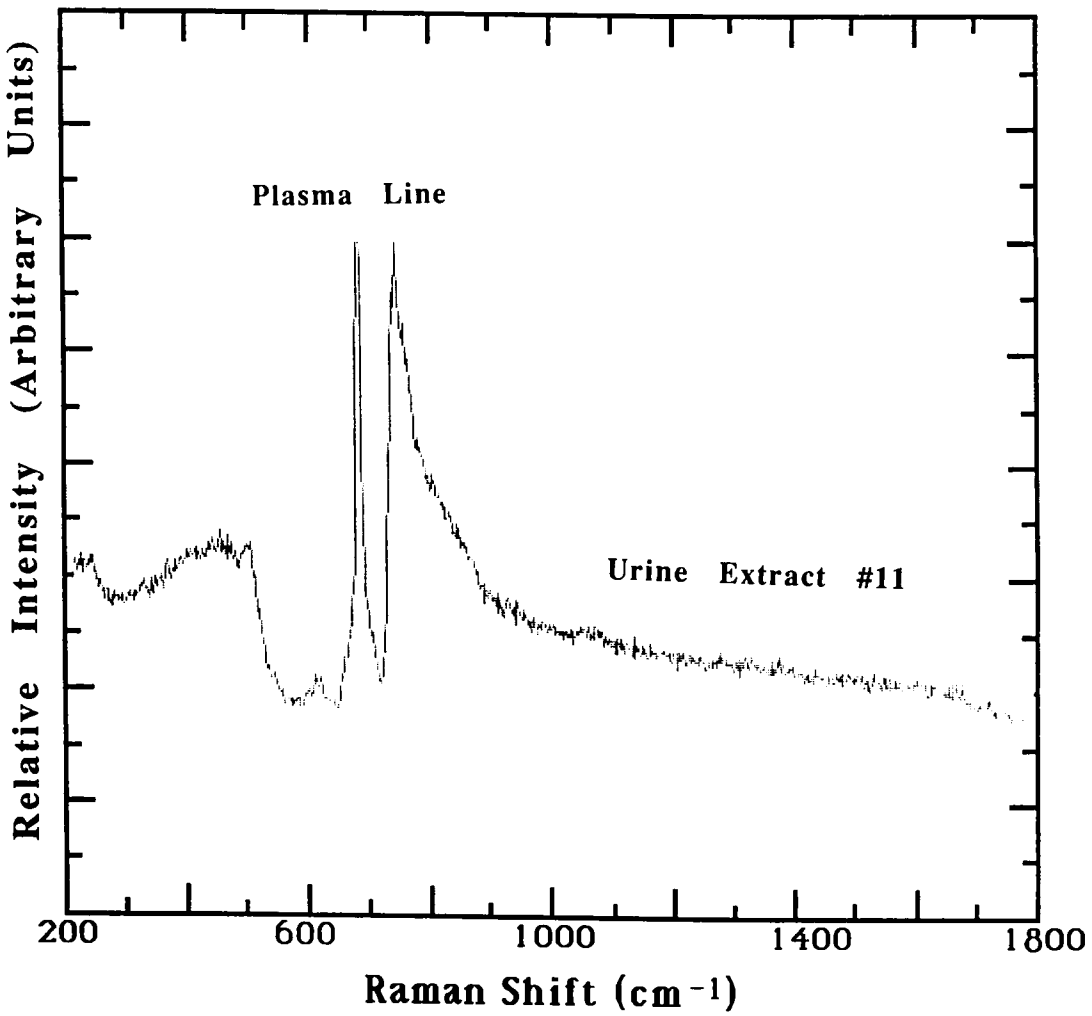

<FIGURE IV-23> SERS Spectrum of Urine Extract #2 Sample was 1.0-μL of sample #2 (0.404-fm aflatoxin/μL). Laser excitation was 70-mW at 647.1-nm.



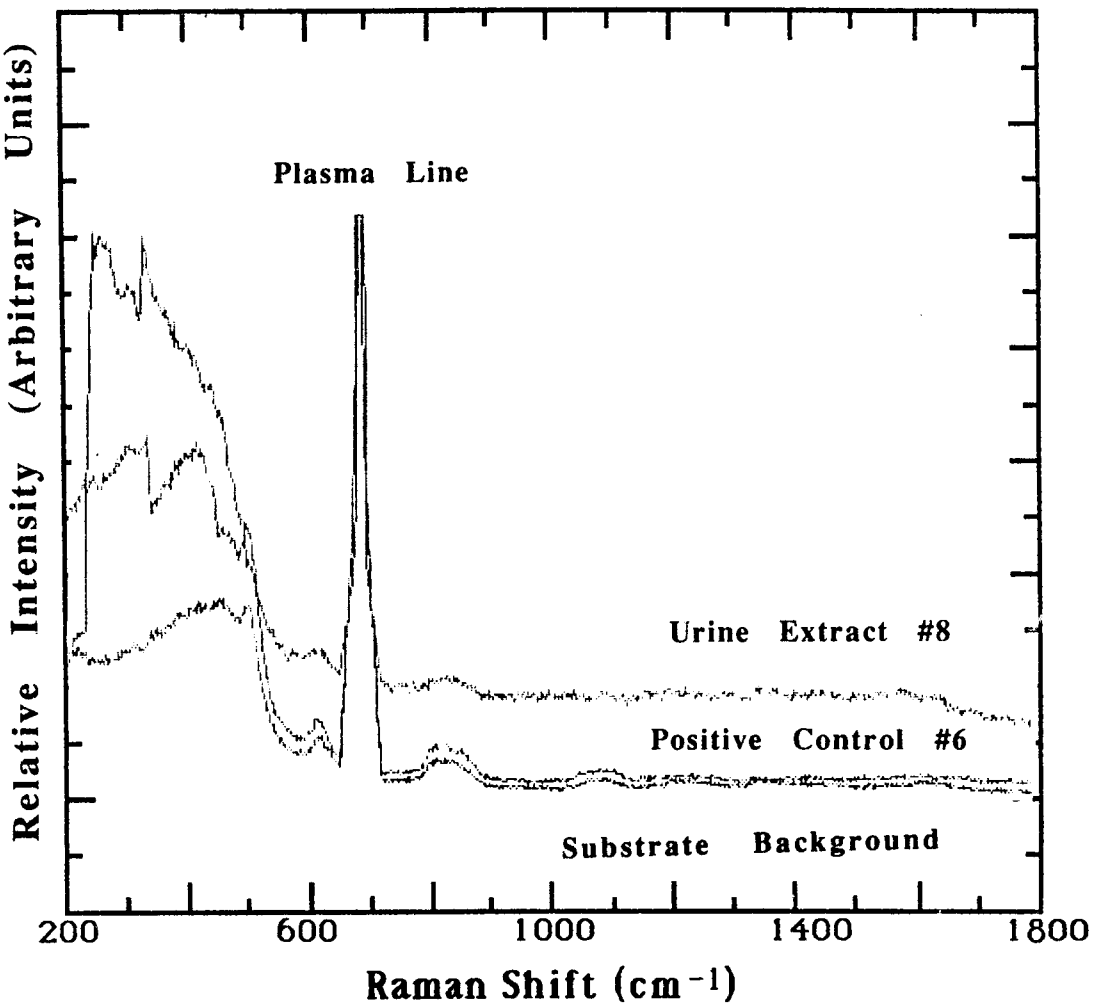

<FIGURE IV-24> A Second SERS Spectrum of Urine Extract #2 Sample was 1.0-μL of sample #2 (0.404-fm aflatoxin/μL). Laser excitation was 70-mW at 647.1-nm.




<FIGURE IV-25> SERS Spectrum of Urine Extract #8 Sample was 1.0-μL of sample #8 (0.732-fm aflatoxin/μL, most concentrated amount of aflatoxin). Laser excitation was 70-mW at 647.1-nm.




<FIGURE IV-26> SERS Spectrum of Urine Extract #11 Sample was 1.0-μL of sample #11 (0.224-fm aflatoxin/μL). Laser excitation was 70-mW at 647.1-nm.

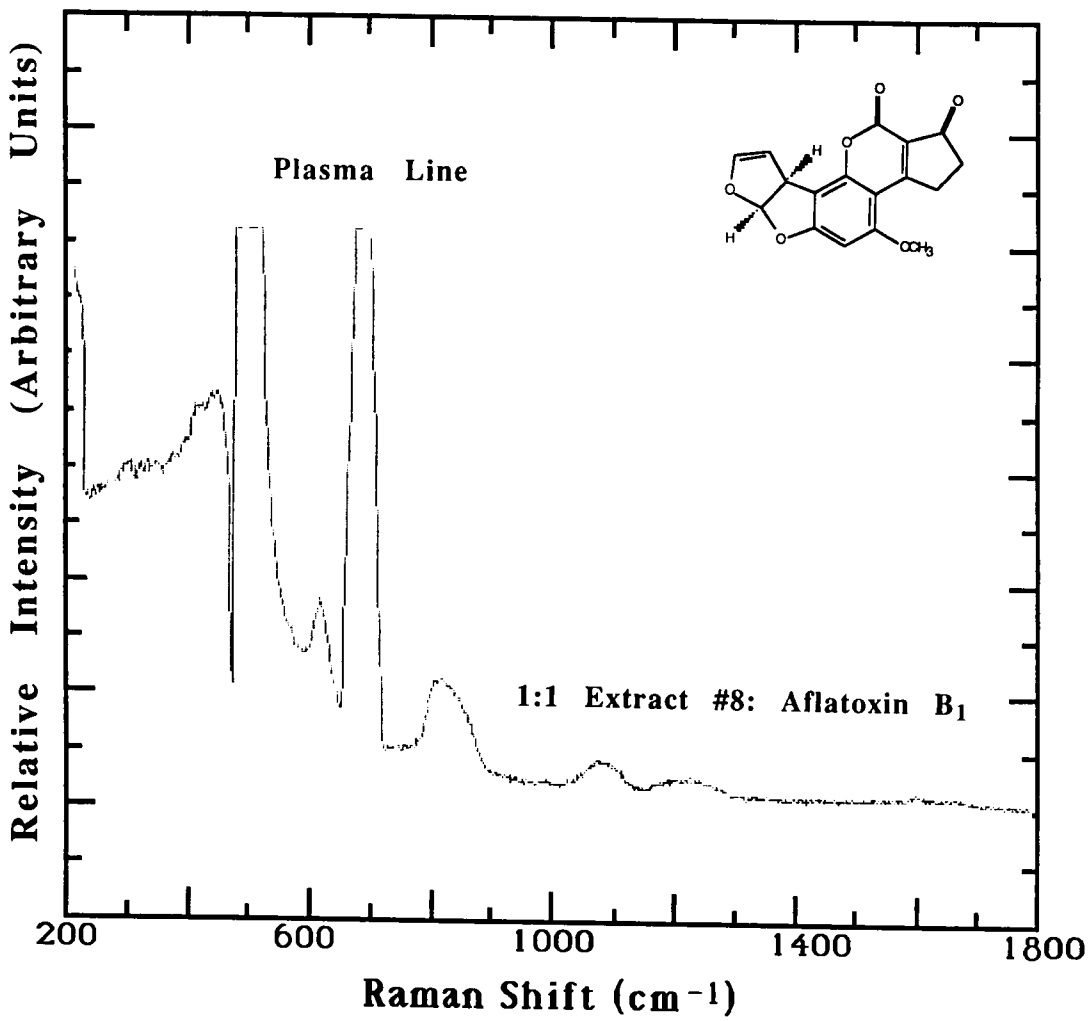



**<FIGURE IV-27> SERS Spectra of High Aflatoxin Level Extract #8, Positive Control #6, and the Substrate Background** Spectra are of (From Right, Top to Bottom) 1.0-μL of sample #8 (0.732-fm aflatoxin/μL), 1.0-μL of sample #6 (5.0-fm aflatoxin/μL), and substrate background. Note the plasma line at 670 $cm^{-1}$. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.

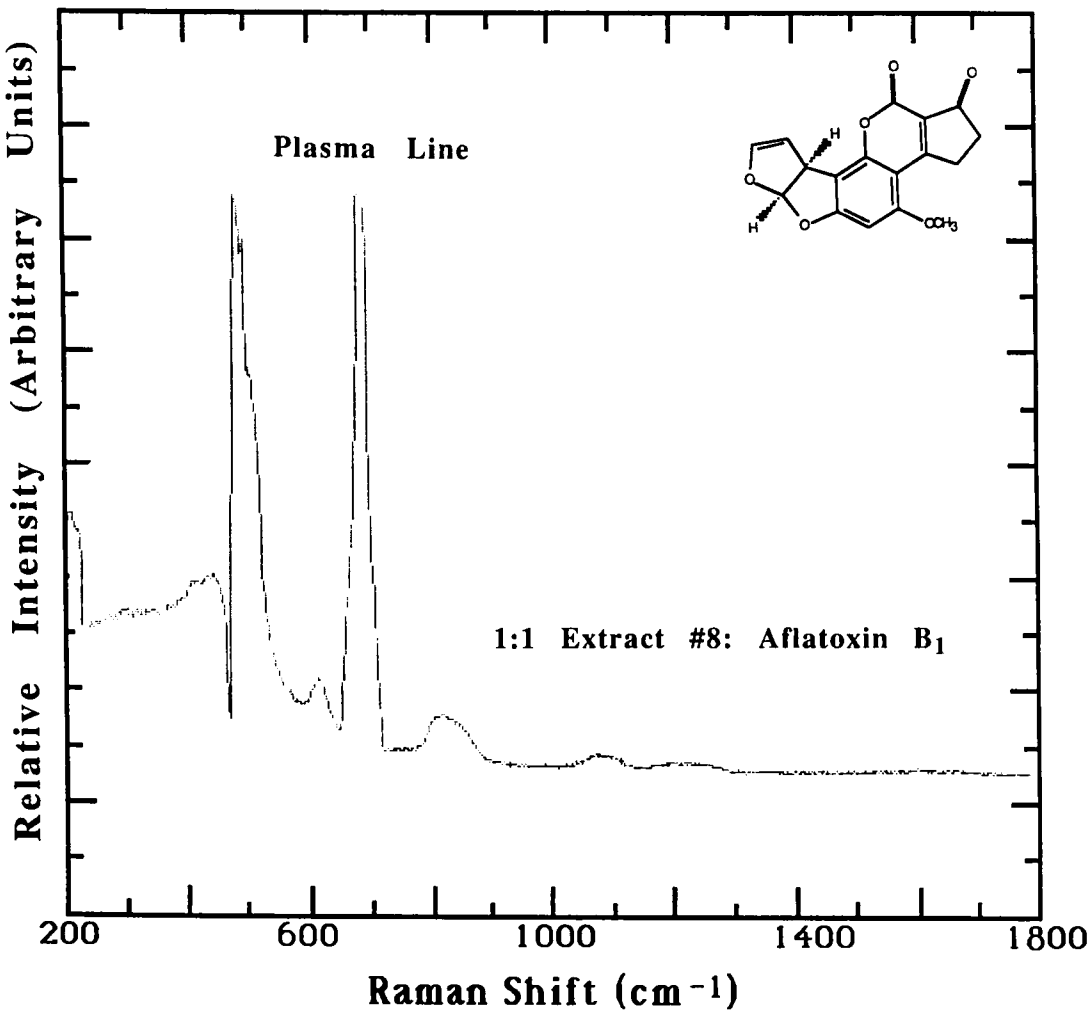
An attempt was made to dope a urine extract with aflatoxin $B_1$ to elicit a SERS signal. The extract chosen for this experiment was sample #8, the extract already containing the highest level of aflatoxin. The aflatoxin sample used was aqueous because the addition of any alcohol to the urine extracts would have caused precipitation of the sample. Representative spectra from this experiment are shown in Figure IV-28 and Figure IV-29. No spectral bands which could be attributed to aflatoxin were observed even though the final concentration of aflatoxin $B_1$ doped into the urine was within the limit observable using SERS with a pure solution of aflatoxin $B_1$. It is likely that aquisition of vibrational information from aflatoxin $B_1$ was prevented by an interaction between the proteins and other large molecular components in the urine and either the substrate or the aflatoxin. The results of attempts to dope aflatoxin $B_1$ into the molecularly complex urine extracts mirror the results of efforts to use SERS to detect ethenocytosine or benzo[a]pyrene in plasma or serum. Spectra of normally SERS-active chemicals rendered inactive in complex matrices may be used as evidence that the high molecular weight constituents may inhibit vibration of the chemical bonds in the sample molecules or that insoluble species in a mixture may suppress SERS scattering.

### *Summary*

Unfortunately, no reproducible data were obtained from these urine extracts. It might have been desirable to have had urine from humans not exposed to aflatoxin processed with the other samples as a further experimental control. Better results could have been expected if the samples




**<FIGURE IV-28> SERS Spectrum of Urine Extract #8 Doped with Aqueous Aflatoxin $B_1$** Sample was 1.0-μL of a 1:1(v/v) solution of sample #8 (0.732-fm aflatoxin/μL, highest aflatoxin level extract):0.16-mg/mL aflatoxin $B_1$ in water. Laser excitation was 50-mW at 647.1-nm.




**<FIGURE IV-29> A Second SERS Spectrum of Extract #8 Doped with Aqueous Aflatoxin $B_1$** Sample was 1.0-μL of a 1:1 (v/v) solution of sample #8 (0.732-fm aflatoxin/μL):0.16-mg/mL aflatoxin $B_1$ in water. Laser excitation was 50-mW at 647.1-nm.

could have been dissolved entirely in water, ethanol, or some other alcohol (although any alcohol would have precipitated the samples). Previous studies (Section II and Section III) demonstrated that SERS samples dissolved specifically in phosphate-buffered saline (PBS) do not generally yield valuable SERS spectra, probably from some ionic interaction between the solvent and the silver on the substrate surface. Even when the samples and positive controls were doped to a concentration at which aflatoxin has been detected with SERS, a characteristic flattening of spectra from the PBS occurred and no aflatoxin-specific bands were seen. Perhaps samples such as these urine extracts would be better evaluated by SERS on a different substrate type such as a silver electrode, possibly with a potential difference applied across the samples.

It is important to realize that although the results of this study did not demonstrate the effectiveness of SERS for the analysis of aflatoxin in these valuable real-life samples, neither were the results completely noninformative. A primary consideration in the interpretation of these results is that aflatoxin $B_1$ was not routinely detected at 5.0-fm/μL (the level of the most concentrated positive control -- the most concentrated sample in this study) even in an absolute ethanol solution. Also, few samples examined throughout these experiments could be detected in PBS, even at high sample concentrations.

# CHAPTER 5

# SERS OF ALTERED DNA

## *Introduction*

In addition to the human urine extracts (aflatoxin), valuable samples of DNA that had been exposed to hazardous chemicals were available for SERS analysis. The samples discussed in this chapter were DNA reacted *in vitro* with chloroacetaldehyde to produce DNA adducts, DNA that had been exposed *in vitro* to benz[a]anthracene epoxides, and DNA from humans that had been exposed to benzo[a]pyrene. As will become evident when the results of these studies are discussed, the examination of DNA samples with SERS was considerably more complicated than SERS detection of highly purified genotoxins in a simple aqueous or alcoholic solution.

## *Materials and Methods*

### Instrumentation

A SERS fiberoptic setup was used for these experiments. Laser excitation was at 647.1-nm. Silvered alumina-roughened (4.0%) substrates were used.

### Chemicals

Highly polymerized calf thymus DNA was obtained from Sigma Chemical Company (St. Louis, MO) and was used as received. Chloroacetaldehyde (50% aqueous solution) was purchased from the Aldrich Chemical Corporation (Milwaukee, WI) and was also used without further purification.

DNA/BPDE samples were provided by Dr. Ainsley Weston of the Molecular Epidemiology Section at the Laboratory of Human Carcinogenesis of the National Cancer Institute/National Institutes of Health. Three samples were received, labelled A (1000-μg/mL DNA in 100-μL aqueous solution), B (320-μg/mL DNA in 300-μL aqueous solution), and C (1000-μg/mL DNA in 100-μL aqueous solution). Sample A contained the highest level of BPDE-adducts, sample B contained an intermediate adduct level, and sample C was below the lower detection limit of Dr. Ainsley's system (HPLC/SFS). Sample C was extremely viscuous and difficult to manipulate for SERS analysis.

DNA epoxide samples were provided by Dr. Mayo Uziel of the Health and Safety Research Division of Oak Ridge National Laboratory (Oak Ridge, TN). The epoxides were originally obtained from the National Cancer Institute and were later dissolved in 100-mL absolute ethanol and frozen. An undetermined amount of epoxide remained in the samples at the time of the SERS experiment. The DNA stock solution was prepared in 1987 at 3.2 units of absorbance at 260-nm per milliliter and was frozen until use.

Procedures

An experiment was performed in which chloroacetaldehyde was reacted with DNA to produce etheno DNA adducts, adapted for SERS from a 1986 paper by M. A. Bedell, M. C. Dyroff, G. Doerjer, and J. A. Swenberg (11). 30-mg of highly polymerized calf thymus DNA was dissolved in 0.1-M sodium phosphate (pH 7.0) to give 2 mg/mL of DNA. Chloroacetaldhyde was added to a final concentration of 0.5%. The mixture was incubated at 37° C for 24 hours. Cold 5-M NaCl (2% volume) and cold absolute ethanol (60% volume) were added to the mixture. The sample was then placed on dry ice (-70° C) for one hour. Next,

the mixture was centrifuged at 0° C for 30 minutes at 10000 rpm. The supernatant was discarded. Water was added to bring the total volume to 1.0-mL. The addition of salt and ethanol and centrifugation steps were repeated twice more. The resulting DNA was dissolved in 2.0-mL water and frozen at -20° C until use.

0.1-mL of each benzanthracene epoxide (concentration unknown) was added to 3.0-mL aliquots of the DNA solution. The mixtures were sealed and allowed to stand for 24 hours at 23° C. Each sample was then passed through an activated reversed phase cartridge (SepPak C-18 swelled with methanol and washed with 5-mL water, Waters Corporation, Milford, MA) to remove unreacted reagents and hydrolysis products. The cartridge was washed with 1.0-mL water. The wash was added to the initial effluent for a total volume of 6-mL. Samples were stored in well-sealed, amber bottles. Samples examined in this study are shown in Figure IV-30 and Figure IV-31. Sample compositions are listed below:

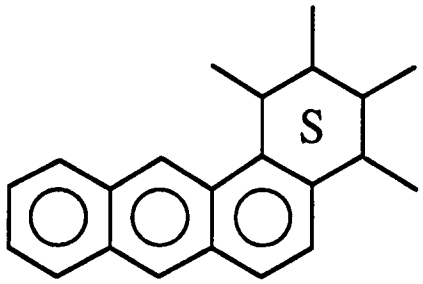
Control #1: benzo[a]anthracene-trans-10,11-dihydrodiol-8,9-epoxide (anti)
NCI#L0181 MRI#433 Lot#CR33-2-8 Batch 01

Control #2: benz[a]anthracene-trans-3,4-dihydrodiol-1,2-epoxide (anti)
NCI#L0189 MRI#431 Lot#6635WB-74 Batch 02

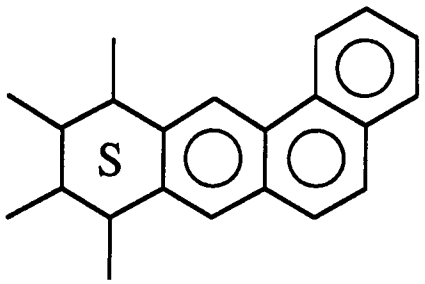
Control #3: 3-methylcholanthrene-11,12-dihydroepoxide
NCI#L0121 MRI#541 Lot#CR45-18-12 Batch 01

Control DNA (received no adduct treatment)

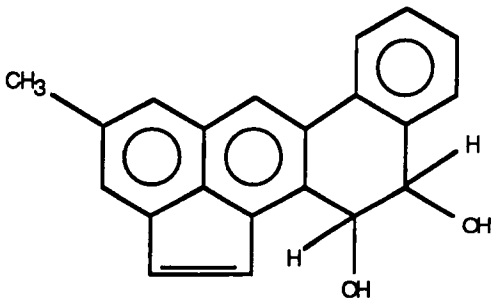
Control #1



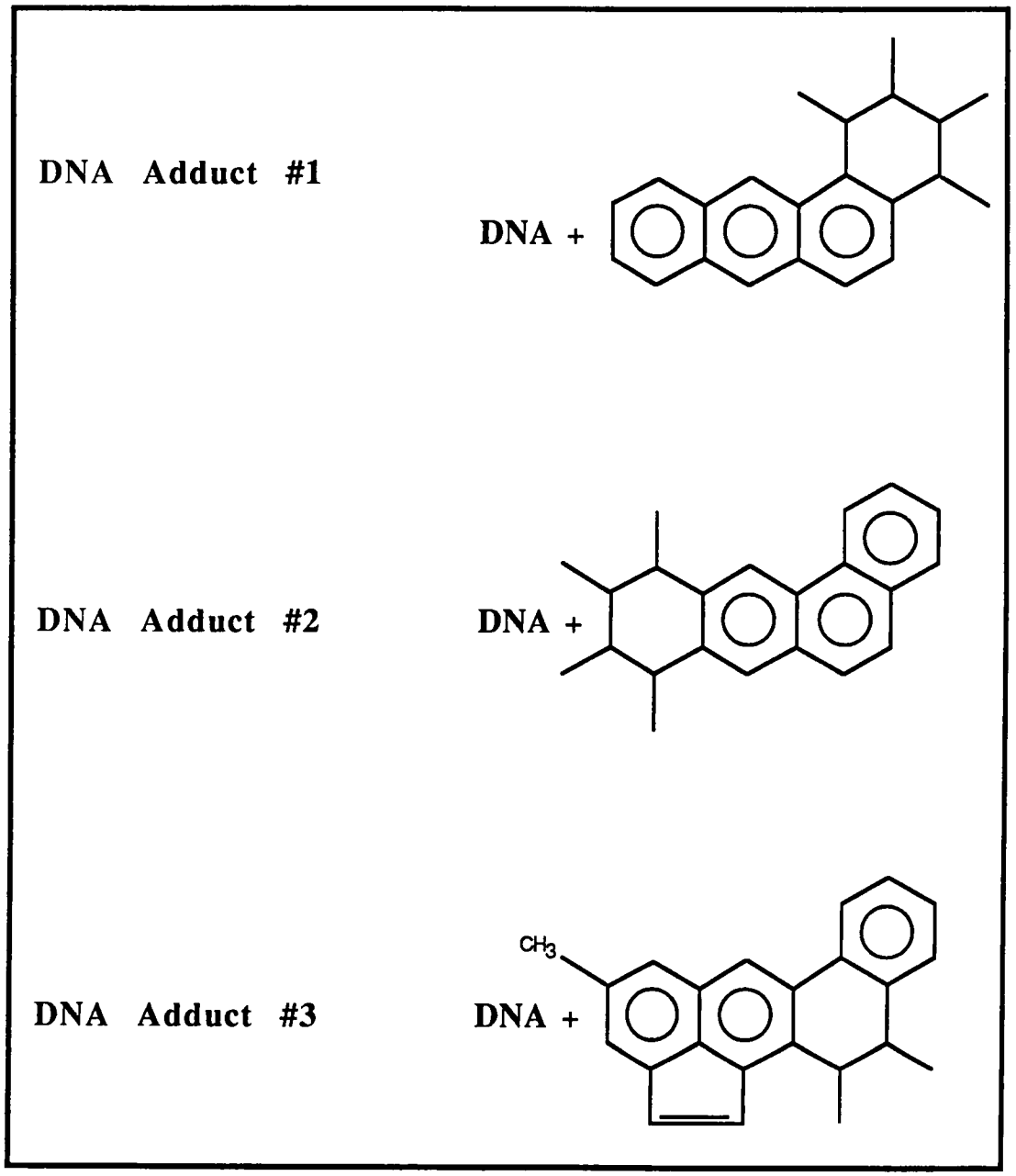

Control #2




Control #3




<FIGURE IV-30> Structures of the Benz[a]Anthracene Epoxide Control Samples




<FIGURE IV-31> Structures of the Benz[a]Anthracene Epoxide DNA Adducts

Adduct #1: benzo[a]anthracene-trans-10,11-dihydrodiol-8,9-epoxide (anti) + DNA

Adduct #2: benz[a]anthracene-trans-3,4-dihydrodiol-1,2-epoxide (anti) + DNA

Adduct #3: 3-methylcholanthrene-11,12-dihydroepoxide + DNA

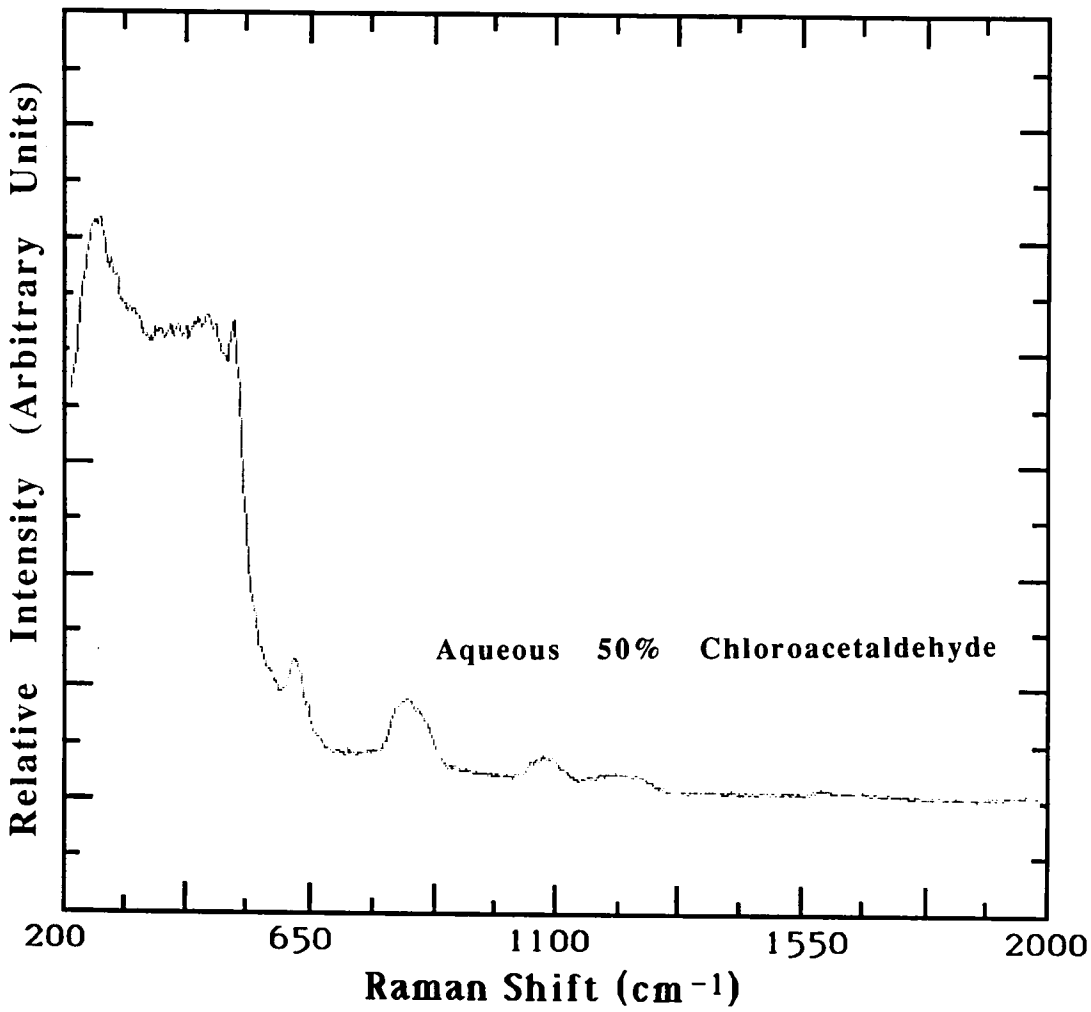
The BPDE/DNA adducts and benz[a]anthracene epoxides were frozen until use and analyzed without further purification or modification. SERS samples were 1.0-μL in volume and were allowed to dry before exposure to the laser beam.

Prior to examination of sample molecules, the optical setup was carefully optimized by maximizing the spectral peak intensities from a dilute solution of 3,$N^4$-ethenocytosine.

## *Results and Discussion*

### Chloroacetaldehyde Reacted with DNA

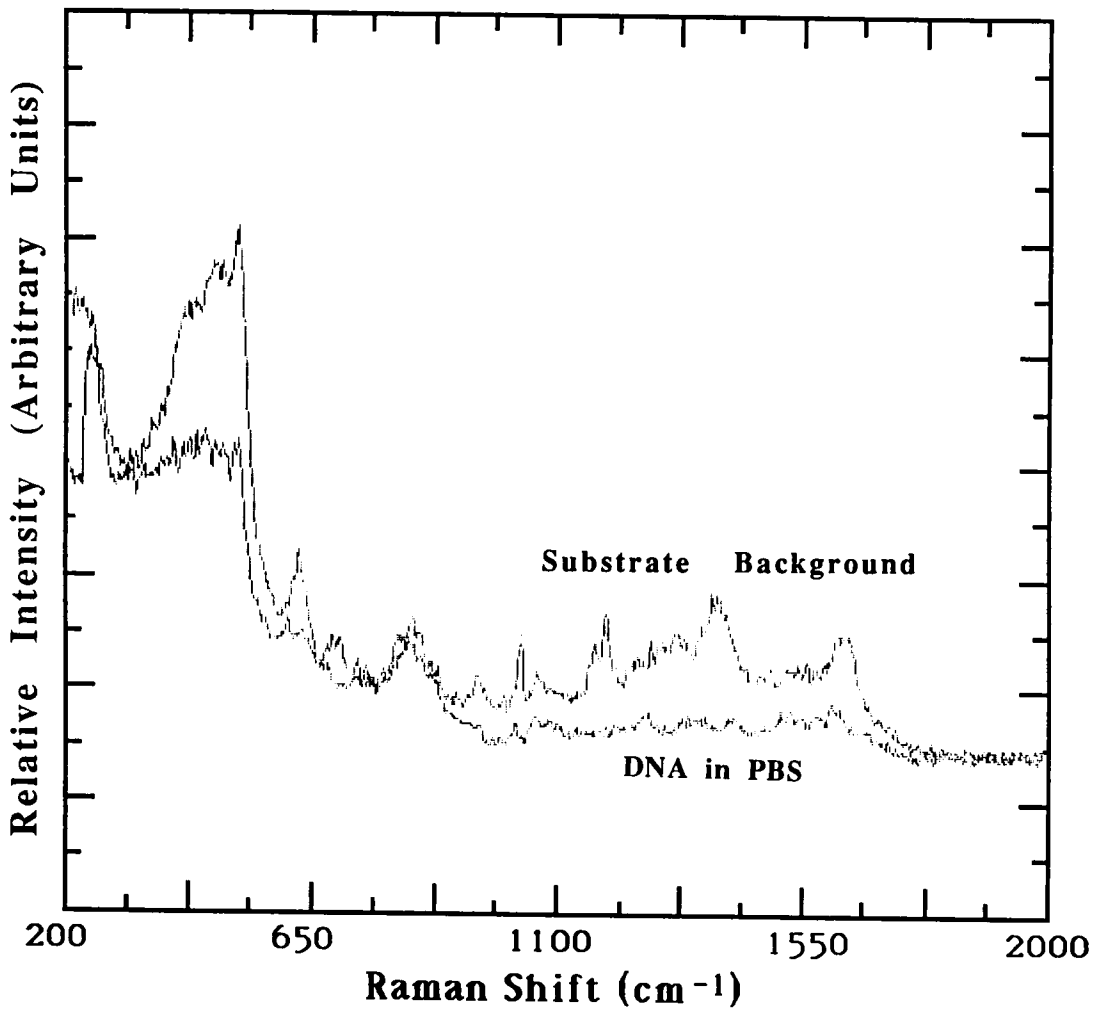
A SERS spectrum of chloroacetaldehyde is given in Figure IV-32. No spectral signature was obtained under these experimental conditions, although it is noteworthy that the chloroacetaldehyde discolored (turned the substrate the yellow color of chloroacetaldehyde) and apparently disrupted the substrate silver surface. Control spectra of the substrate background, calf



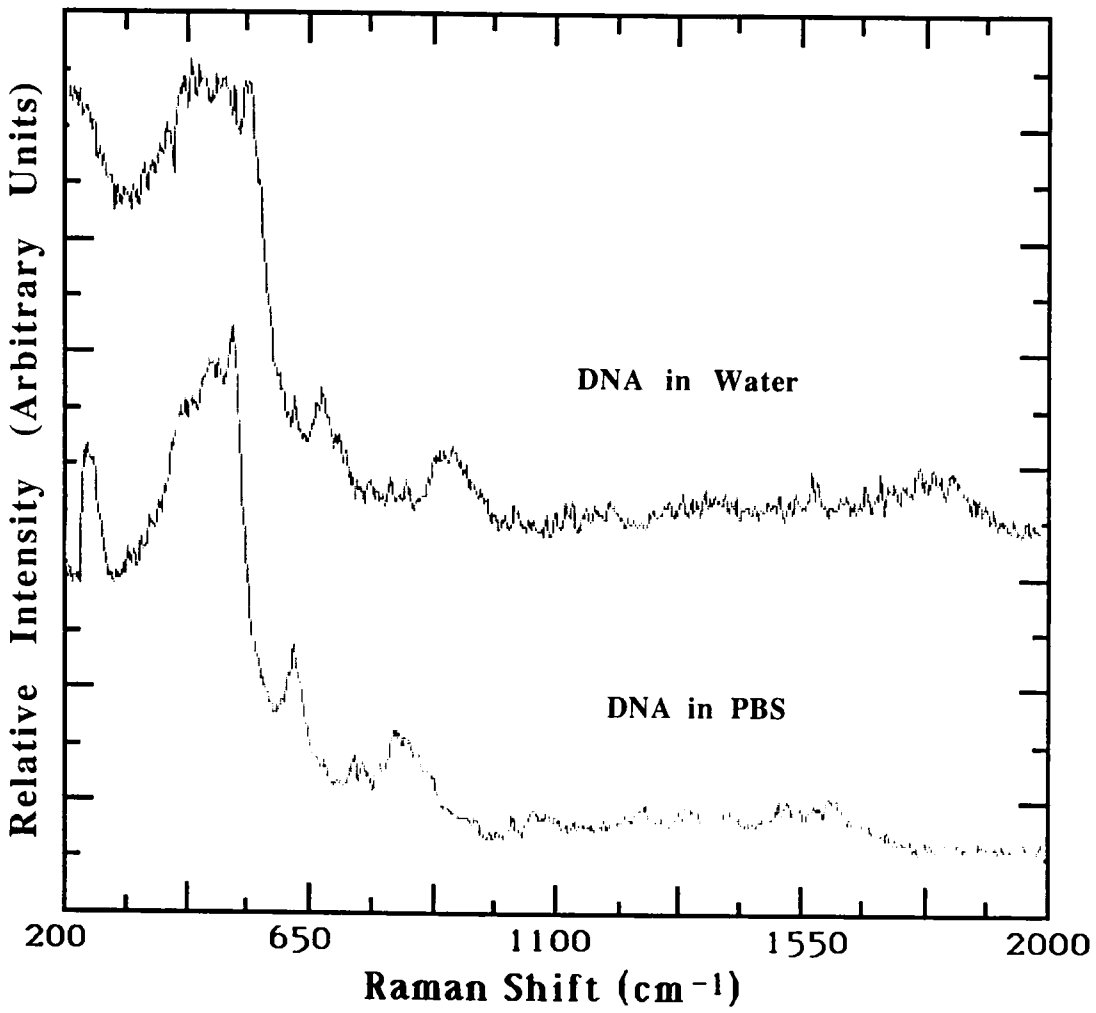

**<FIGURE IV-32> SERS Spectrum of 50% Aqueous Chloroacetaldehyde** Sample was 1.0-μL. Laser excitation was 60-mW at 647.1-nm.

thymus DNA in water, and calf thymus DNA in PBS are shown in Figure IV-33 and Figure IV-34. No intense spectral bands were evident for DNA in either water or PBS in this experiment. (It is noteworthy that SERS spectral bands from DNA may have been resolved had a substrate other than the silvered, microparticle-roughened type been used for these experiments.) A SERS spectrum (Figure IV-35) was also taken of the DNA, CAA, and PBS immediately after mixing (zero reaction time). As expected, no SERS bands except those corresponding to fiberoptic background were observed. No detailed structure was evident in the 900 $cm^{-1}$ to 1800 $cm^{-1}$ spectral region. This spectral flattening is characteristic and indicative of the PBS in the solution.

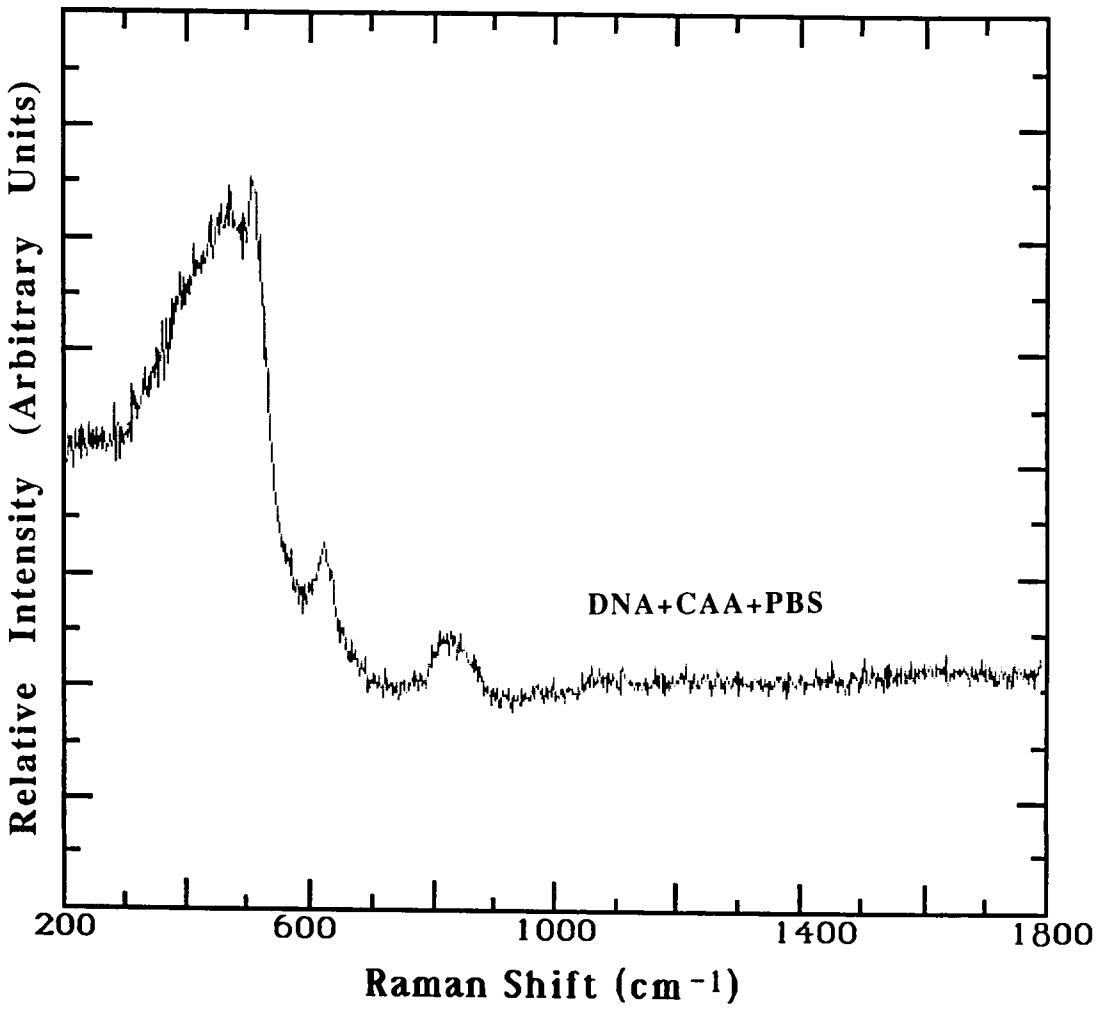
Spectra of the DNA after reaction with CAA and washing are shown in Figure IV-36 (no dilution factor, reacted DNA diluted with water, ethenocytosine control), Figure IV-37 (ethenocytosine control, reacted DNA diluted with water, substrate background), and Figure IV-38 (DNA diluted with ethanol, precipitant formed). It is likely that no spectral detail was available from Figure IV-38 as a result of the precipitation of the DNA upon addition of the ethanol. The reacted, undiluted DNA of Figure IV-36 was very viscuous, but was not solid. Spectra from the undiluted DNA and the aqueous DNA exhibited broad bands where nucleic acid peaks would be expected to appear. The spectral bands from Figure IV-36 and Figure IV-37 could not be assigned to any specific purine or pyrimidine, altered or otherwise. Some spectral bands apparently corresponded to 3,$N^4$-ethenocytosine, although guanine should be alkylated to the greatest extent by the reaction with CAA. The total expected base substitution for the reaction was about $1/10^6$ bases. Individual substitutions should appear with much lower frequency than $1/10^6$ bases. The most significant result of this experiment was that the reacted DNA could be



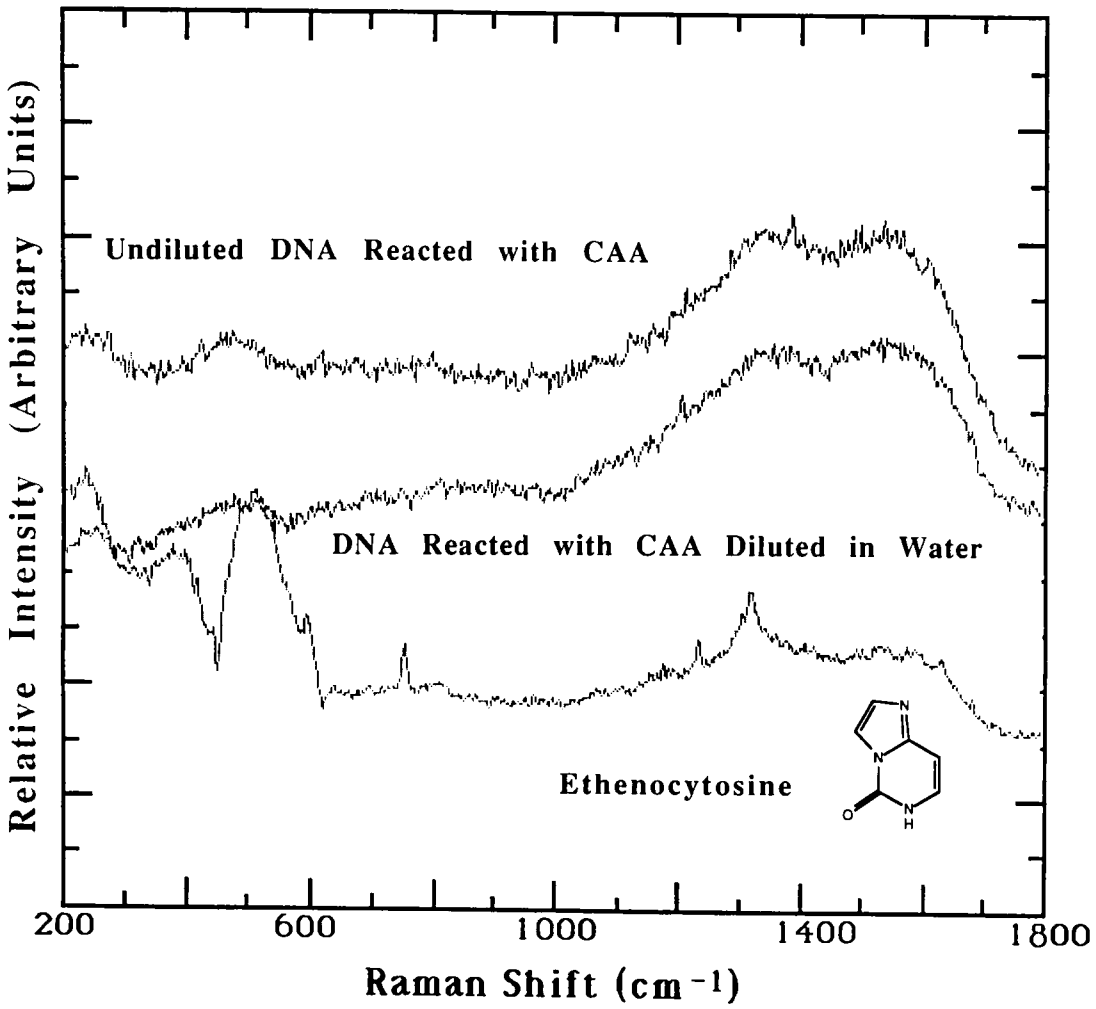

**<FIGURE IV-33> SERS Spectra of the Substrate Background and DNA in PBS** Sample was 1.0-μL of 2-mg/mL highly polymerized calf thymus DNA in PBS (wet spot). Laser excitation was 60-mW at 647.1-nm.



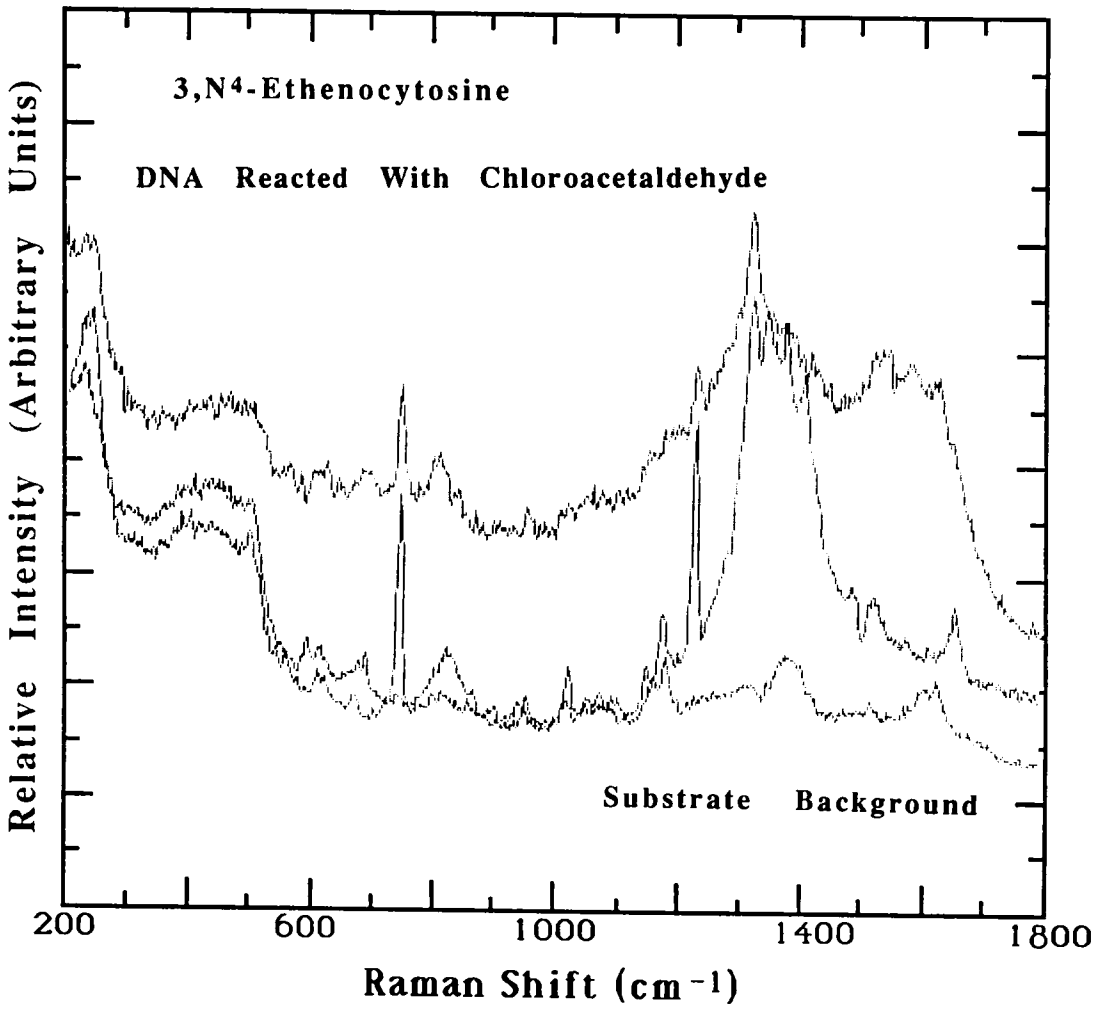

**<FIGURE IV-34> SERS Spectra of DNA in Water and DNA in PBS** Spectra are of (Top) 1.0-μL of a 7-mg/mL solution of highly polymerized calf thymus DNA in water and (Bottom) 1.0-μL of a 2-mg/mL solution of highly polymerized calf thymus DNA in PBS (wet spot). Laser excitation was 60-mW at 647.1-nm.



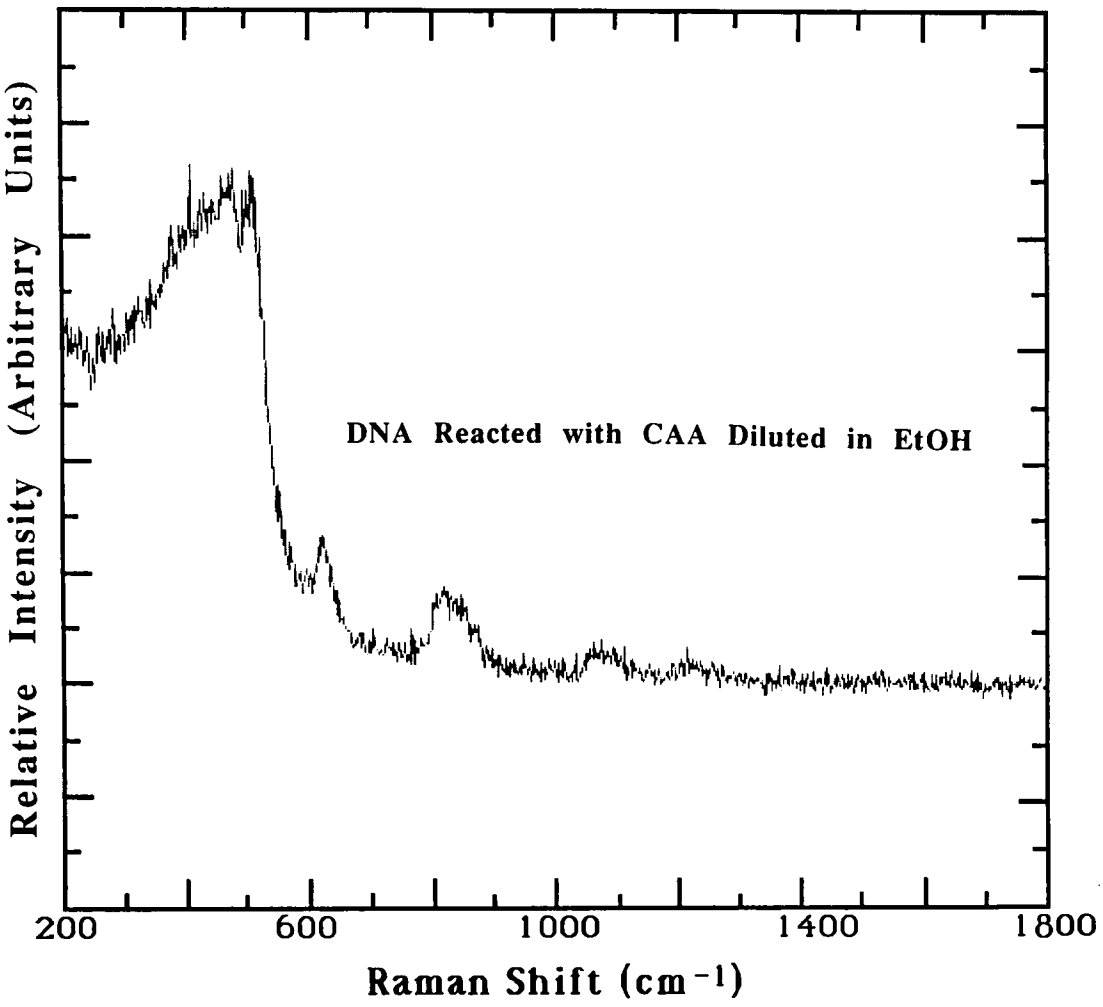

<FIGURE IV-35> SERS Spectrum of the DNA+Chloroacetaldehyde+PBS Mixture Sample was 1.0-μL of the mixture, scanned with SERS immediately after mixing. Laser excitation was 50-mW at 647.1-nm.




**<FIGURE IV-36> SERS Spectra of Undiluted DNA Reacted with CAA, DNA Reacted with CAA Diluted in Water, and 3,N$^4$-Ethenocytosine** Spectra are of 1.0-μL of (From Right, Top to Bottom) undiluted calf thymus DNA following reaction with CAA, DNA reacted with CAA and diluted in 1.0-mL water, and a 1x10$^{-3}$-M solution of 3,N$^4$-ethenocytosine in 1:1 (v/v) EtOH:$H_2O$. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.




**<FIGURE IV-37> SERS Spectra of 3,N4-Ethenocytosine, DNA Reacted with CAA Diluted in Water, and the Substrate Background** Spectra are of (Top) 1.0-µL of a 1.93-mg/mL solution of ethenocytosine in water, (Center) 1.0-µL of calf thymus DNA reacted with CAA and diluted in 1.0-mL water, and (Bottom) substrate background. Laser excitation was 60-mW at 647.1-nm. Spectra were printed to scale relative to each other.



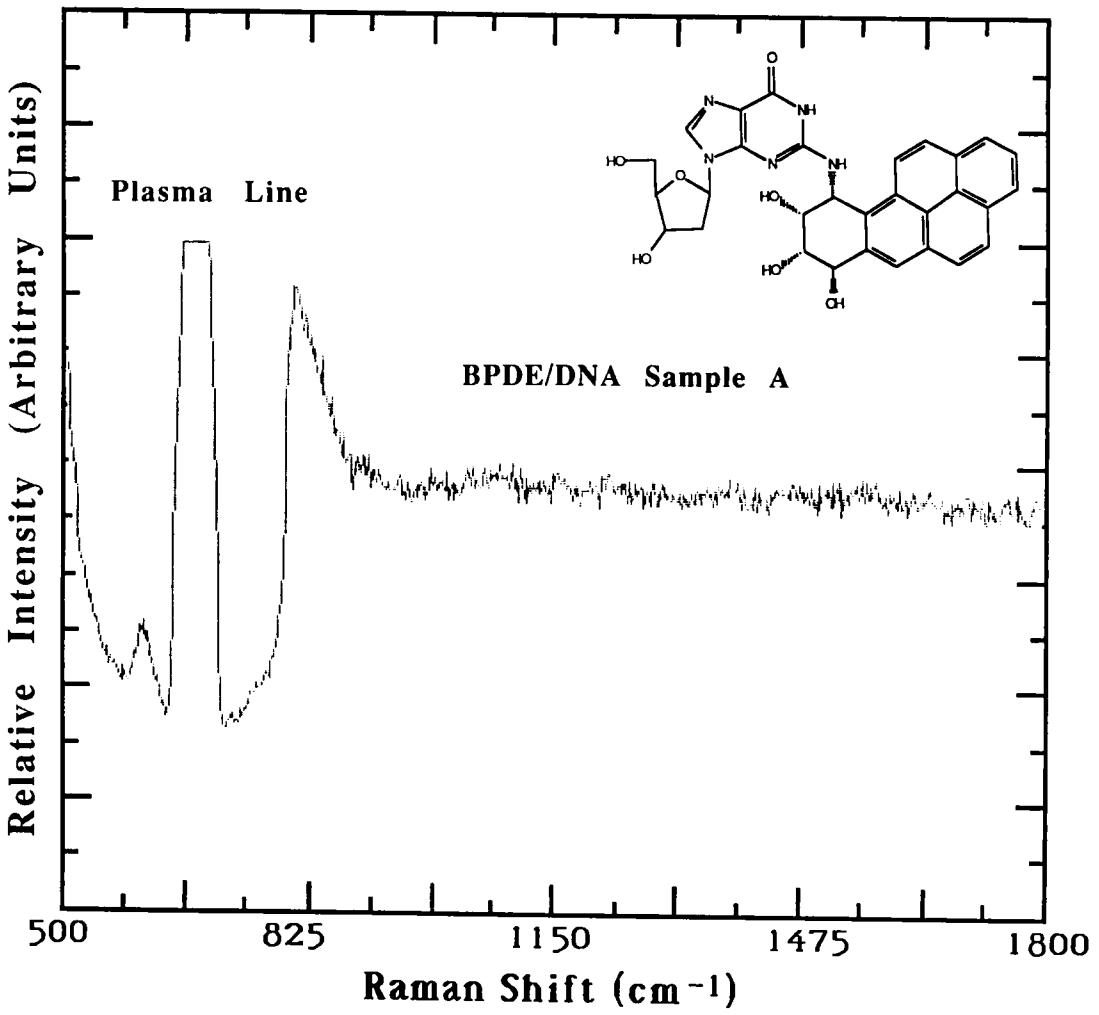

**<FIGURE IV-38> SERS Spectrum of DNA Following Reaction with Chloroacetaldehyde and Diluted in Ethanol** Sample was 1.0-μL. A precipitant formed when 1.0-mL EtOH was added to the total reacted DNA. Laser excitation was 50-mW at 647.1-nm.

*clearly* distinguished from any of the negative controls. Also, SERS bands correlating to the bands of 3,$N^4$-ethenocytosine were found in the chloroacetaldehye-reacted DNA.

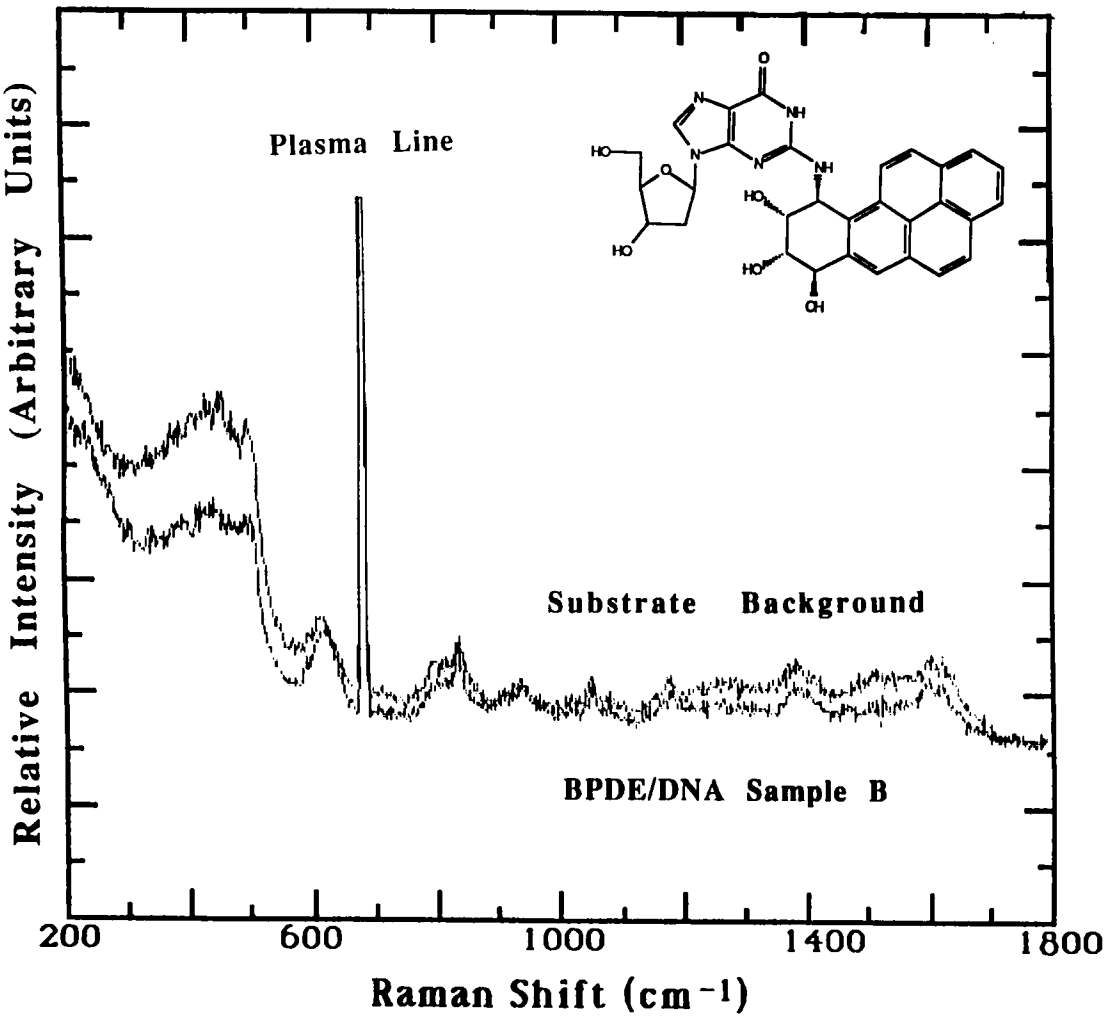
Human BPDE/DNA Adducts

The three samples examined in this study were all extremely viscuous, although the substituted bases were very rare (concentrated DNA, dilute analyte). Representative spectra for the three samples are given in Figure IV-39 (Sample A, 1000-μg/mL DNA in 100-μL water), Figure IV-40 (Sample B, 320-μg/mL DNA in 300-μL water), and Figure IV-41 (Sample C, 1000-μg/mL DNA in 100-μL water). Spectra resembling Figure IV-39 were obtained for all three samples and may be indicative of sample (DNA) overloading. A clean spectrum (e.g. Figure IV-40 and Figure IV-41) was not obtained for Sample A, which contained the highest level of total DNA. Samples B and C exhibited poor signal-to-noise ratios. While spectra from these samples were not entirely coincident with the substrate background, neither could true spectral signatures be attributed to the samples. These spectra did, however, reveal more detail than did the BPDE/DNA adduct examined in Section III. It was not possible to substract the background noise from spectra of Sample B or Sample C because the sample spectra had lower photon counts than did the substrate spectrum.

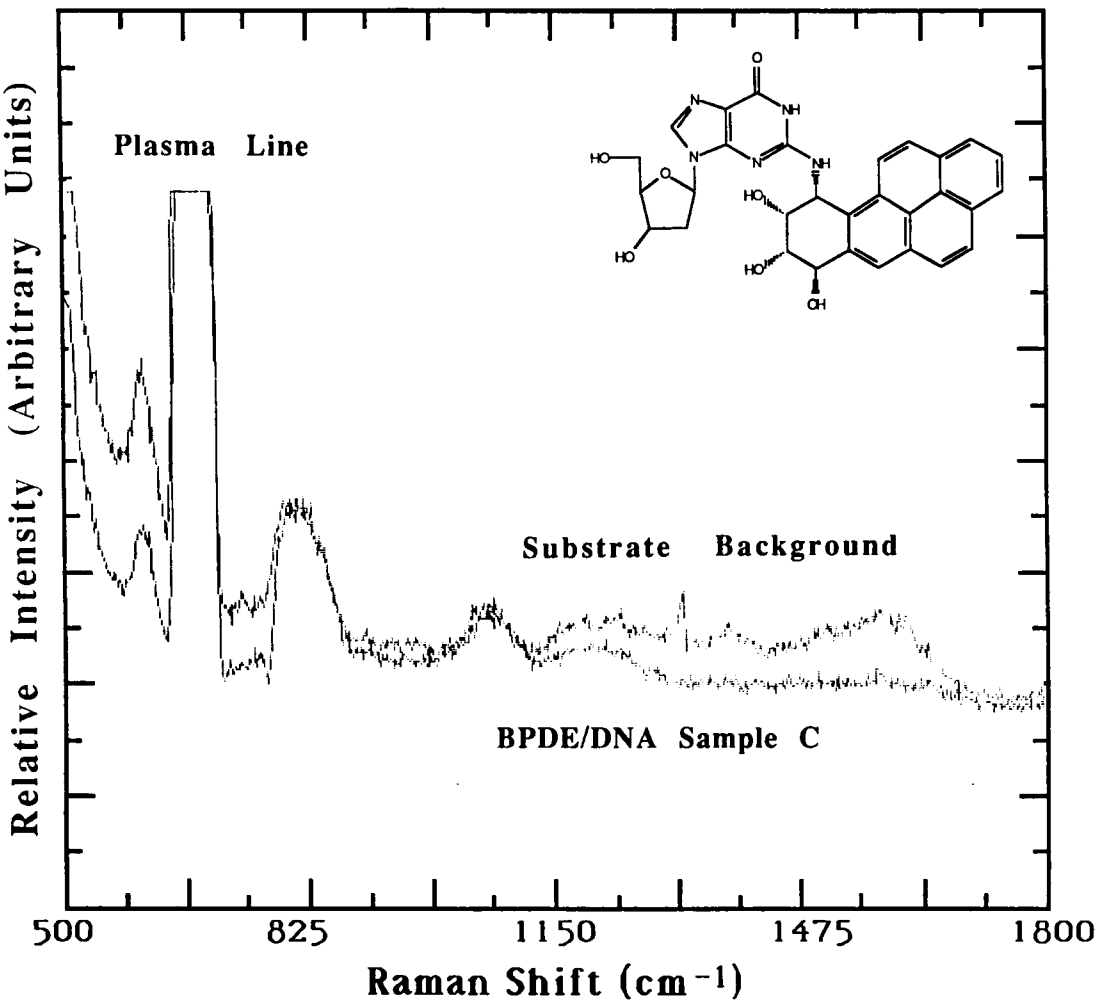
A study was performed in which Sample B (320-μg/mL DNA in 300-μL water) was diluted 1:1 with water to reduce the spectral resolution problems that may have resulted from sample overloading. A SERS spectrum of the results of this study is shown in Figure IV-42. While the BPDE/DNA:water sample differed from the substrate background, no sharp SERS bands attributable to BPDE were seen. The DNA concentration was probably still high



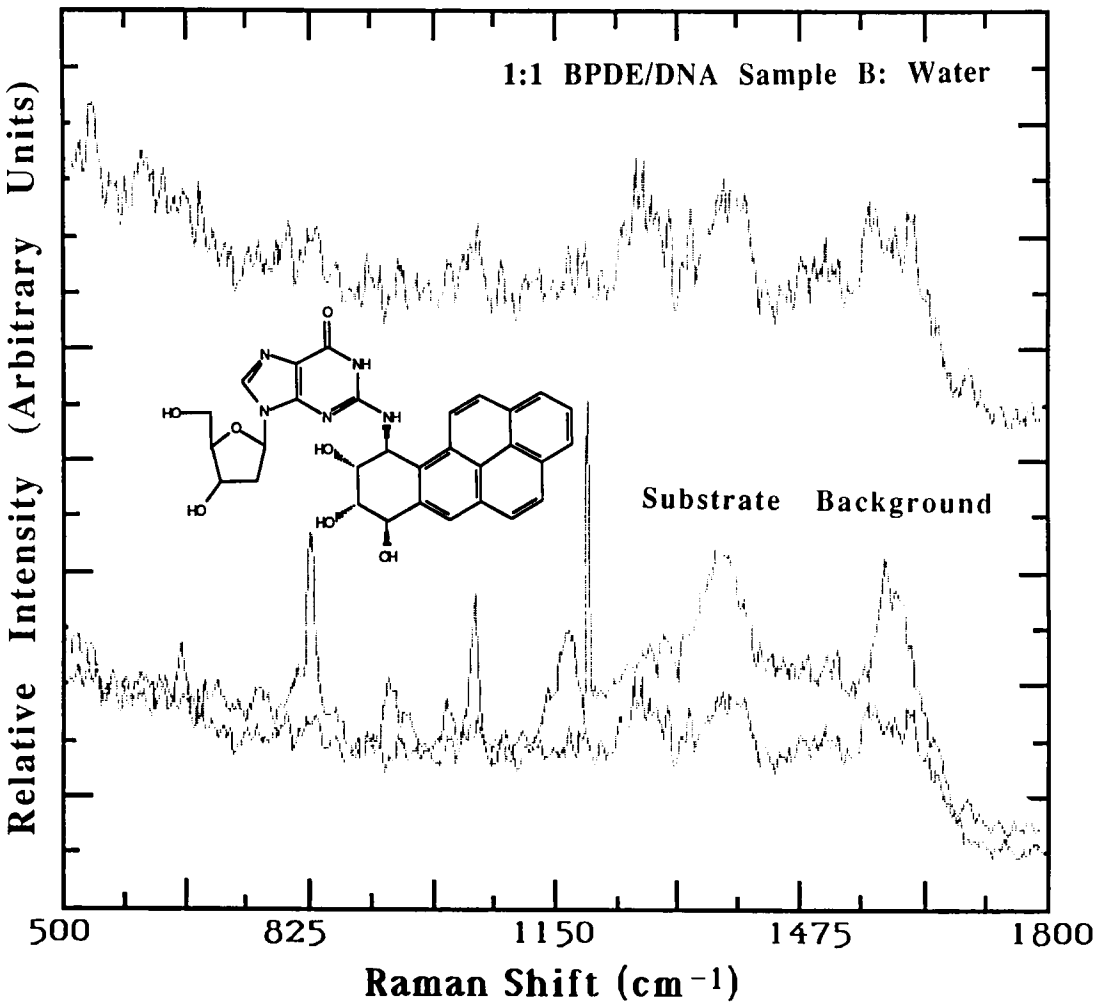

<FIGURE IV-39> SERS Spectrum of BPDE/DNA Sample A Sample was 1.0-μL of sample A (1000-μg/mL DNA in 100-μL water). Laser excitation was 60-mW at 647.1-nm.




**<FIGURE IV-40> SERS Spectra of the Substrate Background and BPDE/DNA Sample B** Sample was 1.0-µL of sample B (320-µg/mL DNA in 300-µL water). Laser excitation was 55-mW at 647.1-nm. Spectra were printed to scale relative to each other.




**<FIGURE IV-41> SERS Spectra of the Substrate Background and BPDE/DNA Sample C** Sample was 1.0-µL of sample C (1000-µg/mL DNA in 100-µL water). Laser excitation was 60-mW at 647.1-nm.



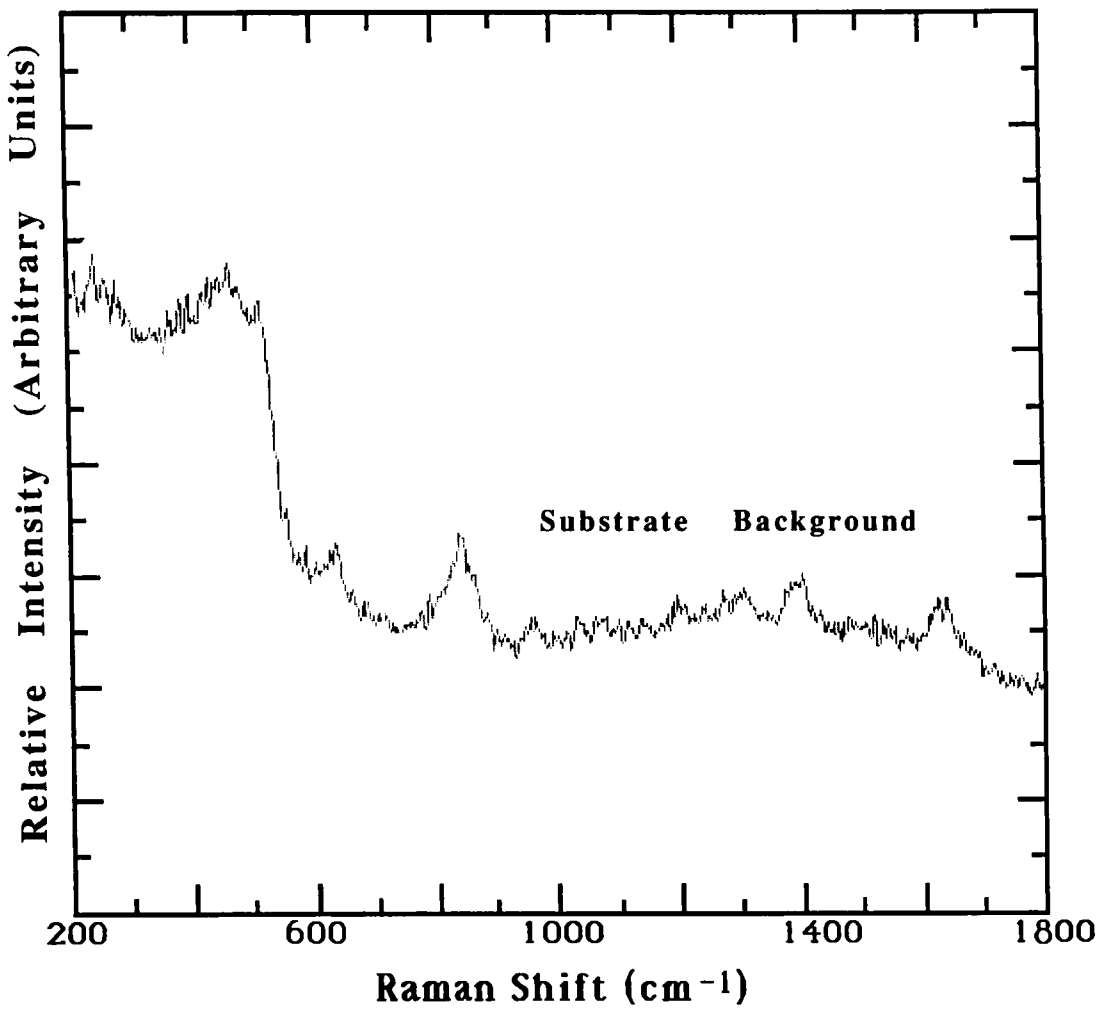

<FIGURE IV-42> SERS Spectra of the Substrate Background and Diluted BPDE/DNA Sample B Sample was 1.0-μL of 1:1 sample B:water. Laser excitation was 70-mW at 616-nm (Ar+ laser with dye head) using a direct excitation/collection optical setup.

enough to cause problems with the substrate effectiveness. For this spectrum, the laser excitation was 616-nm from the argon-ion laser with tunable dyehead (647.1-nm not available; argon-ion blue and green lines were too energetic). The sharp substrate background bands probably resulted from partial laser burnthrough of the 616-nm narrow bandpass filter.

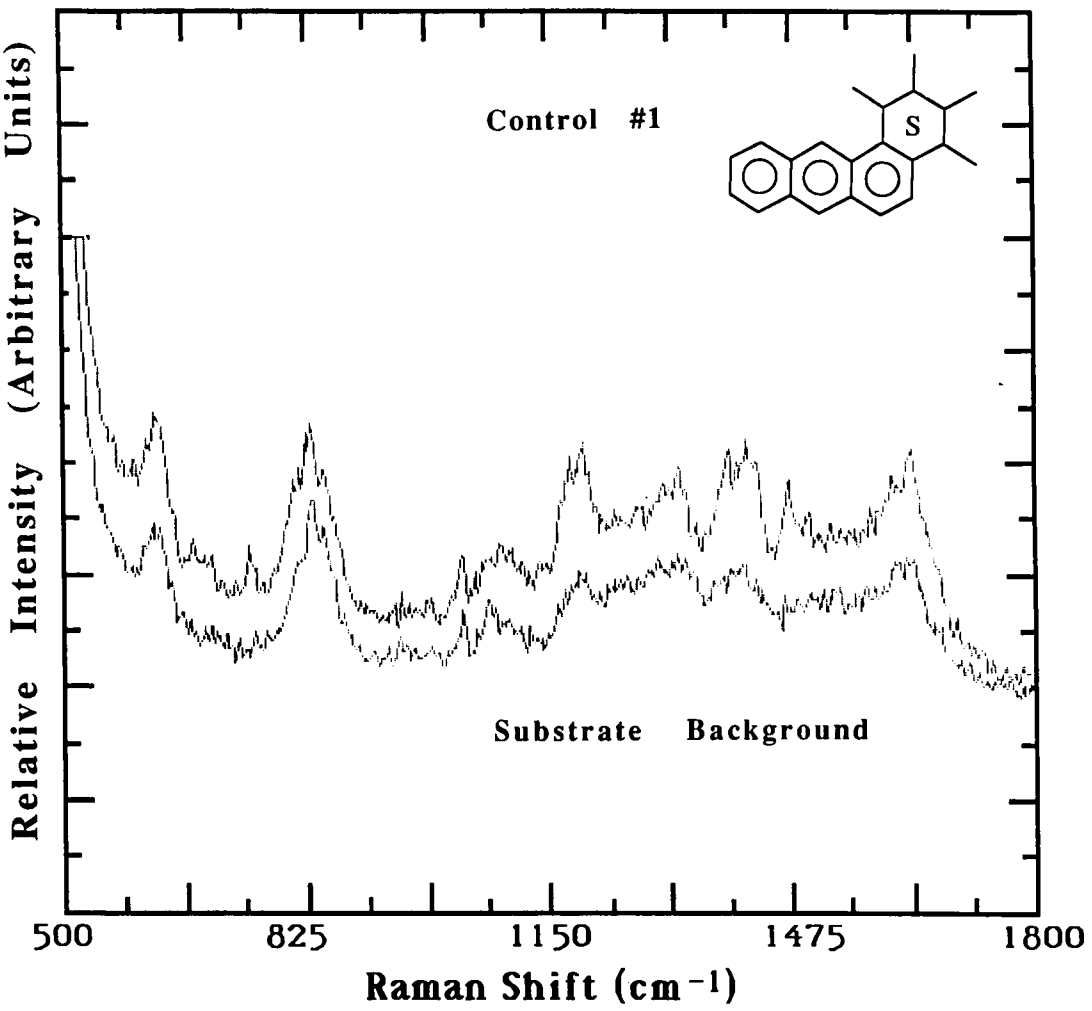
Benz[a]anthracene Epoxides Reacted with DNA

A substrate background spectrum for this experiment is given in Figure IV-43. Spectra of the epoxide controls are shown in Figure IV-44 (Control #1), Figure IV-45 (Control #2), and Figure IV-46 (Control #3). The three epoxide controls are compared with the substrate background in Figure IV-47. All controls could be distinguished from the background, although the signal-to-noise ratios were generally low. Spectra comparing only Controls #2 and #3 with the background are shown in Figure IV-48. The essential difference between spectra of Controls #2 and #3 is a band appearing near 1400 $cm^{-1}$ for Control #2.

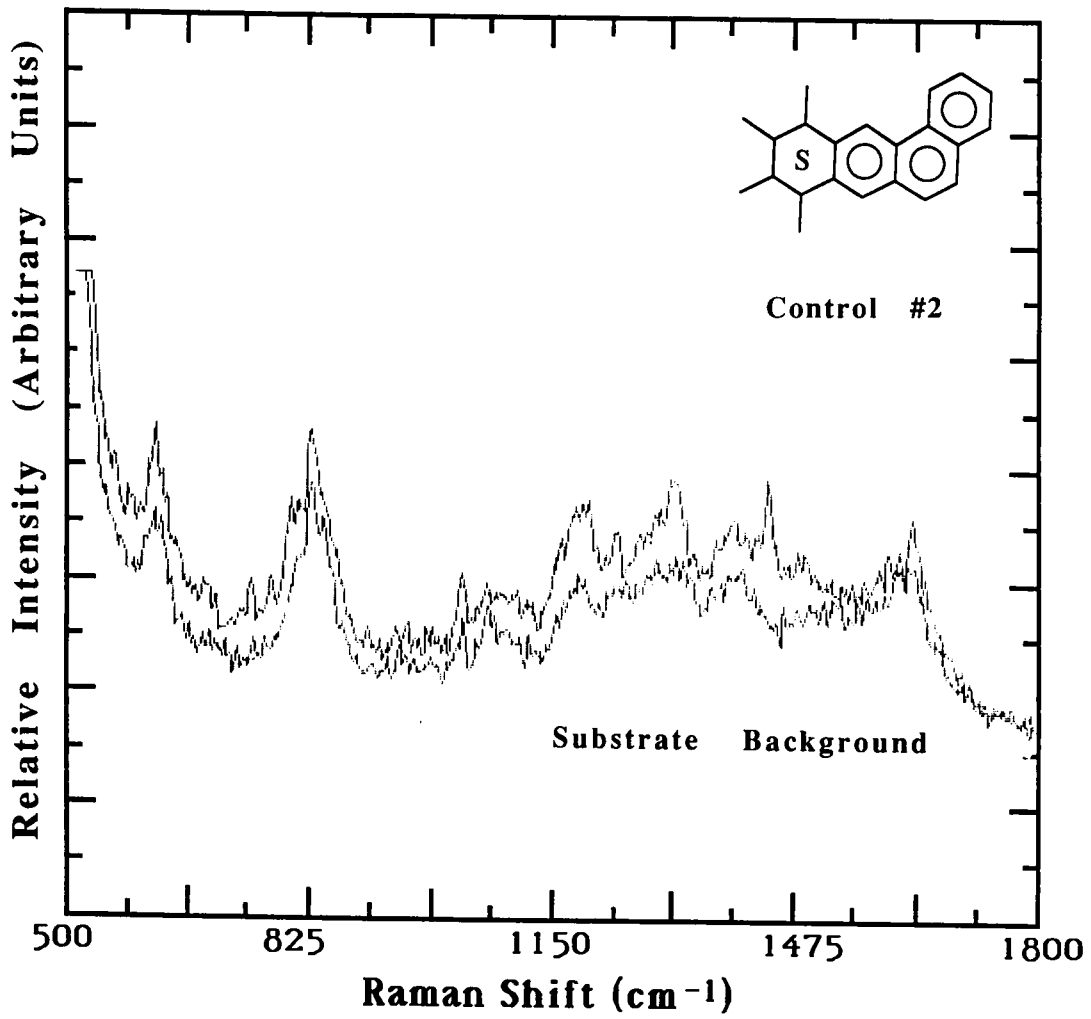
The control samples were very visuous and difficult to manipulate for examination with SERS. While there was little evidence of sample overloading (as seen or the BPDE/DNA adduct samples) a control solution was serially diluted in an attempt to produce a clearer, more meaningful spectrum. Control #3 (3-methylcholanthrene-11,12-dihydroepoxide) was selected for the dilution study because it was available in a larger quantity than were the other epoxide samples. Sample spectra of Control #3 diluted with water are given in Figure IV-49 (1:1 Control #3:water), Figure IV-50 (1:10 Control #3:water), Figure IV-51 (1:1000 and 1:100 Control #3:water), Figure IV-52 ($1:1x10^5$ and $1:1x10^4$ Control #3:water), and Figure IV-53 ($1:1x10^6$ Control#3:water). Dilution of the control



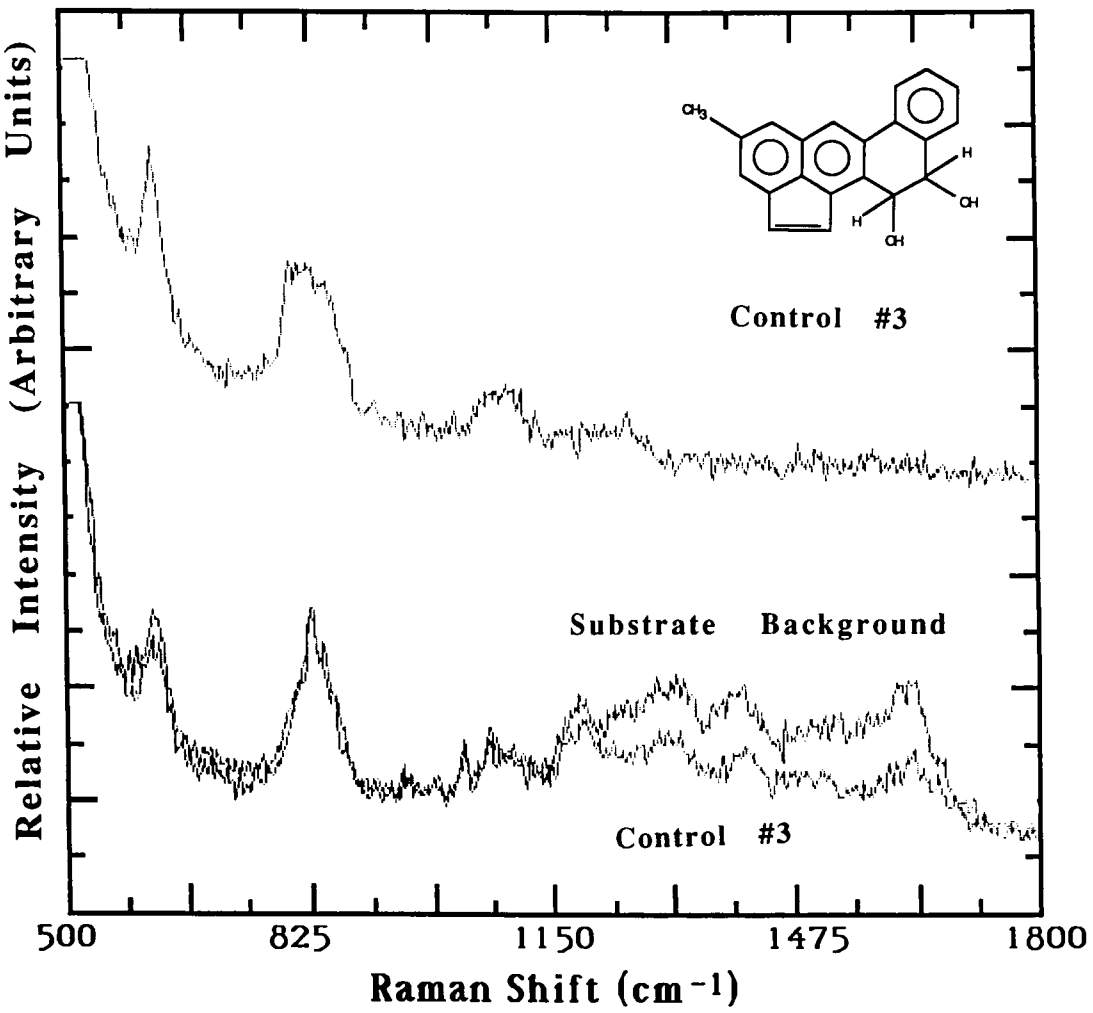

**<FIGURE IV-43> SERS Spectrum of the Alumina-Type Substrate Background for the Benz[a]anthracene Epoxide Study** Laser excitation was 50-mW at 647.1-nm.



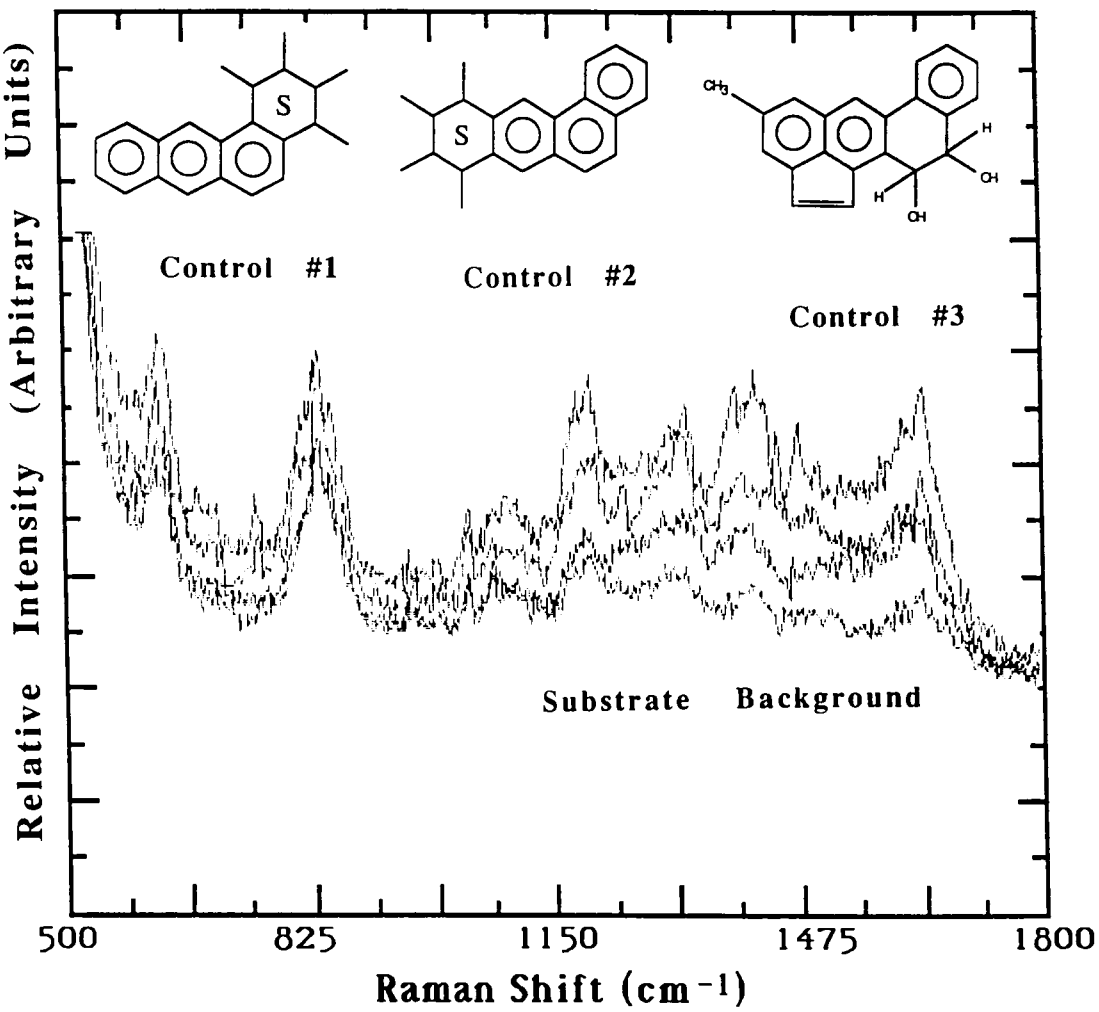

**<FIGURE IV-44> SERS Spectra of Benz[a]anthracene Epoxide Control #1 and the Substrate Background** Sample was 1.0-μL of a benzo[a]-anthracene-trans-10,11-dihydrodiol-8,9-epoxide solution. Laser excitation was 70-mW at 647.1-nm. Spectra were printed to scale relative to each other.



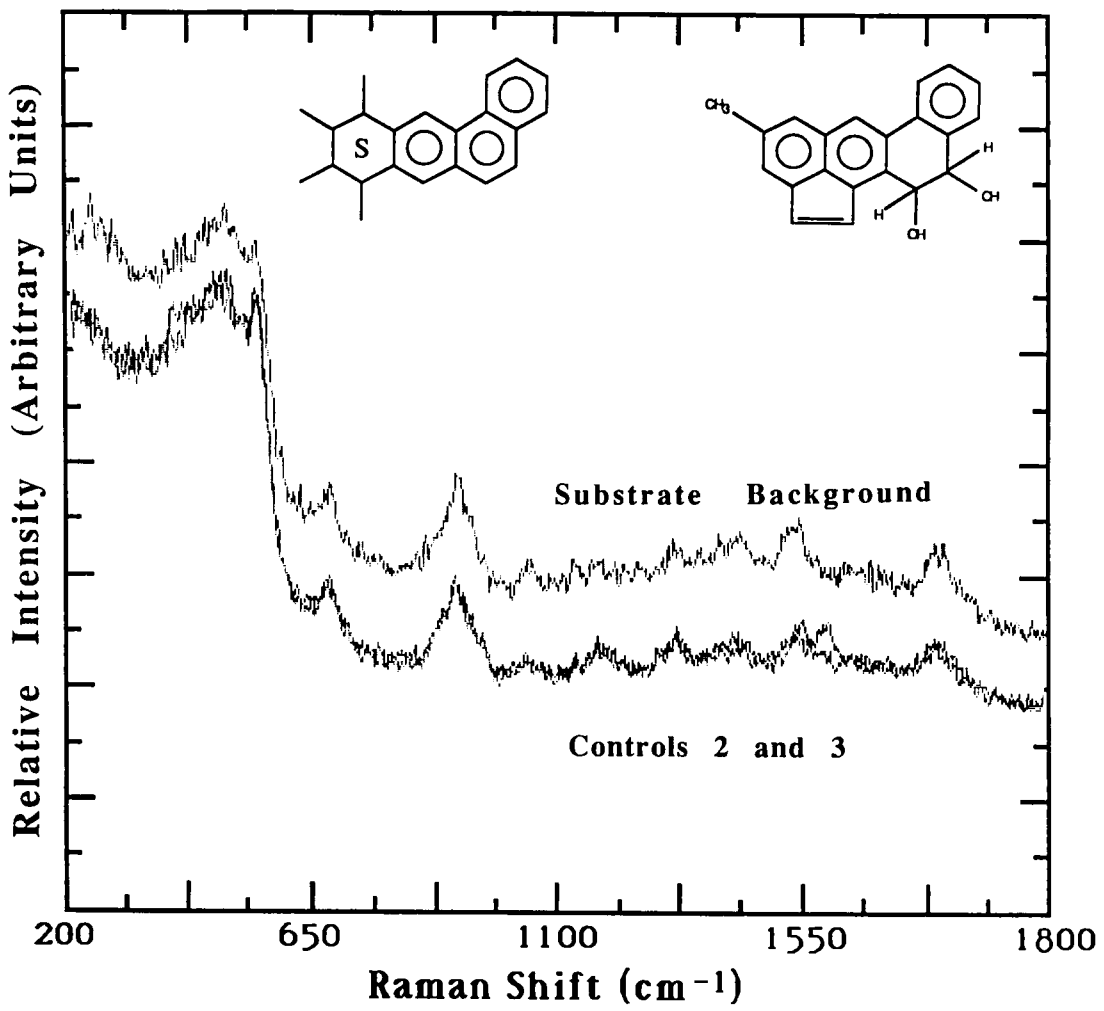

<FIGURE IV-45> SERS Spectra of Benz[a]anthracene Epoxide Control #2 and the Substrate Background Sample was 1.0-μL of a benz[a]-anthracene-trans-3,4-dihydrodiol-1,2-epoxide solution. Laser excitation was 70-mW at 647.1-nm.



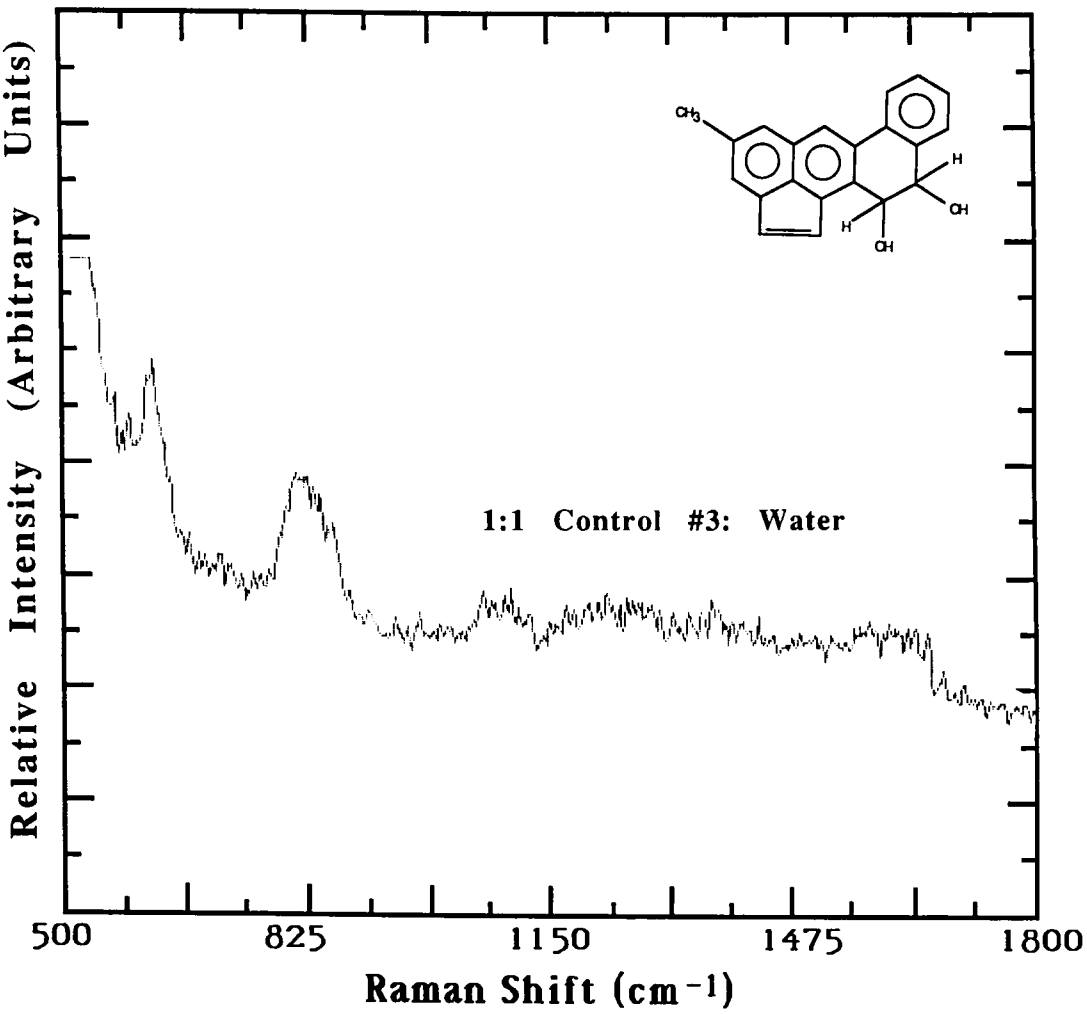

<FIGURE IV-46> SERS Spectra of Benz[a]anthracene Epoxide Control #3 and the Substrate Background Spectra are of (Top) 1.0-μL of control #3 (3-methylcholanthrene-11,12-dihydroepoxide), (Center and Bottom) the substrate background, and control #3. Laser excitation was 50-mW at 647.1-nm. Center and Bottom spectra were printed to scale relative to each other.



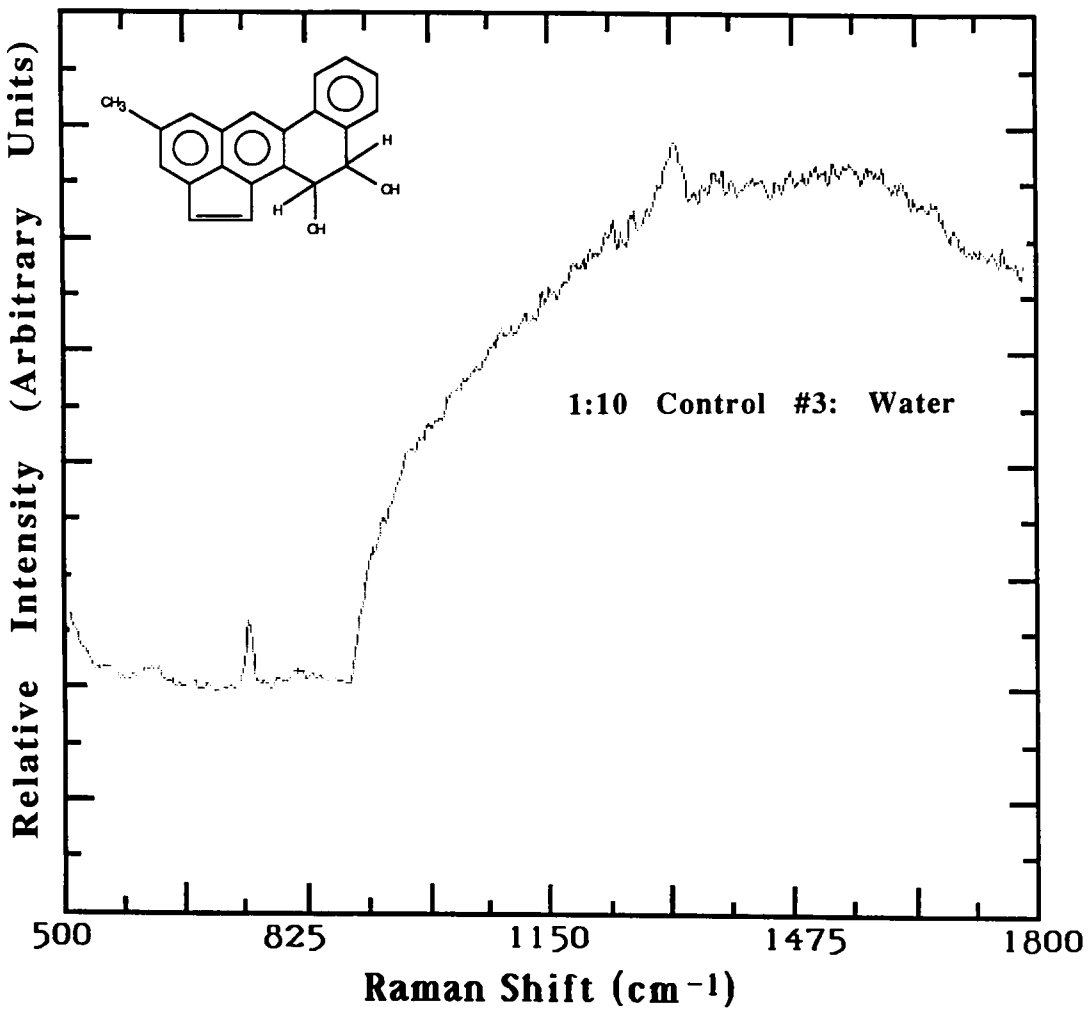

<FIGURE IV-47> **SERS Spectra of Benz[a]anthracene Epoxide Controls and the Substrate Background** Spectra are of control #1, control #2, control #3, and the substrate background. Laser excitation was 70-mW at 647.1-nm. Spectra were printed to scale relative to each other.



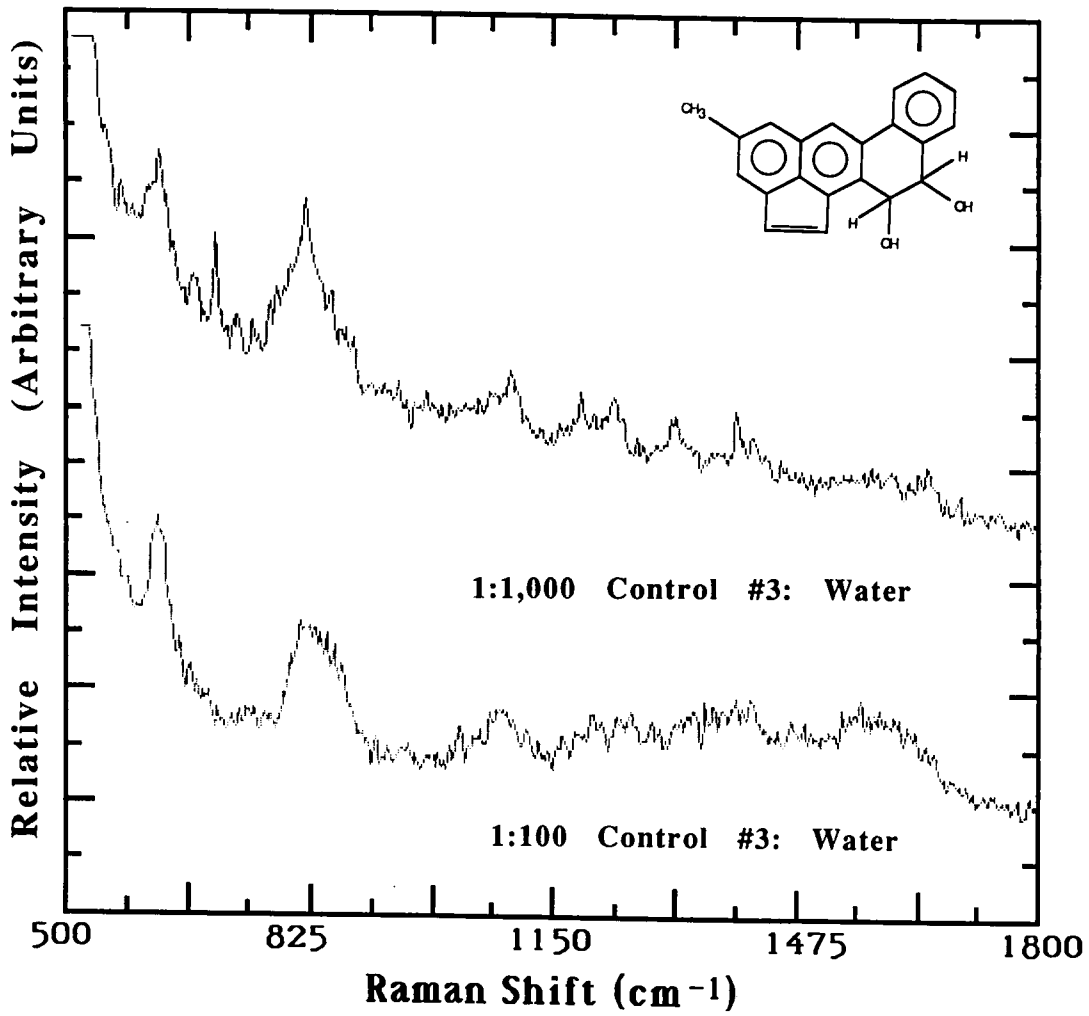

**<FIGURE IV-48> SERS Spectra of the Substrate Background and Benz[a]anthracene Controls #2 and #3** Control #2 has the spectral band near 1400 $cm^{-1}$. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.



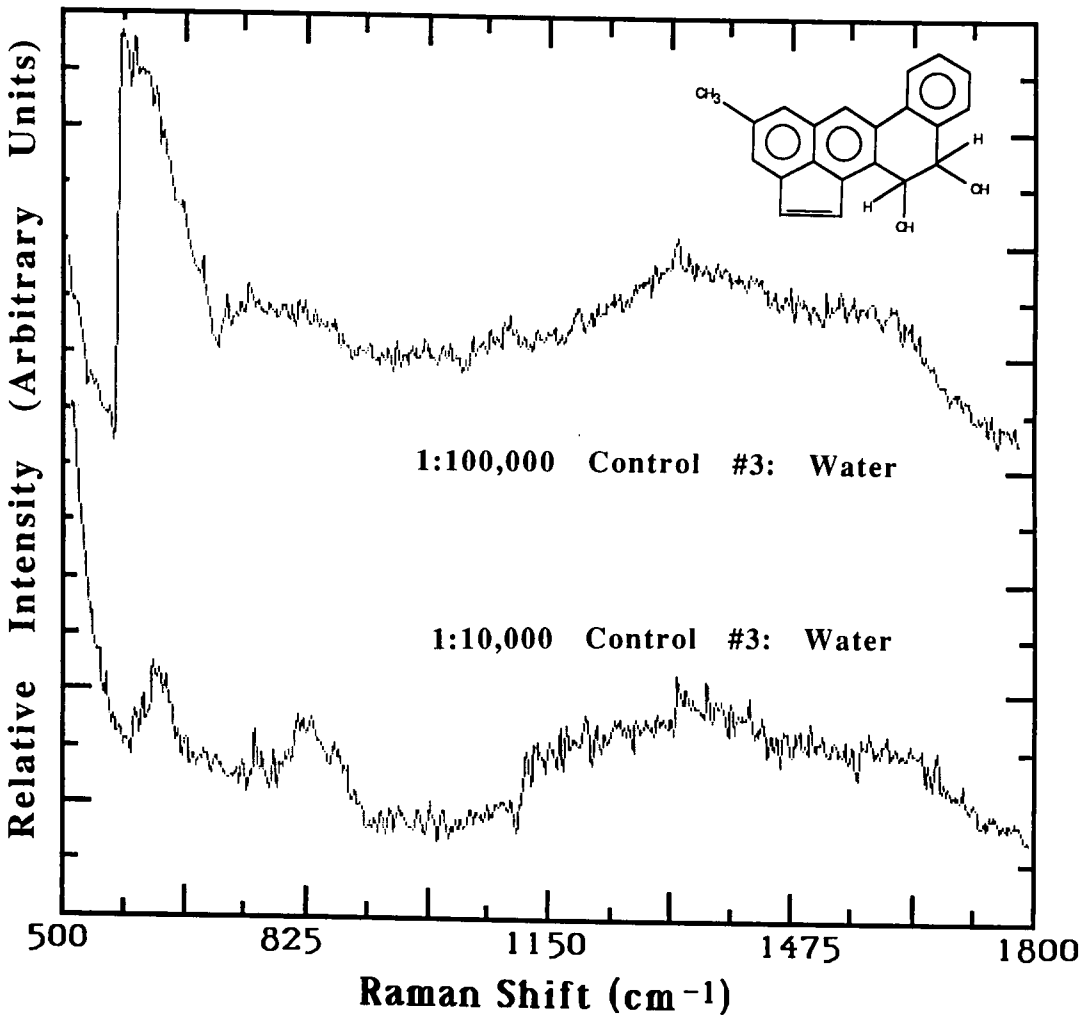

**<FIGURE IV-49> SERS Spectrum of 1:1 (v/v) Control #3:Water** Sample was 1.0-µL. Laser excitation was 50-mW at 647.1-nm.



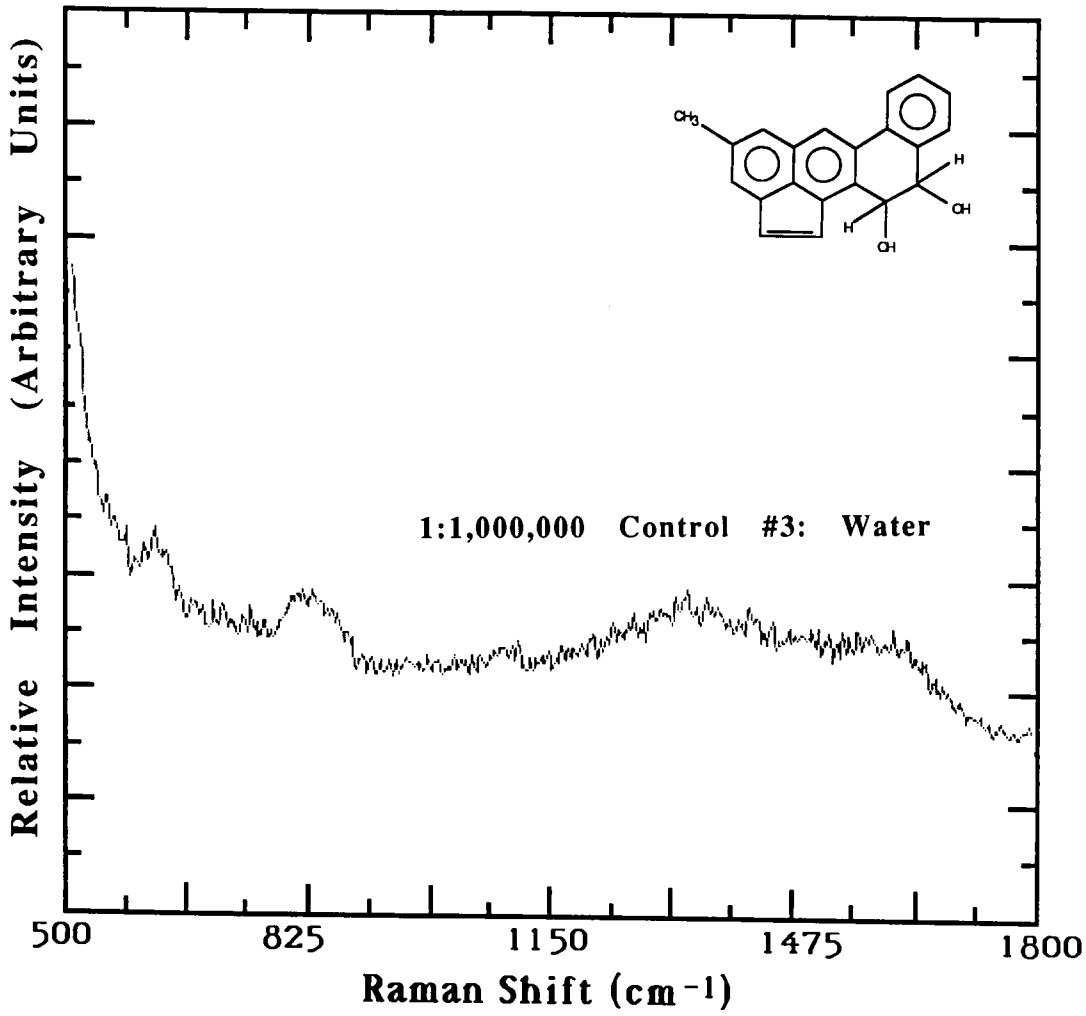

**<FIGURE IV-50> SERS Spectrum of 1:10 (v/v) Control #3:Water** Sample was 1.0-μL. Laser excitation was 50-mW at 647.1-nm.




<FIGURE IV-51> SERS Spectra of 1:1000 Control #3:Water and 1:100 Control #3:Water Samples were each 1.0-µL. Laser excitation was 50-mW at 647.1-nm.




<FIGURE IV-52> SERS Spectra of 1:100,000 Control #3:Water and 1:10,000 Control #3:Water Sampes were each 1.0-µL. Laser excitation was 50-mW at 647.1-nm.




**<FIGURE IV-53> SERS Spectrum of 1:1,000,000 Control #3:Water** Sample was 1.0-μL. Laser excitation was 50-mW at 647.1-nm.

sample did not appreciably affect the quality of the SERS spectra.

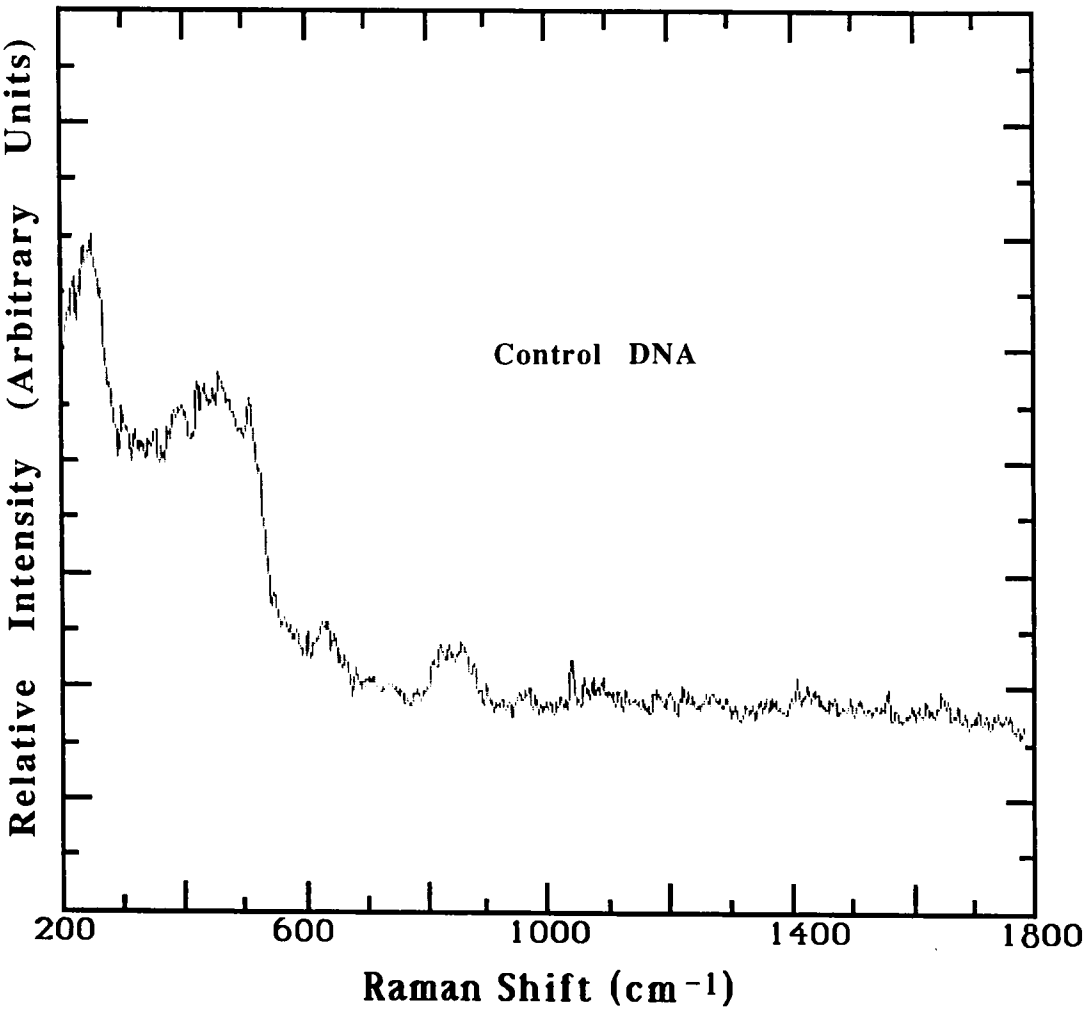
A SERS spectrum of the control DNA for the benz[a]anthracene epoxide study is shown in Figure IV-54. This DNA was not exposed to any epoxide. As expected, the DNA exhibited no SERS bands under these conditions.

Spectra for the DNA exposed to the epoxides (DNA adduct samples) are presented in Figure IV-55 (Adduct #1), Figure IV-56 (Adduct #2), and Figure IV-57 (Adduct #3). A comparison of the adducts with the control DNA is shown in Figure IV-58. All adduct samples could be clearly distinguished from the unaltered DNA, although the adducts could not really be distinguished from each other. The adducts are contrasted with the substrate background in Figure IV-59. The spectra of the adducts clearly differed from the background spectrum. The scale of Figure IV-59 was expanded to show greater spectral detail in Figure IV-60. SERS spectra obtained from the three adduct samples are shown in Figure IV-61.

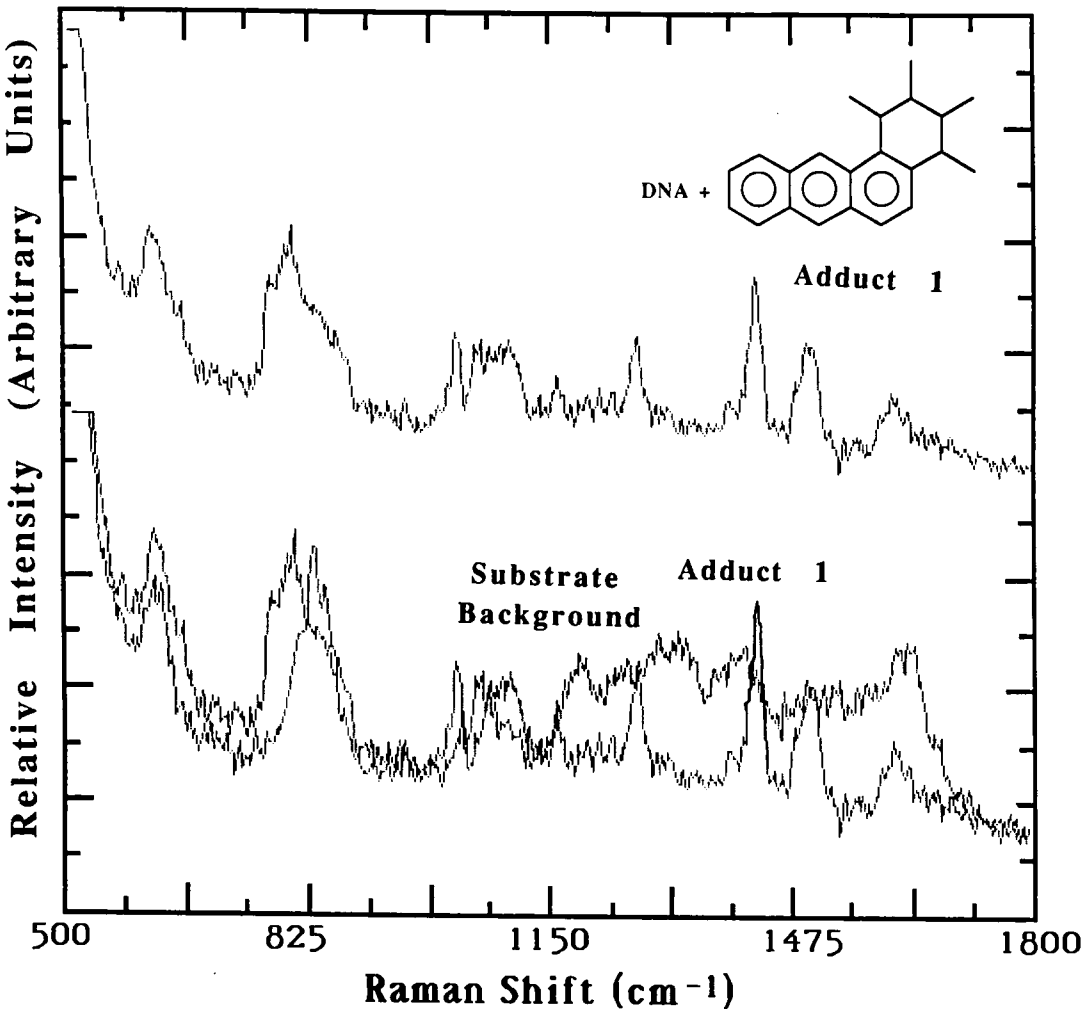
The spectra of the control epoxides could, to a certain extent, be distinguished. The spectral traces of the DNA adducts formed from exposure to the same three epoxides could not be differentiated except through direct comparison of the adducts. The minute differences in the wavenumbers of the SERS bands of the adducts might permit identification of the adducts when all three may be compared, but it is unlikely that a definitive chemical assignment could be made to any one sample without comparison spectra.

### *Summary*

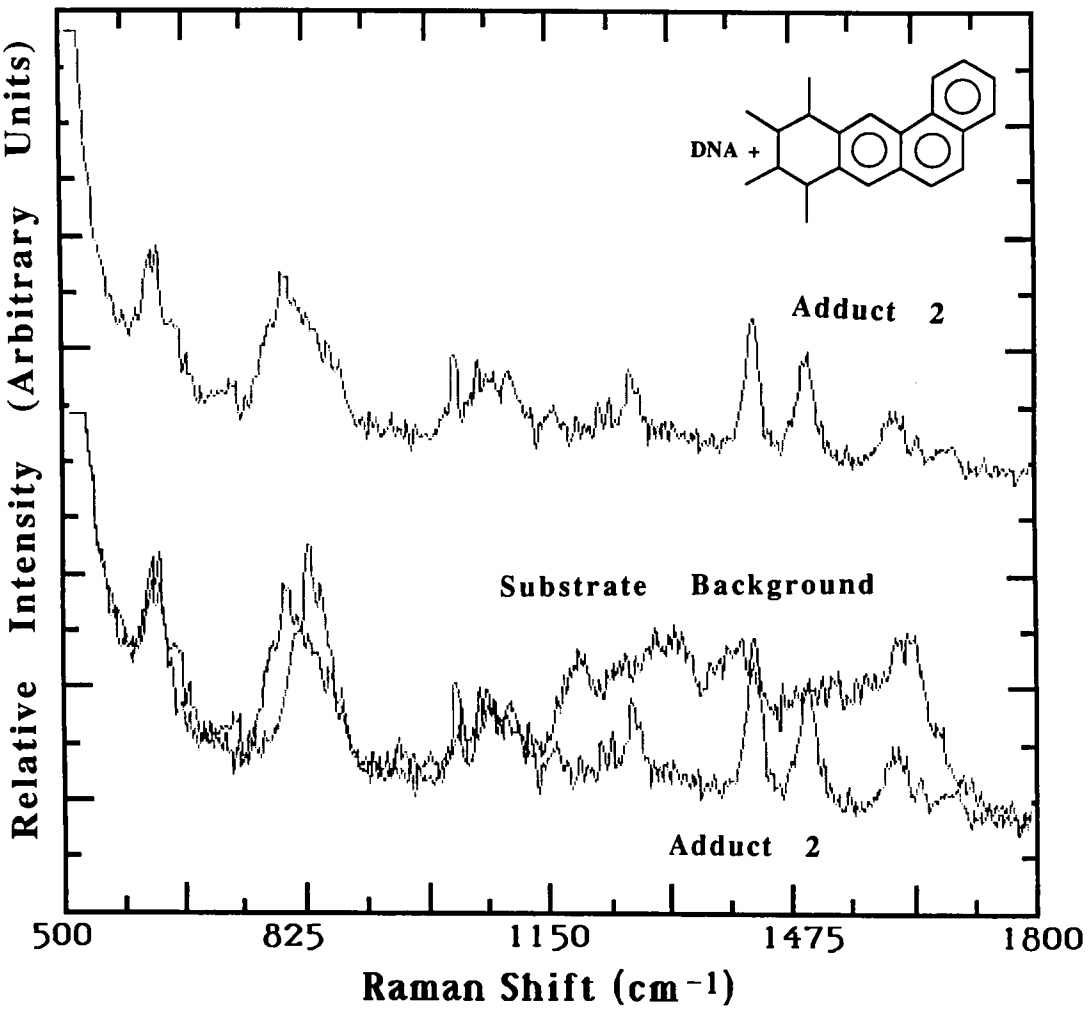
A true test of the effectiveness of SERS as applied to biomedical problems is its usefulness in detecting SERS-active chemicals in valuable, real-life



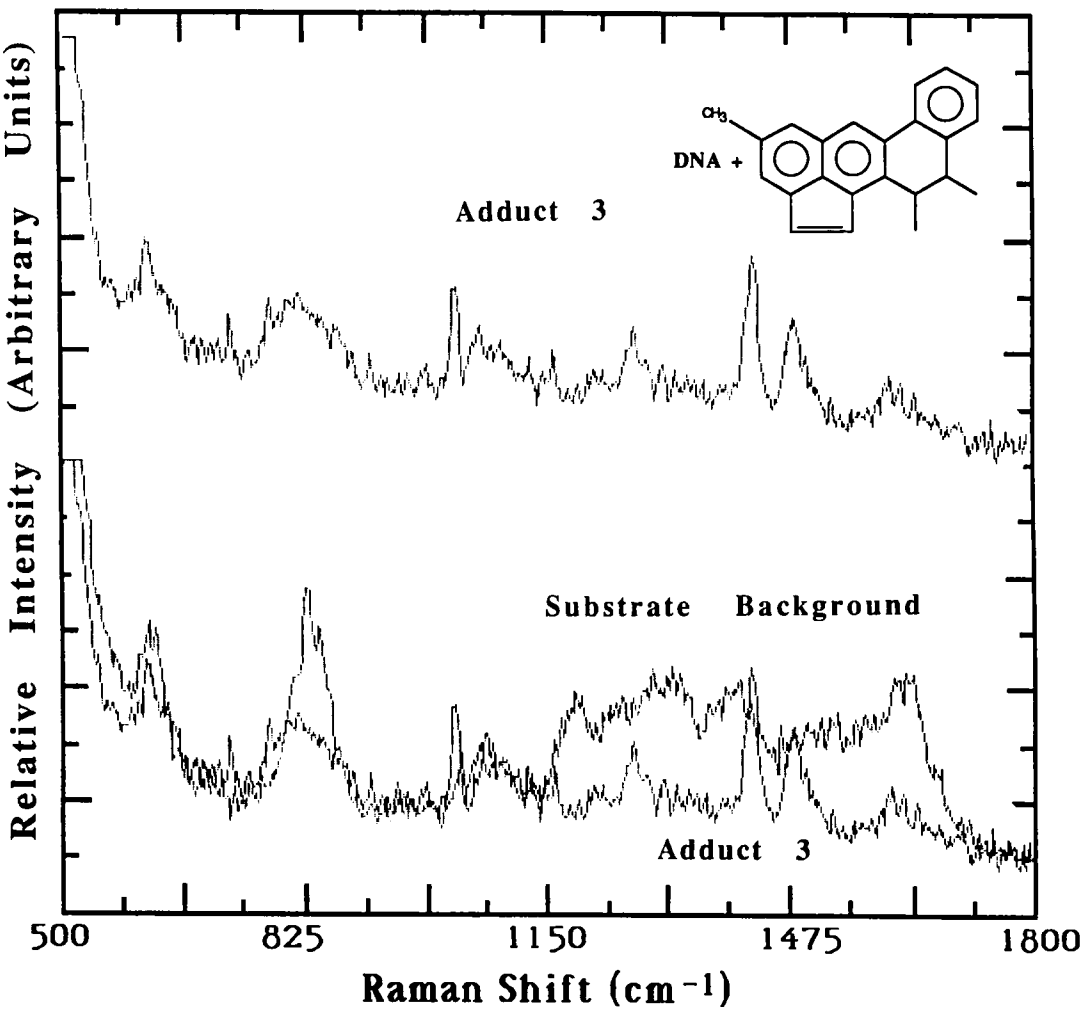

**<FIGURE IV-54> SERS Spectra of the Control DNA for the Benz[a]anthracene Epoxide Study** Sample was 1.0-μL. Laser excitation was 50-mW at 647.1-nm.



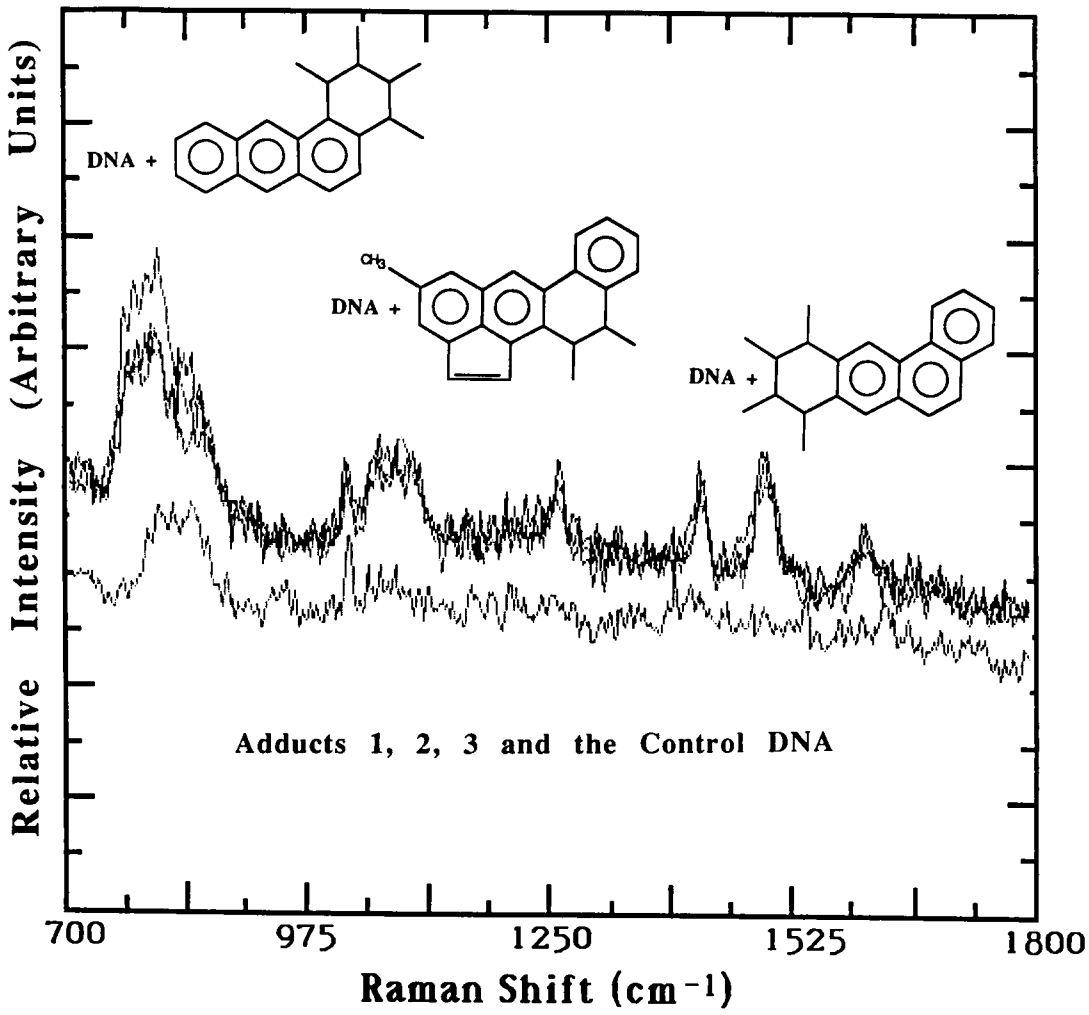

**<FIGURE IV-55> SERS Spectra of Benz[a]anthracene Epoxide DNA Adduct #1 and the Substrate Background** Spectra are of (Top) 1.0-μL adduct #1 (DNA + benzo[a]anthracene-trans-10,11-dihydrodiol-8,9-epoxide), (Center and Bottom) substrate background, and adduct #1. Laser excitation was 70-mW at 647.1-nm. Center and Bottom spectra were printed to scale relative to each other.



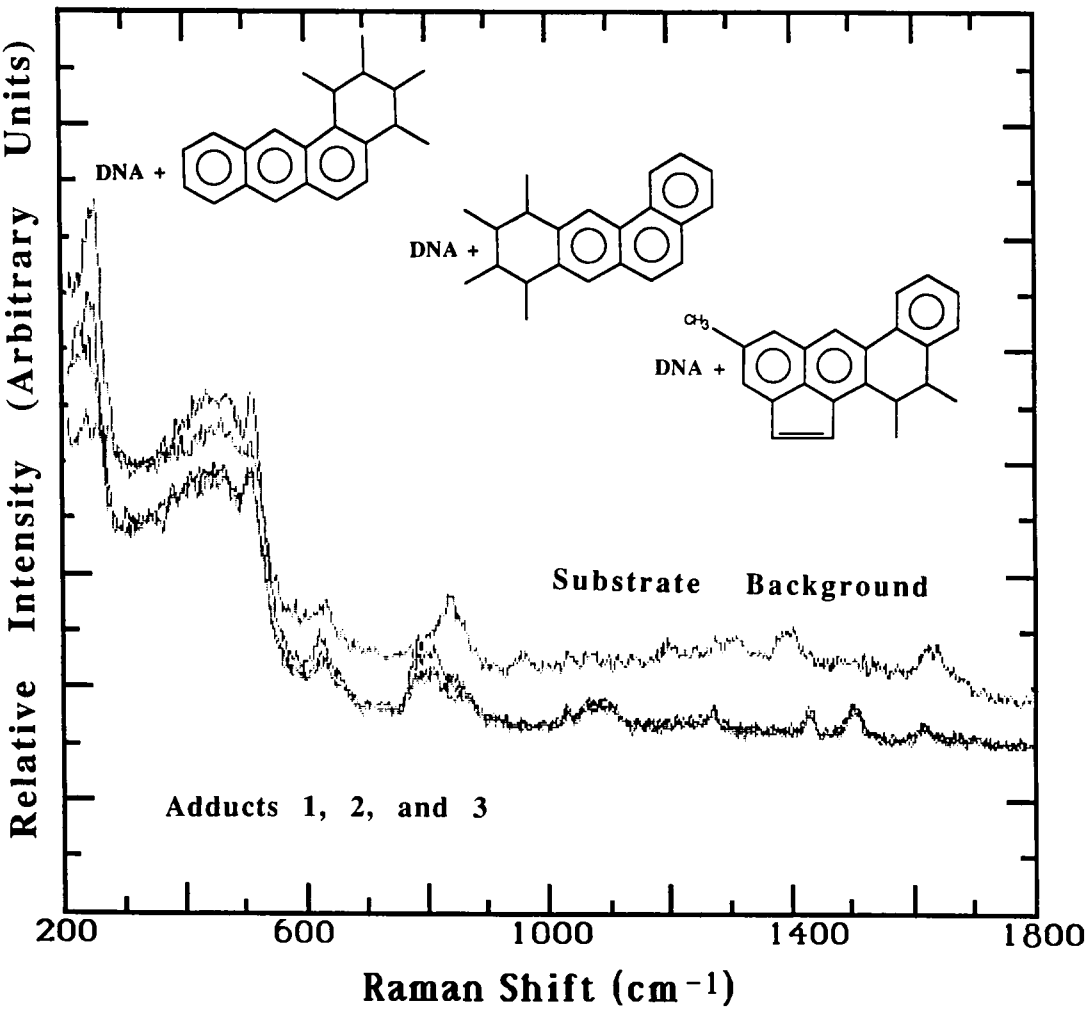

<FIGURE IV-56> SERS Spectra of Benz[a]anthracene Epoxide DNA Adduct #2 and the Substrate Background Spectra are of (Top) 1.0-μL of adduct #2 (DNA + benz[a]anthracene-trans-3,4-dihydrodiol-1,2-epoxide), (Center and Bottom) the substrate background, and adduct #2. Laser excitation was 70-mW at 647.1-nm. Center and Bottom spectra were printed to scale relative to each other.



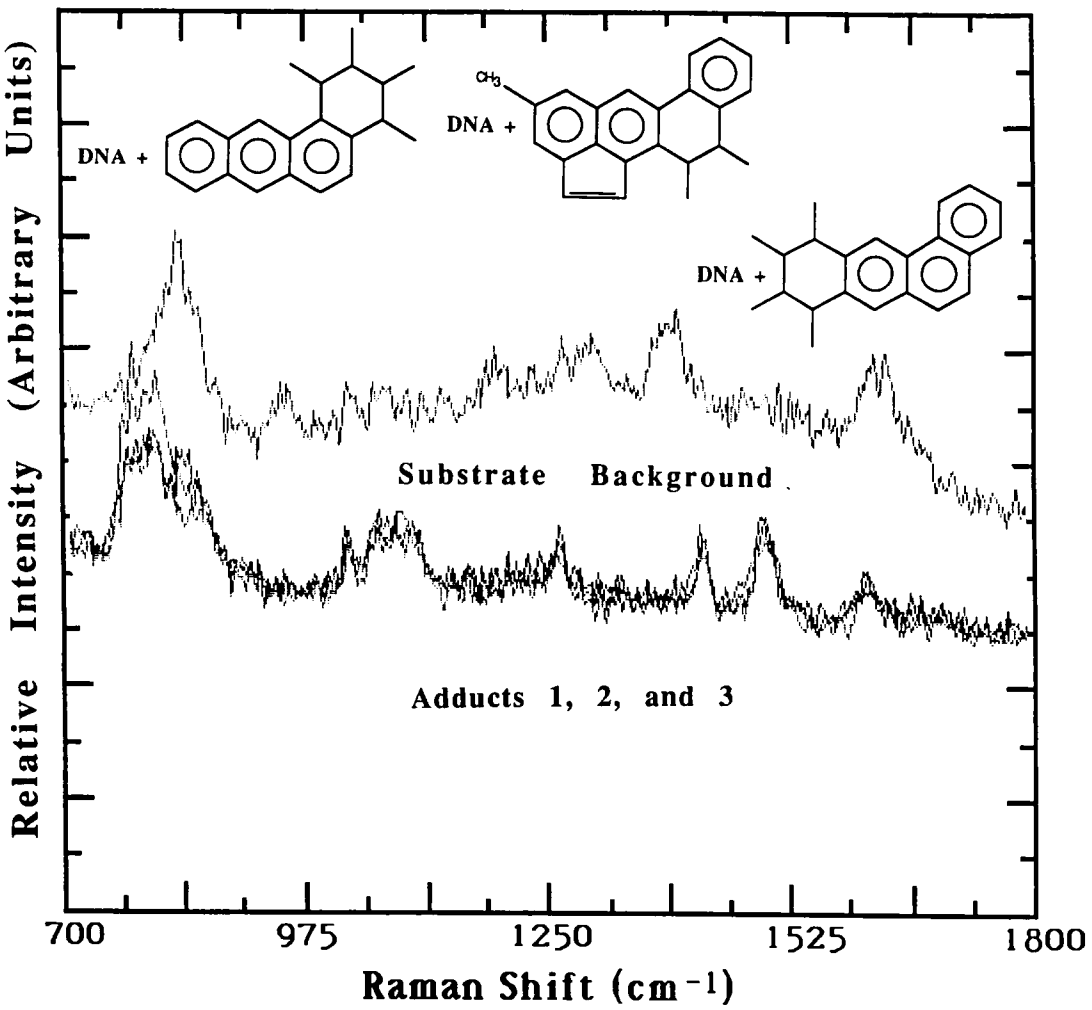

**<FIGURE IV-57> SERS Spectra of Benz[a]anthracene Epoxide DNA Adduct #3 and the Substrate Background** Spectra are of (Top) 1.0-μL of adduct #3 (DNA + 3-methylcholanthrene-11,12-dihydroepoxide), (Center and Bottom) substrate background, and adduct #3. Laser excitation was 70-mW at 647.1-nm. Center and Bottom spectra were printed to scale relative to each other.



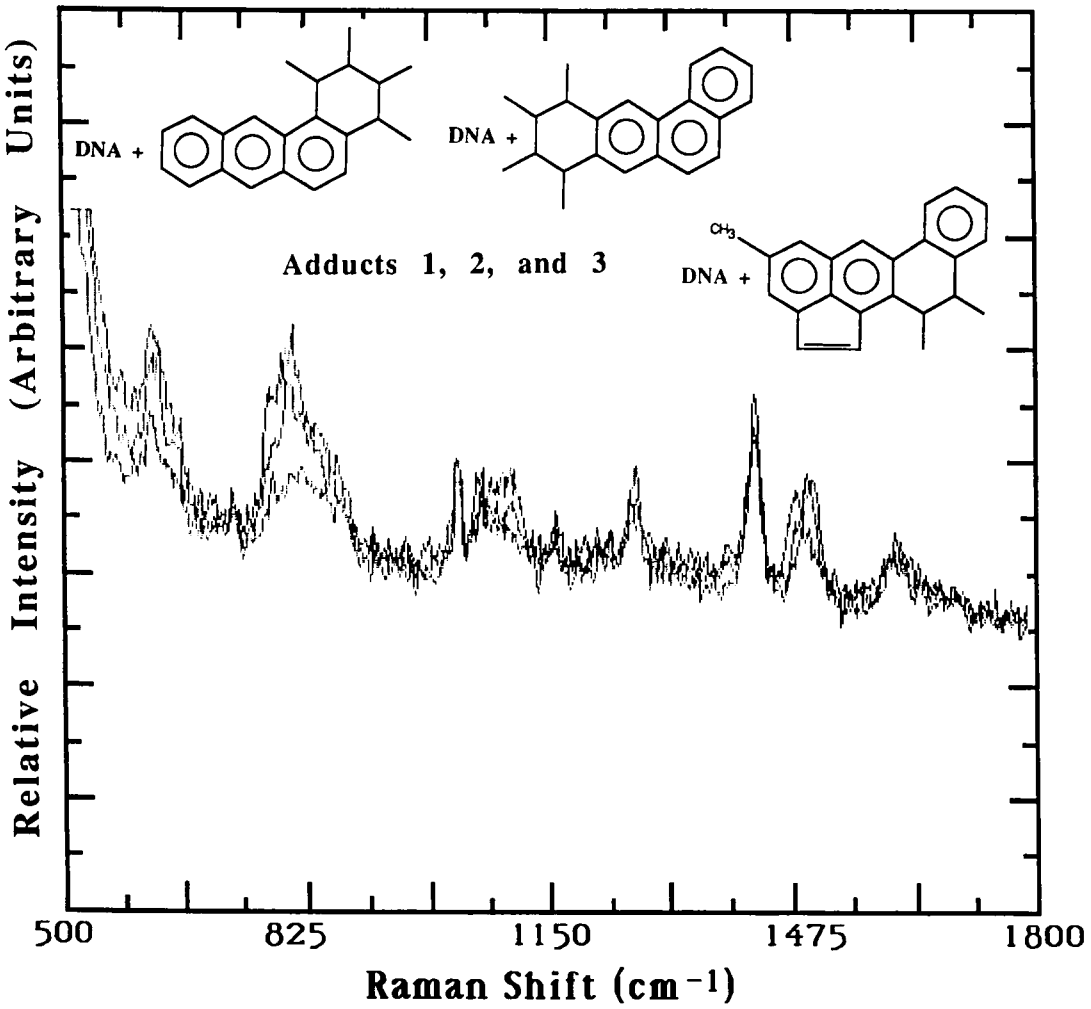

**<FIGURE IV-58> SERS Spectra of the Benz[a]anthracene Epoxide DNA Adducts and the Control DNA** The top three spectra are adducts #1, #2, and #3. The bottom spectrum is of the control DNA. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.




**<FIGURE IV-59> SERS Spectra of the Substrate Background and the Benz[a]anthracene Epoxide DNA Adducts** The top spectrum is of the substrate background. The bottom spectra are of adducts #1, #2, and #3. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.




<FIGURE IV-60> SERS Spectra of the Substrate Background and Three Adduct Samples Expanded to Show Detail The top spectrum is the substrate background. The bottom spectra are of adducts #1, #2, and #3. Laser excitation was 50-mW at 647.1-nm. Spectra were printed to scale relative to each other.




**<FIGURE IV-61> SERS Spectra of the Benz[a]anthracene Epoxide DNA Adducts** Spectra are of adducts #1, #2, and #3. Laser excitation was 70-mW at 647.1-nm. Spectra were printed to scale relative to each other.

samples. The examination of DNA samples is especially promising for SERS because both normal and modified components of DNA, as well as helical DNA may be detected and distinguished from each other. This is not true of other spectroscopic techniques, such as fluorescence, which can detect altered purines or pyrimidines, but not the original species found in DNA. SERS effectiveness is not compromised by the use of aqueous solutions, so a wide range of biological samples may be studied.

The BPDE/DNA samples could not be expected to exhibit SERS spectra as spectacular as those obtained from the DNA/CAA reaction for two reasons. The first and primary reason is that the BPDE/DNA samples were DNA obtained from humans and this DNA was not as highly purified as the highly polymerized calf thymus DNA eventually acquired from the DNA/CAA reaction. As has been demonstrated by the results of these experiments, sample purity has a definite effect on the quality of resultant SERS spectra. The second reason the BPDE/DNA samples were not expected to produce spectra of the same quality as those of the DNA/CAA experiment is that the etheno DNA-adducts could be detected by SERS at extremely low concentration levels. While B[a]P, BPT, and BPDE may all be detected with SERS, they have not been detected at the nanomole level at which 3,$N^4$-ethenocytosine was detected. It is possible that the tentative ethenocytosine signal seen in Figure IV-36 and Figure IV-37 is real and that the ethenocytosine scattering is more intense than the scattering off alkyl-guanine, the predominant altered base from the DNA/CAA reaction.

The results of the benz[a]anthracene epoxide study indicated that SERS may be used to distinguish the epoxides from the background noise and, to a certain extent, from each other. The adducted DNA could be distinguished from

the background noise and the unaltered DNA. The DNA adduct spectra were very similar to each other and to the epoxides used to induce the adduct formation.

The importance of these experimental results was that SERS was demonstrated to be an effective means of discriminating between intact DNA and DNA that has been exposed to and altered by a hazardous chemical. In addition, SERS could often be used to distinguish the altered DNA from the chemical that induced the damage (e.g. chloroacetaldehyde/etheno DNA adducts, benzo[a]pyrene/BPDE-DNA adducts).

# CHAPTER 6

# CONCLUSIONS

SERS may be a valuable technique for the analysis of biological samples because of its chemical group specificity, use of minute sample volumes, and excellent sensitivity for many chemicals. It is unlikely that a SERS biosensor could incorporate all of the advantages of a highly-optimized SERS optical setup for use in an *in vivo* system. While the SERS fiberoptic setup shown in Figure I-4 (p. 18) was able to detect biologically-important substances such as genotoxins and their DNA adducts, these molecules were often at the detection limit of the technique. At present, the signal losses inherent in a biosensor from the use of long optical fibers and an inferior SERS substrate preclude the risk of its use in animal tissues. These studies have shown that one type of risk to organic molecules and tissue from the SERS substrate (toxicity of the silver) may be minimized by coating the substrate (glass plate or optical fiber) with a thin agarose gel. The agarose matrix did not interfere with the substrate effectiveness, but enhanced its sensitivity. Other polymer coatings or semi-permeable membranes may be found to have enhancing effects on substrate effectiveness.

The results of the SERS examination of molecules in complex mixtures such as plasma, serum, urine, and incorporated into DNA (all studied in Section IV) contrast greatly against results of SERS applied to pure samples in simple solutions (Section III). The results of the examination of aflatoxin in human urine extracts were inconclusive because the spectra were not reproducible (possibly from sample overloading, high molecular weight contaminants, low analyte concentration, or use of PBS as a solvent). While SERS could be used to

detect a difference between pure plasma or serum and plasma or serum infused with toxins, SERS could not detect intense spectral bands from the sample molecules.

The sample spectra were more informative when SERS was used to scan purified DNA adducts. While no SERS bands were elicited from the pure DNA, the genotoxins and adducts frequently yielded useful spectra. In all instances, the adducts could be distinguished from the substrate background. SERS was shown to have great potential for assessing the hazards of exposure to low levels of toxins because it could detect minimal alteration of the DNA molecule.

# SECTION IV

# REFERENCES

## REFERENCES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# PART V: CONCLUSION

# CHAPTER 1

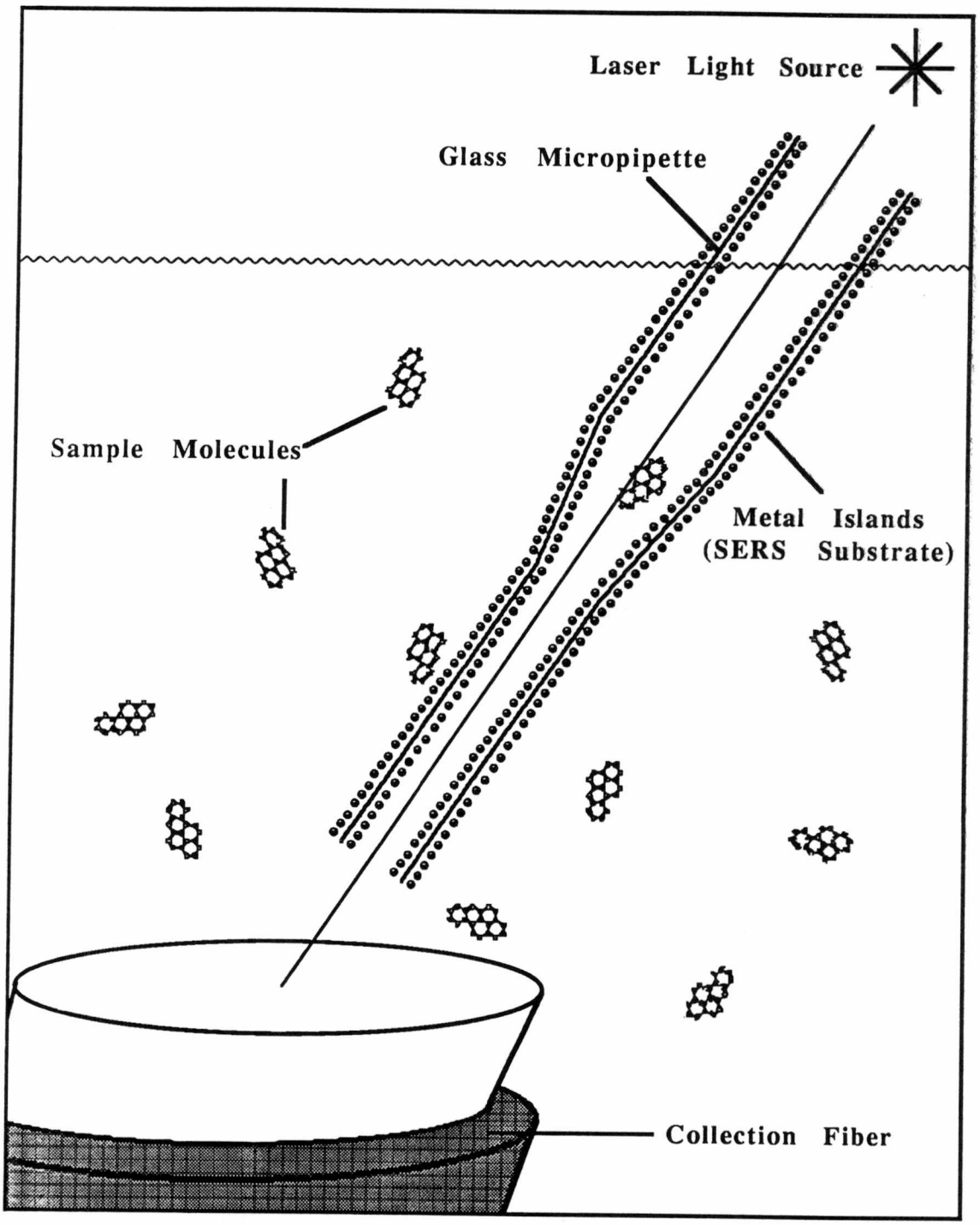
# FUTURE DIRECTIONS

## *Introductory Remarks*

Many possibilities exist for performing SERS other than with a conventional optical setup. Some of the options for expanding the capabilities of SERS are described in this section. These possibilities were examined only cursorily, as a sort of feasibility study to determine their practical value for SERS now and to gauge the extent of time needed to bring them to a useful status. Some of these adaptations to a conventional SERS optical setup have already been successfullly implemented.

## *SERS Microlight*

An alternative SERS substrate would be a SERS microlight, diagrammed in Figure V-1. A microlight is a light source smaller than an optical wavelength. It is ideally capable of propagating a light beam through dimensions of one nanometer. The technique used for production of the microlights was adapted from a method outlined in a paper by Lieberman *et al.* (1). Note that Lieberman *et al.* grew miniaturized chemical crystals in their micropipettes (microlight inner diameter of 100-nm or less); microcrystals were not introduced into the pipettes used in this study. In these SERS experiments, a microlight was produced by heating a glass pipette in the flame of a bunsen burner and then allowing gravity or a vacuum to draw the glass into a hollow tube with a very small bore. Ideally, the bore should be less than




<FIGURE V-1> Diagram of a SERS Microlight

ten microns in diameter for useful application to SERS. Both Pyrex and Kimax pipettes were tested, with the Kimax pipettes yielding microlights with a smaller bore.

A microlight was made into a SERS substrate by either thermally evaporating a metal such as silver onto its surface (inner surface not coated) or chemically depositing a metal onto the inner and outer surfaces of the glass. A method for chemically depositing silver metal onto glass is outlined in Figure V-2. In all of these studies, silver was used preferentially over other metals because it is known to produce the most sensitive SERS substrates. A scanning electron (SEM) micrograph of a silvered Kimax microlight is presented in Figure V-3. Notice that the bore size of this microlight was not as small as would be desirable. One of the greatest problems in working with the microlights was that they were extremely fragile and were invisible to the human eye. The glass tip was brittle and easily broken and was too small to be seen with the naked eye. The location of the microlight end could not be predicted because the tip was flexible (similar to an optical fiber). Handling of the microlight was further complicated by the need to avoid touching the silver surface, which was easily removed or deformed. These difficulties would have to be overcome if the microlights were to be applied to a typical SERS experiment.

For SERS, laser excitation was focused into the microlight, where it interacted with the silver and sample. The microlight could be filled with a sample (solid crystals such as anthracene, liquid, or gas), immersed in a liquid sample, or doped with a sample solution. Scattered light was collected with an optical fiber placed near the sample.

In the experiments where microlights were tested as SERS substrates no

Tollen's Reagent (0.037-M)

Prepared by dissolving 2.0-g $AgNO_3$ in 300-mL water. 0.7-M $NH_4OH$ is added dropwise to the silver nitrate solution until a dark brown precipitant forms. Dropwise addition of $NH_4OH$ is continued until the precipitant dissolves. The solution is diluted with water to 500-mL total volume.

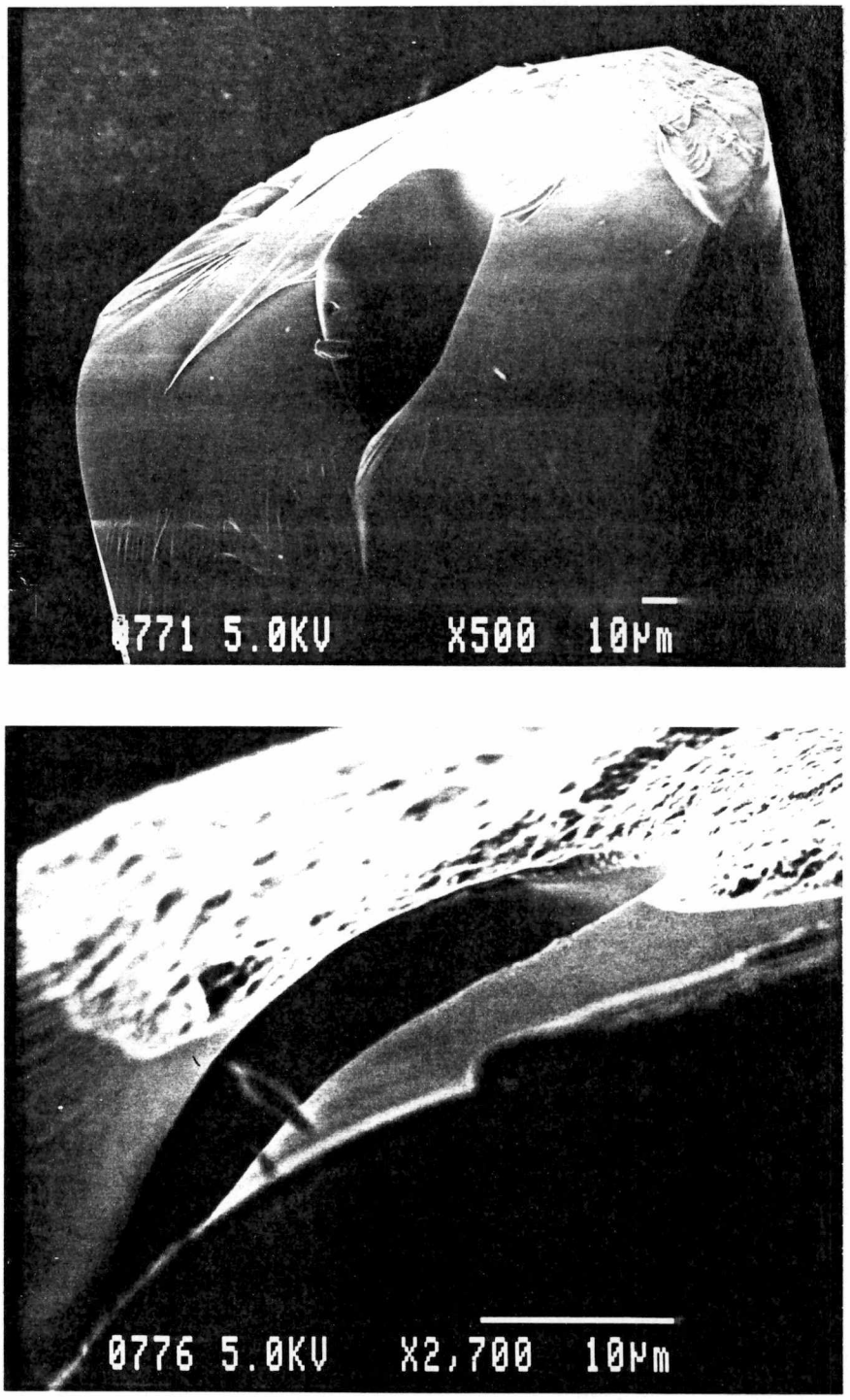
Rochelle Salt Solution (Potassium Sodium Tartrate)

Prepared by dissolving 1.0-g AgNO3 and 1.34-g Rochelle salt in 100-mL water. Solution is brought to a boil and filtered. When cool, the mixture is diluted with water to 500-mL total volume.

Preparation of SERS Substrates

Glass to be coated (alumina-roughened or otherwise) is placed in the bottom of a glass beaker. In another beaker, 3.0-mL each of Tollen's solution and Rochelle salt solution are well-mixed. The mixture is then poured over the substrates in the first beaker. The glass plates are removed after 3-5 minutes. The plates are rinsed with water and stored in a dessicator until use for SERS.

**<FIGURE V-2> One Method for the Chemical Deposition of Silver**




**<FIGURE V-3> Scanning Electron Micrographs of SERS Microlights** Note that these photographs were not taken of the ultimate tip of the microlights. The ends of the micropipettes broke off during transport to the microscope.

SERS sample bands were observed. In these tests, 1.0-μL of a $1x10^{-4}$-M solution of para-aminobenzoic acid (PABA) in ethanol was applied to the exterior of a microlight. PABA was selected for this study because it is an intense SERS scattering agent. An SEM analysis of a sample spot showed that the sample solution had disrupted the silver surface. No micrographs are available of this phenomenon because the exposed glass absorbed electrons to an extent precluding the taking of photographs.

The use of a microlight as a SERS substrate could be advantageous for several reasons. Microlights can be produced with dimensions even smaller than most fiberoptics, have a wide range of emission (ultraviolet to red), and may be quickly and simply fabricated. Lieberman *et al.*'s microlight possessed a high exciton generation efficiency. The crystals in their micropipette could be made to lase in a very confined space and so could be applied for molecular analysis of minute samples.

### *Antigen/Antibody Reactions*

One possible application of SERS could be to the study of antigen/antibody reaction systems. Thomas Rohr *et al.* (2) have successfully developed an immunoassay employing SERS to measure the binding between molecules with mutual affinities. An advantage of using a SERS immunoassay rather than a conventional solid-phase immunoassay is that no washing step is necessary, no signal development time is required, and no signal decay over time will occur.

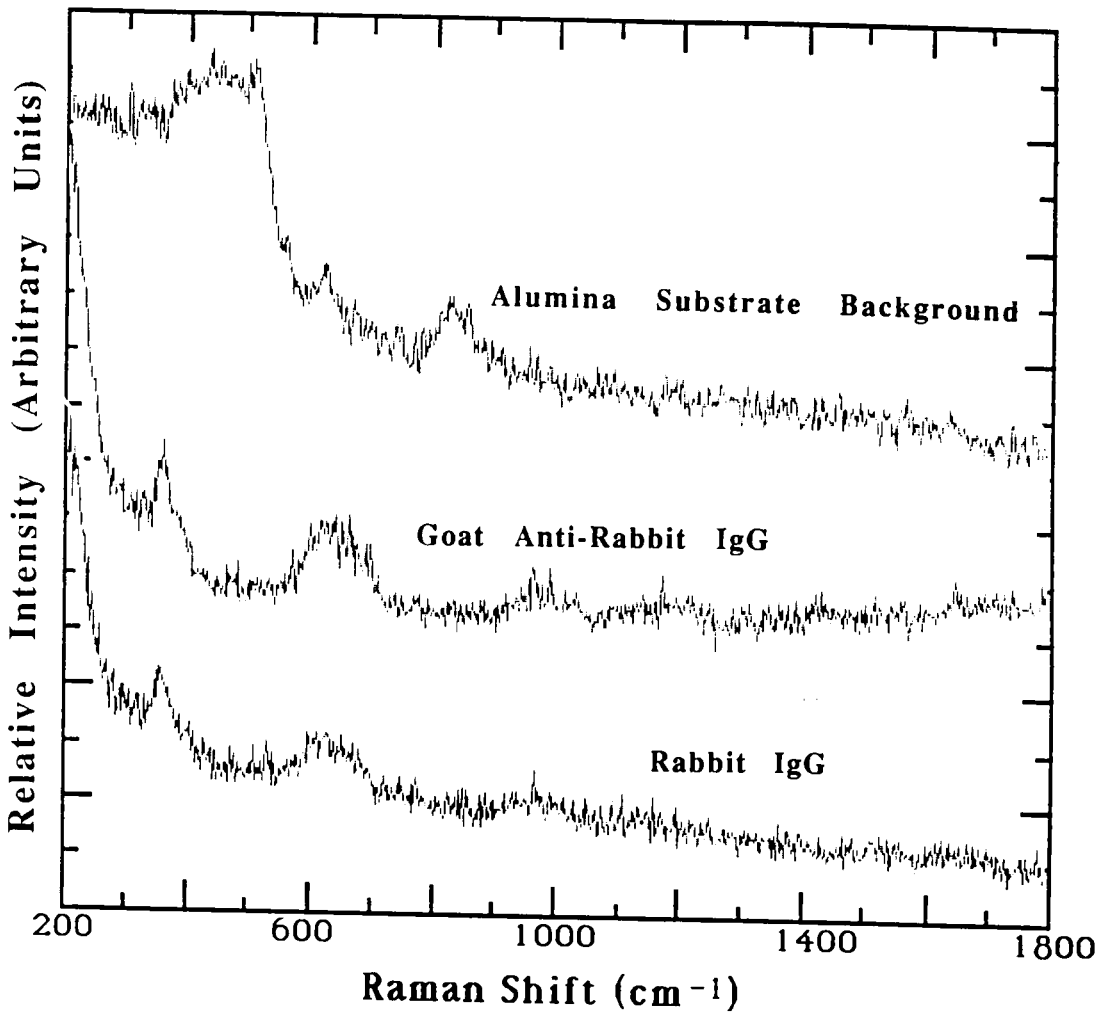
SERS data taken with an optical setup diagrammed in Figure I-4 (p. 18) and using silvered microparticle-roughened substrates suggest that the

antibodies (all obtained from Sigma Immunochemicals, St. Louis, MO) do not exhibit SERS bands. Application of an antibody to a SERS substrate resulted in inhibition of the SERS background noise levels. The background level reduction observed when antibodies were adsorbed onto the substrate surface was similar to that seen when DNA or a body fluid (or any extremely complex sample) was positioned onto the substrate. While the antigens to all of the antibodies in this study were known to be SERS active, no antigen was detected in Ag/Ab reactions, even when there existed a clear excess of antigen in solution. Spectra demonstrating these results are given in Figure V-4 (substrate background and IgG-type antibodies), Figure V-5 (solvent background), Figure V-6 (anti-theophylline, a monoclonal $IgG_1$, ascites), Figure V-7 (anti-dinitrophenol group, a monoclonal mouse IgE), and Figure V-8 (anti-benzo[a]pyrene, a monoclonal mouse IgG, ascites).

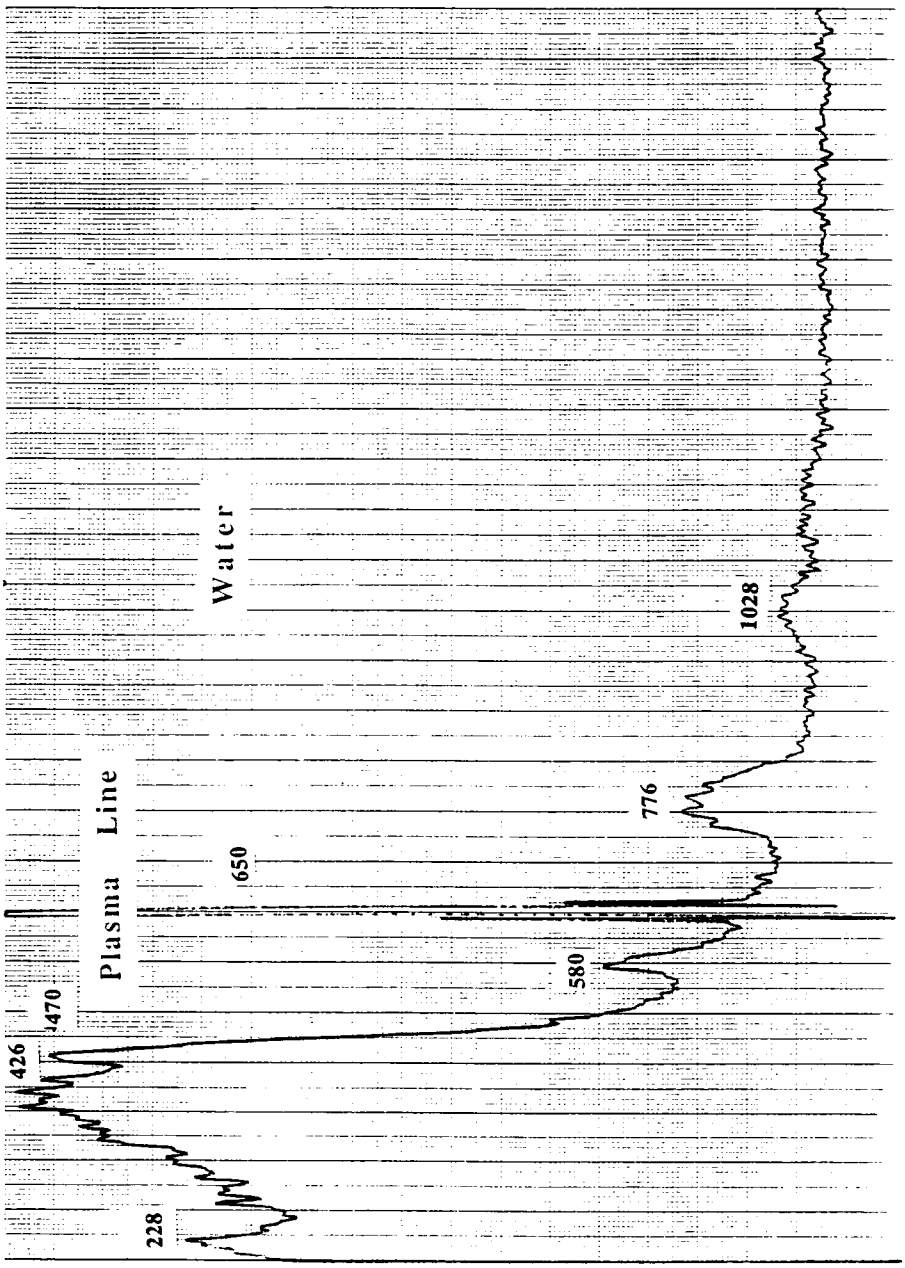
Antibodies may be attached to a SERS substrate through several mechanisms, including embedding in agarose gel as a coating for a substrate. SERS may be used to detect an antigen through analysis of the spectral signal decrease upon antibody binding. With this instrumentation, however, the alteration of SERS spectra upon antigen/antibody binding (as observed by Rohr *et al.*) was not seen.

### *Selectively Permeable Membranes and SERS*

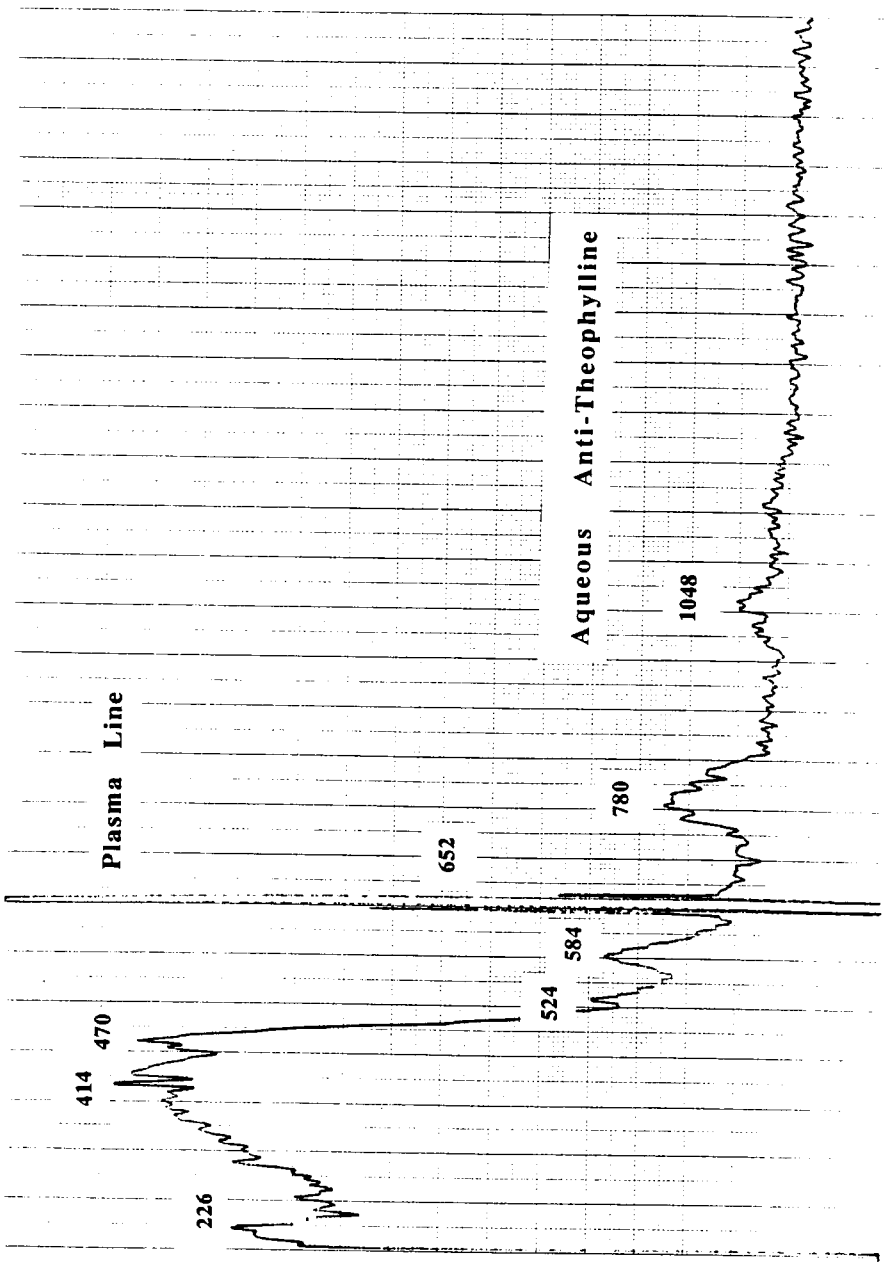
One of the most practical options for detecting samples found in body fluids would be to use a dialysis membrane or other semi-permeable partition that selectively permits sample molecules access to a SERS substrate, while filtering out undesirable molecules. A diagram illustrating the use of such a



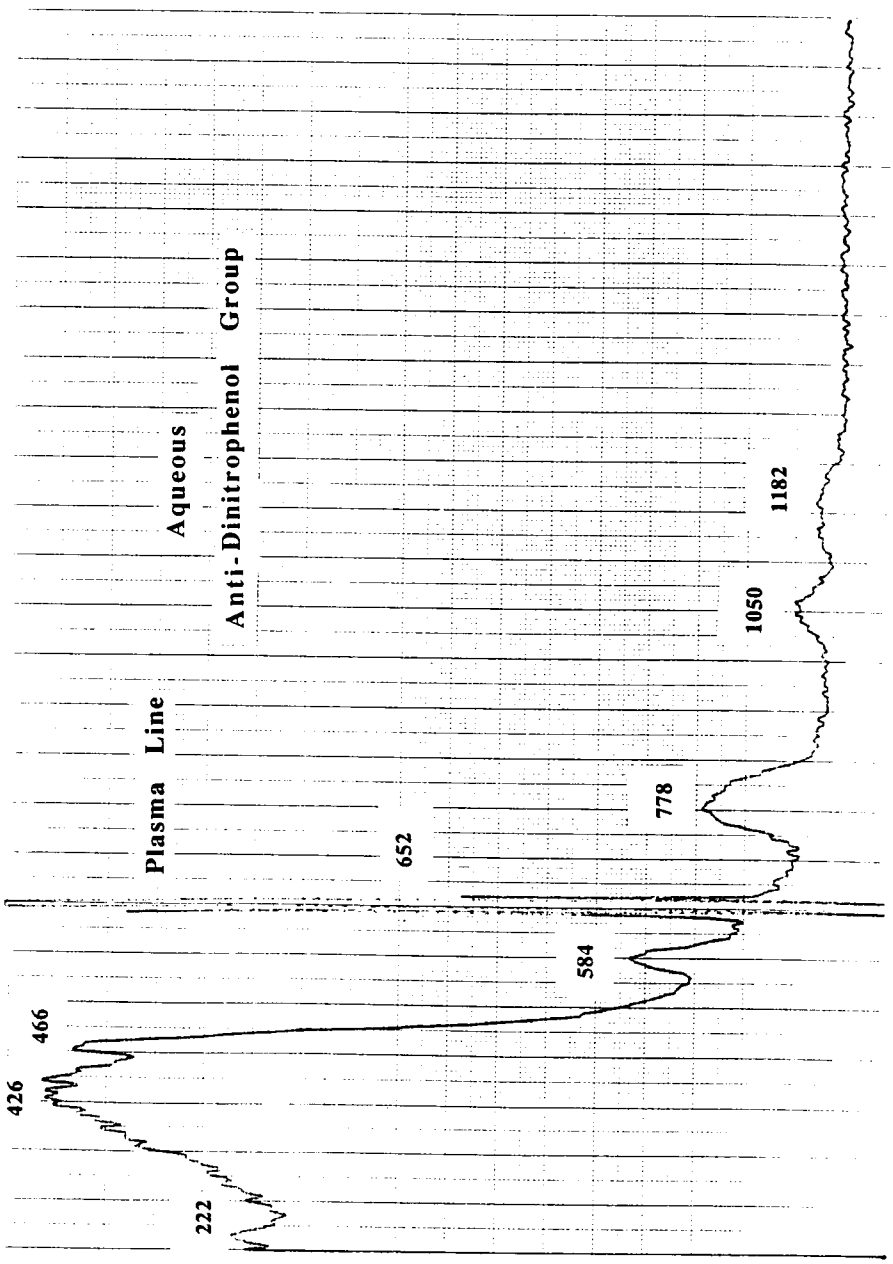

**<FIGURE V-4> SERS Spectra of the Substrate Background, Goat Anti-Rabbit IgG, and Rabbit IgG** Samples were 1.0-μL 2-mg/mL in water (goat anti-rabbit Ab) and 3.0-μL of a 2.5-mg/mL solution of 3:1 (v/v) ethanol:water, with precipitant formation (rabbit Ab). Laser excitation was 70-mW at 647.1-nm.



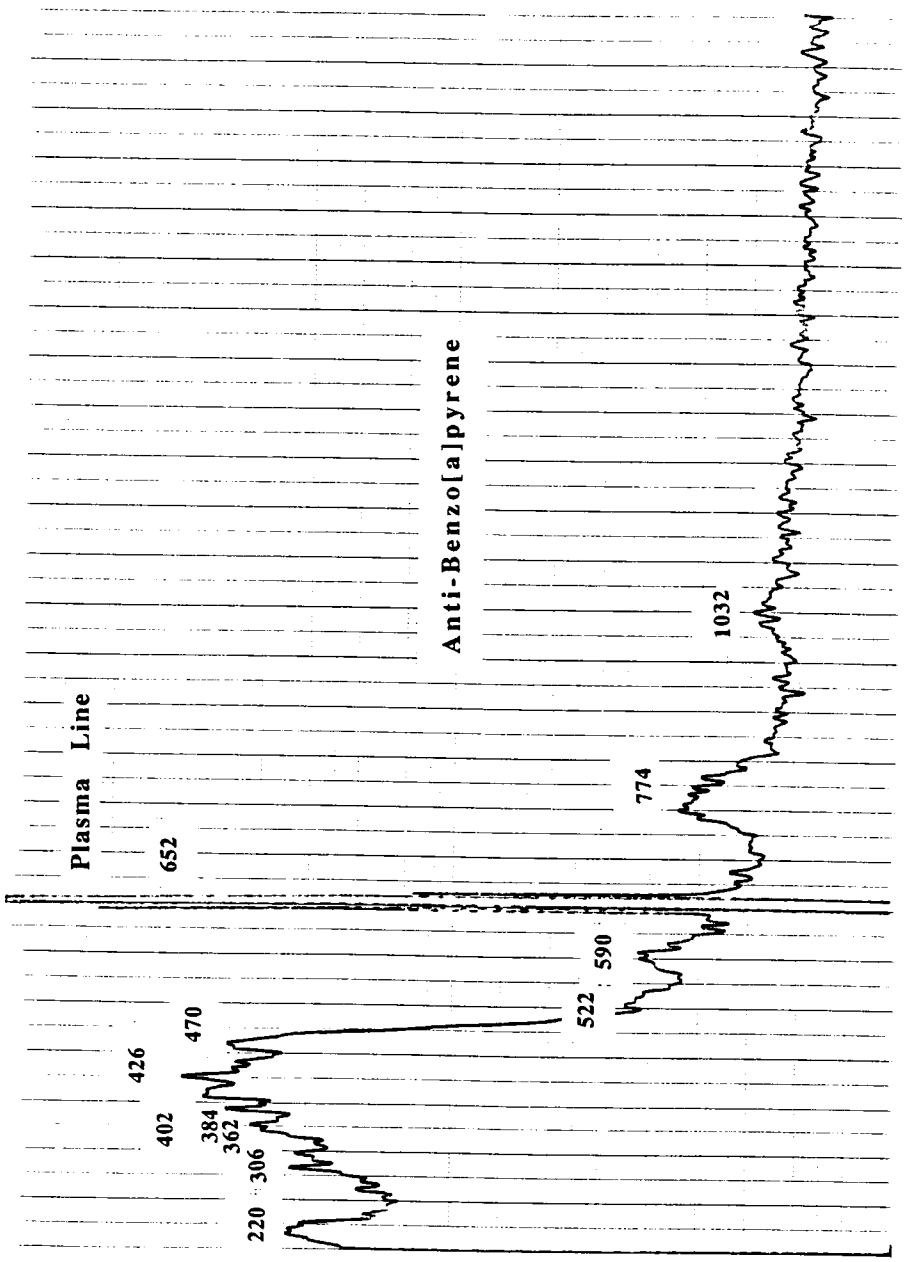

**<FIGURE V-5> SERS Spectrum of 1.0-μL of Water** Spectrum is of relative intensity versus Raman shift (200-1800 $cm^{-1}$). Sample was allowed to evaporated prior to exposure to the excitation beam. Laser excitation was 100-mW at 647.1-nm.




**<FIGURE V-6> SERS Spectrum of 1.0-μL Anti-Theophylline in Water** Sample was a 1:10 (v/v) solution of antibody suspension:water. Spectrum is of relative intensity versus Raman shift (200-1800 $cm^{-1}$). Sample was allowed to evaporated prior to exposure to the excitation beam. Laser excitation was 100-mW at 647.1-nm.




**<FIGURE V-7> SERS Spectrum of 1.0-μL of Anti-Dinitrophenol Group in Water** Sample was a 1:10 (v/v) solution of antibody suspension:water. Spectrum is of relative intensity versus Raman shift (200-1800 $cm^{-1}$). Sample was allowed to evaporate prior to exposure to the excitation beam. Laser excitation was 100-mW at 647.1-nm.



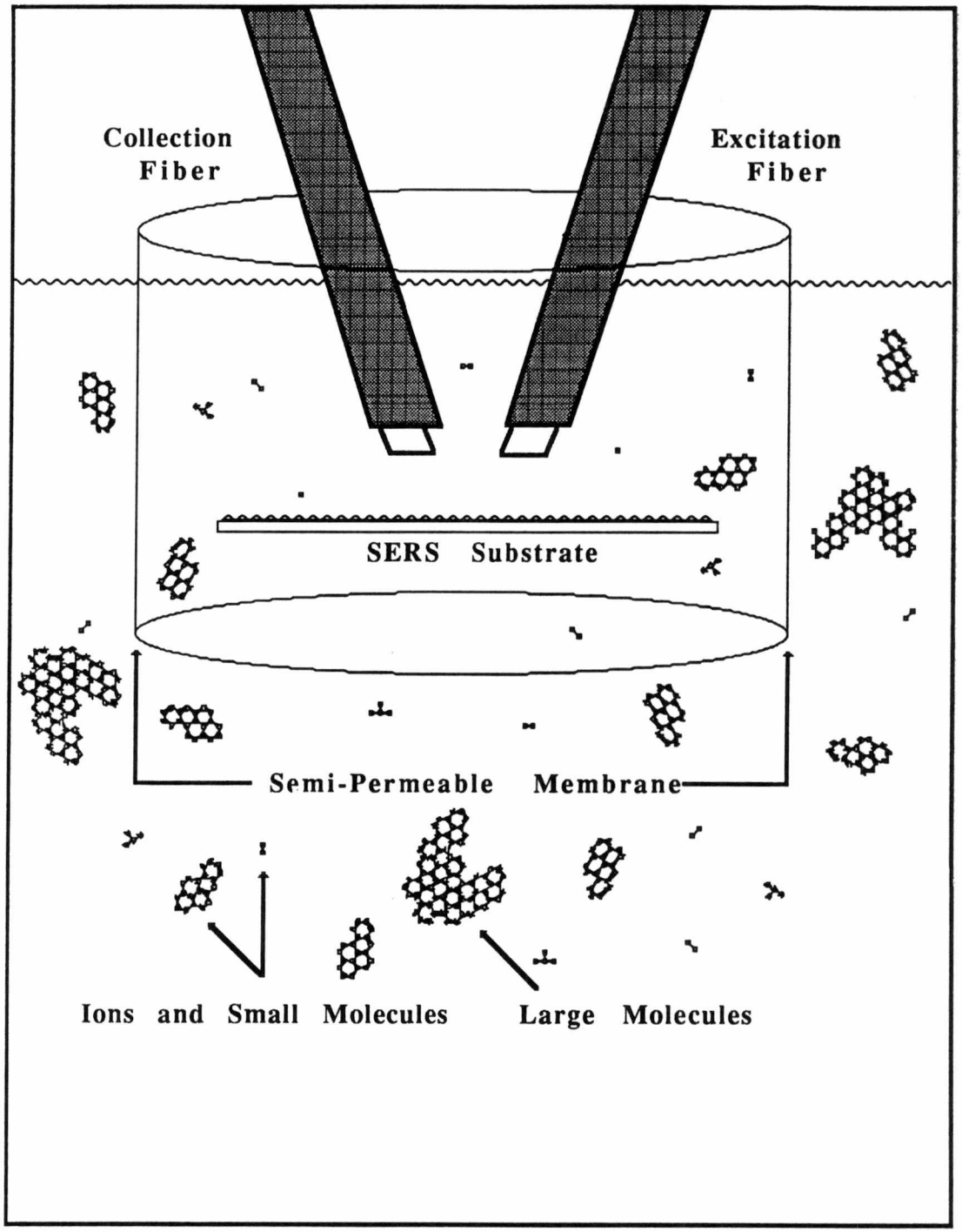

**<FIGURE V-8> SERS Spectrum of 1.0-μL of Anti-Benzo[a]Pyrene Stock Solution** Spectrum is of relative intensity versus Raman shift (200-1800 $cm^{-1}$). Sample was allowed to evaporated prior to exposure to the excitation beam. Laser excitation was 100-mW at 647.1-nm.

membrane for SERS is given in Figure V-9. While a semi-permeable membrane may screen out undesirable proteins and other contaminants, the ion balance in a sample would generally be preserved. The SERS spectra taken under these experimental conditions in a saline solution were poor (with the exception of 1,$N^6$-ethenoadenine). Other SERS researchers have not encountered this difficulty when using ionic solutions and may benefit from the application of a partition for improved analysis of complex biological mixtures.

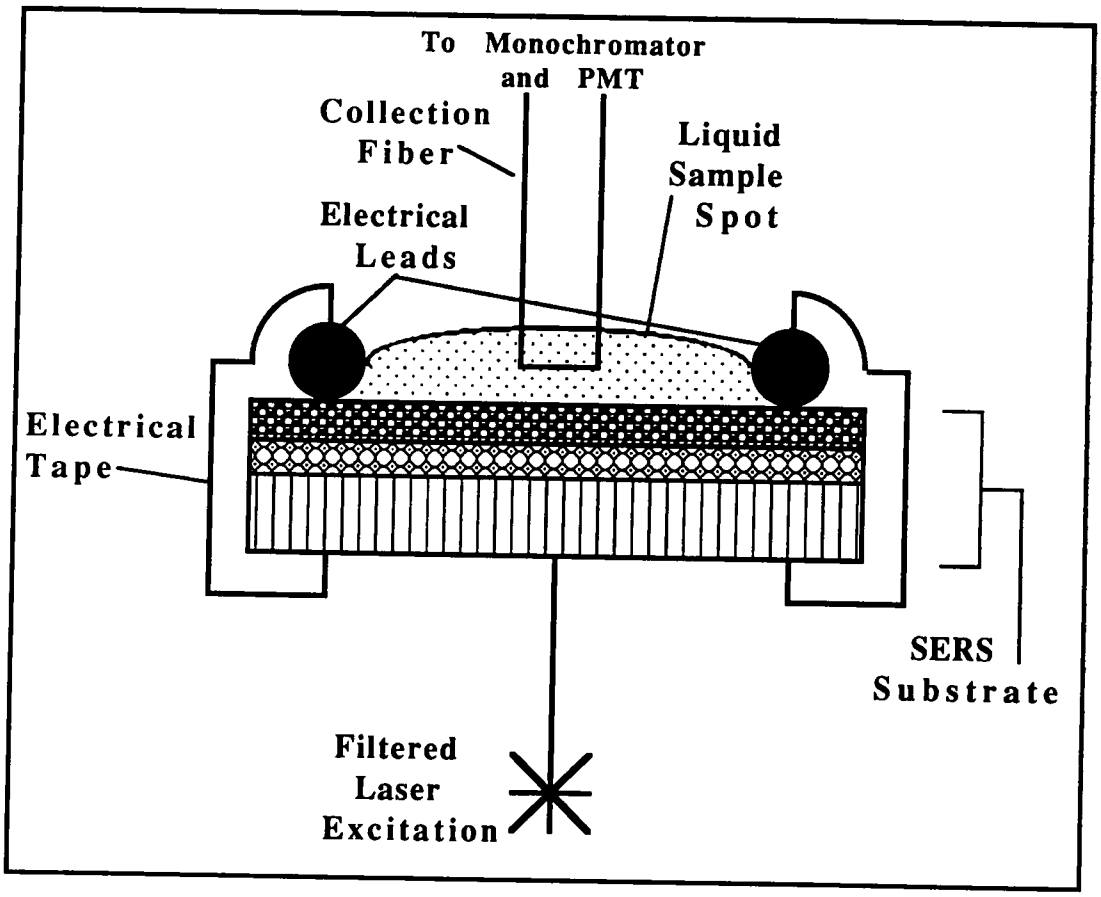
### *Electrochemical SERS*

Another SERS research possibility briefly explored in these experiments was the use of electrochemistry to elicit a spectral response to obtain additional structural information about a molecule. The application of a potential difference across biomolecules adsorbed onto a SERS substrate may elucidate the behavior of these chemicals at charged interfaces such as membranes. Precedents already exist for this type of SERS (3-7). Ervin *et al.* obtained SERS spectra of nucleic acid components adsorbed onto a silver electrode. Ervin's group found the intensities of the nucleic acid vibrational bands to be dependent upon the electrochemical preparation of the electrode, the applied potential, and the nature of the adsorbate species. Similar results have been obtained for pyridine (6) and catecholamine neurotransmitters (7).

In this preliminary experiment, a current was passed across a silvered alumina-roughened SERS substrate. The substrate was prepared by completely coating the region between the cathode and anode with a sample in a conductive liquid solvent (see diagram Figure IV-10.) The sample selected was




<FIGURE V-9> Diagram of SERS Utilizing a Semi-Permeable Membrane for Sample Filtration




<FIGURE V-10> Diagram of a SERS Substrate Prepared for an Electrochemistry Experiment

1,$N^6$-ethenoadenine because it could be detected in an ionic solvent. The solvent used was PBS. All 4.0% alumina-type substrates prepared by the author were found to have a resistance of 7.0-ohms. The resistance dropped to 0.4-ohms when the sample in PBS was added. While this resistance was reproducible for these substrates, it varied for substrates prepared by other researchers (6-ohms to 11-ohms before addition of the ionic sample). This technique could nicely complement the use of semi-permeable membranes for enhanced SERS analysis of biological molecules using a substrate (silver electrode) and optical setup specifically designed for this purpose.

*SERS and Electrophoresis*

Electrophoresis, and especially capillary zone electrophoresis (CZE), has become widely accepted as a high resolution separation technique. Detection systems such as fluorescence and ultraviolet absorption may be supplanted by techniques capable of providing better molecular characterization of the sample molecules. Chen and Morris have developed conventional Raman spectroscopy to meet the need for improved detection systems for electrophoresis (8,9). They have inserted optical fibers into a CZE gel for Raman detection of the separated molecules. The application of SERS to electrophoresis would be considerably more complex. In addition to the problems Chen and Morris encountered in applying Raman spectroscopy to electrophoresis, there would be a need to incorporate a metal or other electron source into the system to provide the Raman cross-scattering enhancement characteristic of SERS.

In the preliminary experiments done with a silvered, alumina-

roughened substrate, a thin polyacrylamide gel containing separated nucleic acids was placed onto the substrate for SERS analysis. It was determined that one of the buffer components (EDTA, a ligand used to remove impurities from the gel and electrophoresis apparatus) covalently attached itself to the silver on the substrate surface and interfered with the scattering enhancement mechanism. When the EDTA was removed from the running buffer the samples did not traverse the gel evenly. No SERS was detected from the molecules in the polyacrylamide gel when the system was run without the EDTA. Possibly a different gel matrix (e.g. agarose) may be used with better results. It remains to be determined whether or not SERS can be used as an electrophoresis detection method.

### *Neural Networks and SERS*

The application of neural networking for the detection, identification, and quantification of SERS peaks may be a reality in the future. The instrumentation and samples in this study were not ideal for neural training because their SERS peaks were at or near the background noise level. Another problem that may be encountered in the training of a neural network would be evaluation of the effects on SERS relative peak intensities and chemical interactions in a complex sample solution. For now, it seems practical to identify and assign SERS peaks manually, using the human talent for pattern recognition to identify weak bands from the background noise.

While the new sensing options presented by the microlight, antigen/antibody reactions, selectively permeable membranes, electrochemistry, electrophoresis, and neural networks are not all available for SERS using a silvered-microparticle substrate and the optical setup of Figure I-4 (p. 18), many of these techniques have been successfully applied by other SERS researchers using other substrates and optics. With these new methods, a realm of possibilities opens for a spectroscopic technique with few practical applications a decade ago. As the number of uses for SERS grows, the instrumentation for a procedure will become ever more specialized. The basic requirements for SERS (i.e. a quasi-monochromatic light source, a plasmon supplier, and a signal detection mechanism) will permit adaptation of the technique to address a number of research problems.

# CHAPTER 2

## SUMMARY AND CONCLUSION

Like standard Raman spectroscopy, SERS may be used to examine and differentiate chemically similar molecules. These molecules may be selectively analyzed in complex mixtures. A great deal of structural information may be obtained about the molecules because the interactions between the unique chemical bonds within the molecules determine the SERS or Raman spectrum. Since it is these chemical bonds which distinguish one substance from another, SERS and Raman spectroscopy elicit unique spectral signatures from molecules. Unlike conventional Raman spectroscopy, SERS may be used to examine dilute biological specimens. Experimentation with biological samples is possible because of SERS cross-scattering enhancement of Raman signals resulting from an interaction betweeen the sample molecules and a roughened, electron-conducting surface known as the SERS substrate.

The variety of substrates available for Raman enhancement is a source of flexibility for the SERS technique. Different substrates may be used to evoke more vibrational information about a substance than would be available using a single substrate type. When the substrate is altered, the relative intensities of the sample spectral bands will usually change; a band that was too weak to be detected with one substrate may be intense if a different substrate is used.

SERS may be a valuable technique for the analysis of biological samples for several reasons. First, minute sample quantities and volumes are required to produce a SERS spectrum. Second, the sample is not destroyed and may be

recovered for additional testing. This is because low laser excitation powers at a variety of wavelengths may be utilized for SERS. An agarose gel matrix may be applied to the substrate surface to prevent damage to the sample through any reaction with the substrate electron source. Third, when optical fibers are integrated into the SERS setup, remote analysis of a sample is possible. Fourth, a variety of solvents may be used with SERS, including water. Also, different phases of matter may be examined. Additional advantages include the low initial and operating costs of the instrumentation required to use the SERS technique and the portability of the setup (fits inside a medium-size suitcase).

In this study SERS was proven to be effective for detecting selected biologically-important molecules. Samples successfully examined included purines and pyrimidines, etheno adducts of adenine and cytosine, carcinogens benzo[a]pyrene, benzo[a]pyrene tetraol and related epoxides, and aflatoxin. SERS analysis of these samples could contribute unique structural information unavailable through utilization of other experimental techniques. The experimental parameters governing the substrate and optical setup were optimized such that the detection limit of SERS rivaled that of sensitive techniques such as fluorescence spectroscopy. The SERS detection limit of 3,$N^4$-ethenocytosine was particularly outstanding (femotomole level), but other samples were also routinely detected at low concentration levels (picomolar quantities). The SERS detection limit was improved when a thin coating of an agarose gel was applied onto the silvered alumina-roughened substrate surface. Other polymer coatings may also have an effect on substrate effectiveness.

Difficulties were encountered when SERS was used to study these

genotoxins in complex biological mixtures such as plasma, serum, and urine. Problems included sample overloading (too many proteins and other large molecules) and damage to the substrate through an interaction between the components of the body fluids and the silver surface. These problems persisted even when the fluids were greatly diluted with an analyte solution. Although detection of SERS-active molecules in body fluids was problematic, valuable SERS spectra of several DNA adducts were obtained. These adducts included alkylated products from DNA exposure to chloroacetaldehyde and DNA adducts from benz[a]anthracene epoxide exposure. For all samples in the DNA adduct study, the adducts could be distinguished from the solvent, unaltered DNA, and the substrate background.

Many of the new possible applications for SERS (microlight, antigen/antibody reactions, semi-permeable membranes, electrochemistry, electrophoresis, and neural networks) are not available for use under these experimental conditions. This is mainly because these parameters were optimized to suit one specific purpose -- sensitive detection of biomolecules at the concentration at which they may be present in an *in vivo* system. This system was found to be capable of detecting extremely low levels of contaminants, but at the cost of low signal-to-noise ratios and a limitation of useful solvents. A different SERS setup using a different substrate type could achieve the aims described in the first part of this section.

Substantial numbers of relevant spectra clearly demonstrated the effectiveness of this type of SERS analysis of genotoxic substances. The selected chemicals were detected even at the low concentration levels at which they would be present in a biological system. Samples containing similar chemical groups could be distinguished through careful comparisons of their

unique spectral signatures. SERS was shown to be a powerful, versatile analytical technique, capable of addressing many research problems through variation and optimization of a multitude of experimental parameters.

# SECTION V

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## REFERENCES


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# VITA

Anne Marie Manasil was born in Burwell, Nebraska on July 7, 1965. She attended Burwell Jr.-Sr. High School, graduating as salutatorian in 1983. Ms. Manasil received a bachelor of arts degree with majors in physics and mathematics from Hastings College, Hastings, Nebraska in 1987. She remained at Hastings College an additional year to improve her knowledge of biology. Anne married Todd Allan Helmenstine on August 13, 1988. In August of 1988 she enrolled in the University of Tennessee - Oak Ridge Graduate School of Biomedical Sciences. In December of 1992 Anne received a Doctor of Philosophy degree in Biomedical Sciences.