

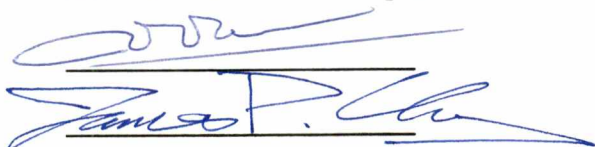
To the Graduate Council:

I am submitting herewith a dissertation written by Wendi Elizabeth Watts entitled "Improved Spectroscopic Methods for the Study of Normal and Neoplastic Cells." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.



Dr. Donita Frazier, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor and
Dean of The Graduate School

**IMPROVED SPECTROSCOPIC METHODS FOR THE STUDY OF
NORMAL AND NEOPLASTIC CELLS**

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Wendi E. Watts
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DEDICATION

This work is dedicated to my parents and brother
James, Jennifer and Kevin

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I would like to sincerely thank the members of my committee:

Dr. Tuan Vo-Dinh, Research Director and Committee Co-chairman

Dr. Donita Frazier, Committee Co-Chairman

Dr. James Chen, Committee Member

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ABSTRACT

The purpose of this study was to utilize fluorescence spectroscopic techniques to differentiate between normal and neoplastic cells.

Specific fluorescent biological compounds, i.e., tryptophan, nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FADH₂) and collagen, were examined in this study in order to determine if the cellular fluorescence could be attributed to them. Rat liver epithelial (RLE) cells, McA-RH7777 rat hepatoma (McA) cells, astrocytes and astrocytoma cells were investigated in this study. The autofluorescence of whole cells and cellular extracts of RLE and McA was studied using conventional and synchronous fluorescence, before and after dialysis. Synchronous fluorescence was used to study the astrocyte and astrocytoma cellular extract after dialysis. Polyacrylamide gel electrophoresis (PAGE) was performed with the polypeptide extracts. A charge-coupled device (CCD) was used to image fluorescent bands illuminated in the gels with a Helium-Cadmium laser.

The use of synchronous fluorescence showed no differences

with the astrocytes and astrocytomas. However, some differences in the spectra of RLE and McA cells led to an attempt to identify the source of the fluorescence. Tryptophan, NADH, FADH₂ and collagen did not appear to be the source of fluorescence differences in the normal or neoplastic cells because the peak positions for the synchronous spectra for the cells did not correspond to peak positions for synchronous measurements of tryptophan, NADH, FADH₂ and collagen. McA cells have a synchronous fluorescence peak that is shifted to shorter wavelengths with respect to the peak position of RLE cells by 4-5nm. Differences in the peak positions are even greater in the cellular extracts. The McA cellular extract exhibits a synchronous fluorescence peak blue-shifted by 10nm to that of the RLE cellular extract. When imaging gels to detect fluorescent bands, a 70-75 kD polypeptide was found to fluoresce in the McA cell line, whereas no fluorescent band was observed in the RLE cell line. No spectral differences were detected in the astrocytes or astrocytomas when performing synchronous fluorescence. No fluorescent bands were detected in astrocytes or astrocytomas when imaging gels to detect fluorescent bands.

In conclusion, conventional, synchronous fluorescence and CCD

imaging have been employed in order to detect differences in the emission of RLE and McA cells. Spectral differences were detected in RLE and McA cells using synchronous fluorescence, and a fluorescent polypeptide detected in the McA cells using CCD imaging. Although these techniques did not detect differences in the other cells studied, it may be possible to utilize synchronous fluorescence excited at other wavelengths to detect differences in other cell lines. Synchronous fluorescence may be an effective way to determine differences in normal and neoplastic cells.

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CHAPTER 1. INTRODUCTION

A. AUTOFLUORESCENCE OF CELLS AND TISSUES

Conventional fluorescence and phosphorescence, synchronous fluorescence and phosphorescence and laser-induced fluorescence are simple and powerful tools used in the analysis of many types of complex samples. Fluorescence occurs when an electron that has been promoted to a higher electronic energy state returns to the ground state by emitting light. Conventional fluorescence includes excitation and emission spectra. Synchronous fluorescence uses both excitation and emission spectra. Phosphorescence occurs when an electron that has been promoted to a higher electronic energy state and has undergone a change in spin direction returns to the ground state by emitting light. A more complete explanation of these techniques can be found beginning on page 14. Luminescence has proven to be effective for applications in biology, chemistry, biochemistry and medicine¹.

Fluorescence assays have long been used as a diagnostic technique for detection of certain important molecules in body fluids. For example, the amino acids tyrosine, tryptophan and

phenylalanine can be measured by fluorescence as well as phosphorescence. It is also possible to detect specific compounds in the complex biochemical matrices of cells and tissues. Exogenous compounds such as photosensitizers that accumulate in tissues can be detected; as well as endogenous macromolecules^{2,3}. The present study focused on detection of specific fluorescent endogenous molecules in normal and neoplastic cells.

To date, many studies have described differences in fluorescent spectra between normal and diseased tissues; however, few have attempted to describe the definitive fluorescent component(s). Zeng⁴ and colleagues reported shifts in the autofluorescence spectra of layers of the skin using a 442 nm He-Cd laser as the excitation source. The lower dermis emitted between 480 to 500 nm, the upper dermis spectra was shifted about 20 nm to the red, and the stratum corneum exhibited very low autofluorescence in the 500 to 520 region. Autofluorescence differences between neoplastic and normal tissues of rodents and human beings have been evaluated⁵⁻⁸. Normal and malignant bronchial tissue autofluorescence has been studied using a krypton ion laser (405 nm), a helium cadmium laser (442 nm) and an argon

ion laser (488 nm)⁹. In all cases⁵⁻⁹, the normal tissue fluorescence intensity was twofold to threefold that of the tumor tissue, but no other significant spectral differences were seen. The tissues all exhibited a broad emission from 480 to 690 nm. Peak positions were the same for normal and tumorous tissues, which included bronchial tissue, lung tissue and colonic tissue, with differences in the intensities of the signals. In contrast, other studies showed excitation spectra of neoplastic samples differed from spectra of normal samples¹⁰. Recently, laser-induced fluorescence has been successfully used for the *in-vivo* diagnosis of cancer of the esophagus in over 100 patients⁷.

Cells are comprised of several different types of fluorescent molecules. Cellular fluorescence was first attributed to nucleic acids¹¹, nucleotides^{11,12} and proteins¹¹⁻¹⁹. Konev confirmed that almost all cells and their major subunits exhibit one excitation maximum and one fluorescence maximum. These maxima are close to those obtained with purified proteins¹⁵. In most cases, the autofluorescence of proteins can be attributed to the presence of the amino acids, tryptophan and tyrosine²⁰. Fluorescence can also be associated with other fluorescent molecules such as coenzymes

(NADH, FADH_2) bound to the protein¹. Spectral differences in normal and diseased tissues described in the above studies could reflect a difference in one or more of these compounds.

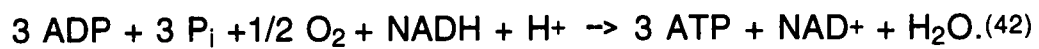
Nicotinamide adenine dinucleotide was first mentioned in the literature by Warburg and Christian in 1931 after establishing that mammalian erythrocytes contain a stable factor that is required for aerobic oxidation of glucose-6-phosphate to 6-phosphogluconic acid, and isolating it from erythrocytes²¹. Later work demonstrated that the compound was formed by the union of 1 molecule of adenine, 2 pentose units, 3 equivalents of phosphoric acid and 1 molecule of the amide of nicotinic acid²².

Nicotinamide adenine dinucleotide occurs in oxidized (NAD^+) and reduced (NADH) forms. Prior to 1960, the nomenclature was diphosphopyridine nucleotide, the oxidized form designated as DPN and the reduced form designated as DPNH²³. The reduced form displays fluorescent properties²⁴⁻²⁷. Fluorescence was used in determining the function of NAD^+/NADH in cellular respiration. Initially, aerobic material maintained a constant fluorescence until the oxygen supply was depleted, at which point the fluorescence intensity increased²⁸.

The nicotinamide component of these coenzymes serves as the transient intermediate carrier of a hydride ion that is enzymatically removed from a substrate by action of certain dehydrogenases^{29, 30,31}. Glycolysis is the central pathway for recovering chemical energy from the breakdown of glucose into pyruvate. In glycolysis, NAD⁺/NADH is used in the transformation of triose phosphates to 3-phosphoglycerate, an intermediate step in the production of acetyl-CoA. NAD⁺/NADH plays a role in cellular respiration by acting as an electron acceptor for dehydrogenases³²⁻³⁵, as well as functioning as an electron acceptor in the fatty acid oxidation cycle³⁶⁻³⁷.

In 1931, Warburg³⁸ reported that rapidly growing tumor cells have a high rate of aerobic glycolysis and low respiration. Warburg proposed that a undefined carcinogenic agent interfered with cell respiration and the cell either died or used a fermentative mechanism for energy production. Since this initial discovery, it has been found that some tumors have normal rates of respiration and that rates of aerobic respiration differ between normal tissues³⁹. Although a unique pattern of energy metabolism is not observed in all tumor cells, a correlation of growth rate with increasing glycolysis is observed in hepatomas⁴⁰.

Bucher and Russman⁴¹ reviewed the interrelationships of NADH, ATP and various enzymes in glycolysis. The NAD⁺:NADH ratio is dependent on the availability of an electron acceptor such as pyruvate whereas pyruvate formation is dependent on ADP availability; and ADP concentration is dependent on the release of a phosphate by ATP. A general equation for this is as follows:



Hypoxia is believed to be a characteristic state of tumors in rapid growth. As indicated in the above equation, ADP, P_i and O₂ can be regulatory. A decrease in O₂ results in a decreased ability of the cell to utilize ADP, P_i, and/or NADH. Aerobic respiration will be impaired and the cell will function only in glycolysis. NADH concentrations should be higher in cells that utilize glycolysis to generate energy and, thus, should be higher in certain tumor cells that use glycolysis than in normal cells or tumor cells that do not⁴². Alternatively, an increase in NADH may result from the increased rate of proliferation of tumor cells compared to normal cells⁴². Cells that have a high proliferation rate presumably have greater energy needs than cells with lower proliferation rates.

Flavin adenine dinucleotide (FAD) was first isolated from

kidney, liver and yeast as a coenzyme of d-amino acid oxidase^{43,44} and its structure deduced from the empirical composition of the material and by stoichiometric analysis of the products of hydrolysis and photolysis (adenine and lumiflavin)⁴⁵.

The fluorescence of FAD and riboflavin were studied and the fluorescence of FAD was found to be much weaker^{46,47}. Unlike NADH, the fluorescence of FAD is not frequently used in its determination; however, enzymatic assays using the apoenzyme of d-amino acid oxidase have been developed⁴⁸.

Similar to nicotinamide adenine dinucleotide, flavin adenine dinucleotide exists in both oxidized (FAD) and reduced (FADH₂) forms. The reduced form of FADH₂ functions as a receptor in oxidation reduction reactions⁴⁹. FADH₂ functions in fatty acid oxidation⁵⁰ as well as in the citric acid cycle⁵¹, which is the main pathway for oxidation of carbohydrates and occurs in virtually all tissues of higher animals⁵². FADH₂ may also be increased in cells having high proliferation rates.

Proteins are composed of polypeptides which are long sequences of amino acids⁵³⁻⁵⁸. Although there are only 20 amino acids, they can be arranged in a number of combinations to make a

variety of different proteins⁵⁹. Protein structure has been determined by x-ray crystallography⁶⁰⁻⁶⁴. Proteins have a wide variety of biological functions but can be grouped into two major types based on structure: fibrous and globular⁶⁵. Globular proteins have polypeptide chains that are tightly folded into compact spherical or globular shapes⁶⁵. Fibrous proteins are long, stringy, water-insoluble molecules with polypeptides centered around one axis^{65,66}. Fibrous proteins most often function in a structural or protective role. Collagen is the most abundant protein in vertebrates, primarily because it is a major source of structural support^{67,68}. Laser-induced fluorescence studies have shown tryptophan, collagen and elastin vary between normal tissues and atherosclerotic tissues⁶⁹.

Recent advances in molecular biology have confirmed the presence of aberrant proteins, tumor markers, that control proliferation and differentiation of tumor cells. The pioneering work in tumor marker research are summarized in Table 170-84. Some of the proteins that are currently being investigated are c-erb2 protein⁸⁵, Pa8-15 protein⁸⁶, Pit-1 protein⁸⁷, CD36 protein⁸⁸, retinoblastoma protein⁸⁹, c-Kit protein⁹⁰, CD44 protein⁹¹, cathepsin

Table 1. Tumor Markers

Marker	First Described	Marker Type	Tumor	Effectiveness
Carcinoembryonal Antigen (CEA)	Gold and Freedman ⁴³	glycoprotein ⁴⁴	metastatic colorectal ^{44,45,46} metastatic breast ^{45,46}	marginal ⁴⁷
Alpha-fetoprotein (AFP)	Bergstrand and Czar ⁴⁸	oncofetal glyco-49 protein	hepatocellular carcinoma ^{50,51} testicular carcinoma ⁵²	
Human chorionic gonadotropin (HCG)		glycoprotein ⁵³	tumors of the placenta ⁵³ and testis	minimal ⁵³
Tissue polypeptide Antigen (TPA)	Bjorklund ⁵⁴	single chain ^{54,55} polypeptide	prostatic, colon, lung, pancreatic and cervical cancer ⁵⁵	marginal ⁵⁵
Tennessee Antigen (Tenagen)	Lovins ⁵⁶	glycoprotein ⁵⁶	lung cancer, colorectal cancer ⁵⁶	undetermined

D⁹², C-myc protein⁹³ and p53 protein⁹⁴. These tumor markers represent either: 1) increased production of endogenous biochemicals, i.e., hormones, enzymes, polyamines; 2) synthesis of biochemicals from previously dormant genes, i.e., oncofetal proteins, cell surface antigens, or enzymes; 3) receptors, i.e., estriol receptor in breast cancer or androgen receptor in prostate cancer; or 4) modification of usual cell or organ function, i.e., gamma-glutamyl transferase (GGT) or 5'-nucleotidase⁹⁵.

Monoclonal antibodies have been developed to examine p53 expression in a wide variety of benign and malignant skin lesions⁹⁶. Benign skin lesions did not express p53 whereas a significant number of malignant lesions showed detectable levels of p53⁹⁷. p53 expression also was reported in squamous cell skin carcinoma by other investigators⁹⁸. Although the role of the protein is not known, abnormalities in the p53 protein are common in a wide range of human tumors⁹⁹, including prostatic cancer¹⁰⁰, early bronchial neoplasia¹⁰¹, basal cell carcinoma¹⁰², tumors of the testis¹⁰³, and ovarian carcinoma¹⁰⁴. Other tumors, including cervical intra-epithelial neoplasia, do not appear to express the p53 protein¹⁰⁵.

The presence of p53 may also be determined by amplification

of p53 DNA obtained from biopsies using polymerase chain reaction-single strand conformation polymorphism analysis and sequencing of p53 DNA¹⁰⁴. This study indicated that mutation of p53 is an early event in head and neck carcinogenesis and precedes signs of overt neoplasia.

The c-myc protein is another protein which is being investigated as a tumor marker. Immunocytochemical analysis of fragments of uterine and cervical tissue showed c-myc protein staining was observed only on microinvasive carcinomas⁹². In another study, c-myc protein was detected in all 19 papillary thyroid carcinomas examined¹⁰⁵. Studies to date suggest the c-myc protein may be less effective as a tumor marker than the p53 protein.

To date, few publications have explained the differences in fluorescent spectra of normal and neoplastic tissues by defining the differences in the macromolecular components. Since tumors frequently outgrow their blood supply, differences in the biochemical matrices of normal and neoplastic tissues could be explained by differences in biochemical pathways secondary to changes in pH and oxygen tension. Alternatively, spectral

differences could reflect the presence of an aberrant protein; i.e., a tumor marker, in neoplastic cells and tissues.

In the present study, conventional and synchronous luminescence were utilized to study differences in normal and neoplastic cells. It was proposed that synchronous luminescence (SL) would allow detection of subtle differences in the biochemical matrices of cells by focusing on the spectra of the specific macromolecule(s) that differed between neoplastic and normal cells. The sensitivity of detection was improved by extraction of fluorescent components, dialysis and gel electrophoresis; and excitation using a laser. Improved detection may allow earlier diagnosis of cancer. This may ultimately lead to a higher survival rate for cancer patients since it is widely recognized that earlier detection correlates with a greater chance of survival for many neoplasms.

B. THEORY OF LUMINESCENCE

Luminescence Spectroscopy

Luminescence spectroscopy is based on the detection of the electromagnetic radiation emitted from a chemical system undergoing a radiative transition between different electronic states. The basic principle of luminescence spectroscopy has been previously described in detail¹⁰⁶. This section briefly discusses some fundamental characteristics relevant to the basic understanding of luminescence.

Most molecules contain an even number of electrons. In the ground state, the electrons fill up various atomic and molecular orbitals in pairs, with their spins in opposite directions in one given orbital (Pauli's exclusion principle)¹⁰⁷. As a consequence, this state has no net electron spin and is defined to be a singlet state. This state has no unpaired electrons and is said to have a multiplicity of 1. Whereas the ground state, S_0 , is generally a singlet, the excited state can be either a singlet or a triplet, depending on the state of the spin of the electron that has been promoted to a higher orbital (Figure 1)^{107,108}. A triplet state is one which has two unpaired

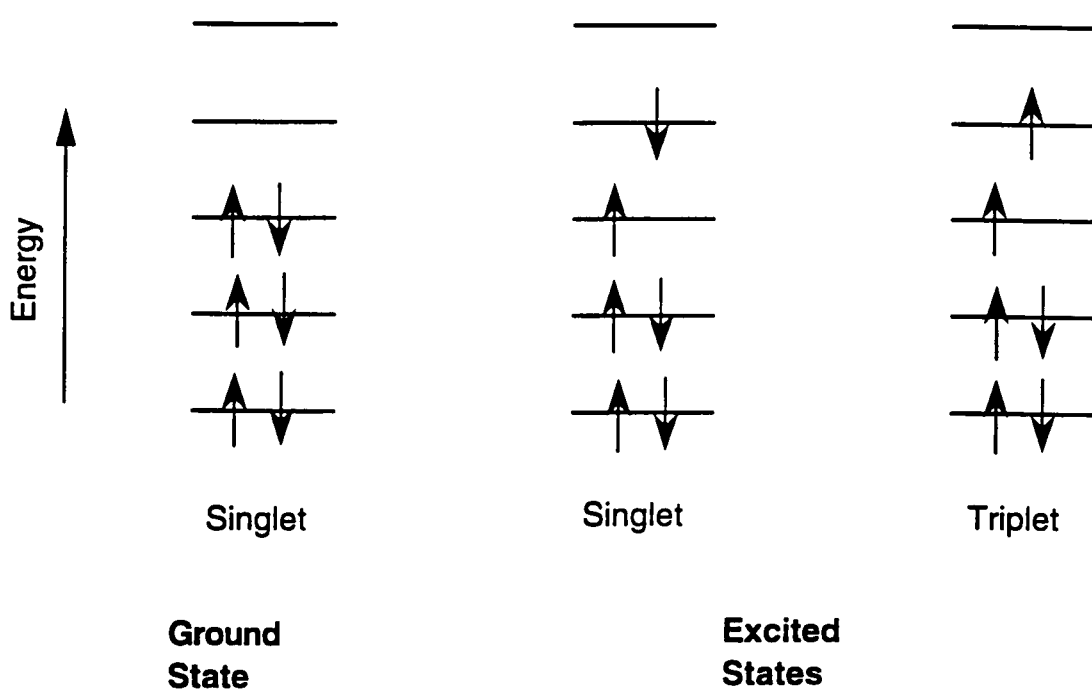
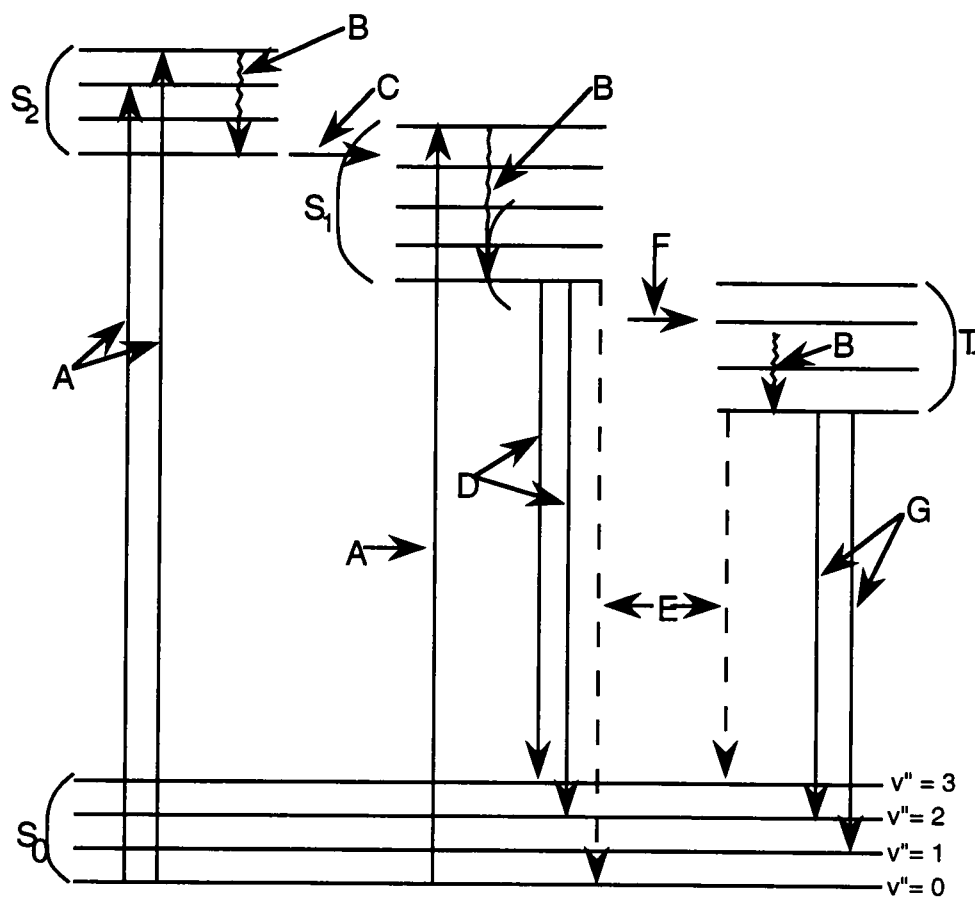


Figure 1. Molecular Orbital Diagram

electrons with equal spin quantum numbers. It has been defined to have a multiplicity of $2S+1$.

The basic processes of fluorescence and phosphorescence are illustrated in the Jablonski diagram in Figure 2. In this figure, S_0 , S_1 , S_2 and T_1 represent the electronic energy levels of a molecule. The state of lowest energy, S_0 , is called the ground state. S_1 , S_2 and T_1 are the excited singlet and triplet states, respectively. Each state is shown with its own set of vibrational levels. When a molecule absorbs energy, an electron is elevated from S_0 to a vibrational level of one of the excited singlet states^{108,111-113}. Molecules quickly lose their excess vibrational energy and will return to the ground vibrational state of the given electron state. This is called vibrational relaxation.

Internal conversion (IC) is a process where there is crossover between two states of the same multiplicity^{112,113}. Multiplicity refers to different electronic states based on quantum theory, i.e., singlet and triplet states. For example, if a molecule is excited to S_2 , it will first lose excess vibrational energy and return to the lowest vibrational state of the 2nd singlet electronic state (S_2). Then through internal conversion, an excited molecule will move to



- A. Absorption
- B. Vibrational Relaxation
- C. Internal Conversion
- D. Fluorescence
- E. External Conversion
- F. Intersystem Crossing
- G. Phosphorescence

v'' represents
vibrational
energy levels

Figure 2. Jablonski Diagram

the 1st singlet electronic state (S_1). Crossover between the two states is likely when the lower vibrational levels of the higher electronic state are approximately the same energy as the higher vibrational levels of the lower electronic state¹¹³. The vibrational energy is quickly lowered to the ground vibrational level. From S_1 , the molecule returns to the ground state by the following processes:

1. There is emission of a photon from S_1 to S_0 . There is no change in multiplicity (spins stay in opposite directions) for this transition. This transition is called fluorescence^{109,113}.
2. Transition takes place to some vibrational level of the triplet state by intersystem crossing (ISC) with change in spin multiplicity (multiplicity changes from 1 to 3). The molecule then relaxes rapidly to the lowest vibronic level of T_1 (vibrational relaxation). From T_1 it returns to the ground state, S_0 , by external conversion, a radiationless deactivation path (ISC process) or by a radiative transition called phosphorescence. External conversion results in a nonradiative loss of energy by the

excited species via transfer of their excess energy to another species. Other species can be solvent molecules or even the sides of the container the excited molecules are in. The energy is lost as heat. For phosphorescence the multiplicity changes from 3 to 1 during the transition¹¹⁴⁻¹¹⁷.

3. Energy loss that did not occur from emission of a photon (fluorescence or phosphorescence) is emitted as heat.

Nature of Fluorescence and Phosphorescence

Transitions between different excited singlet states of the same multiplicity ($S_n \leftrightarrow S_m$ or $T_n \leftrightarrow T_m$) may occur easily, but transitions between pure spin states of different multiplicity ($S_n \leftrightarrow T_m$) are quantum mechanically forbidden by the spin selection rule. Forbidden transitions do occur, however, under certain conditions.

The excitation level obtained by a species upon absorption of a photon is determined by the molecular structure of the species, the environment the species is in and the energy of the photon being absorbed. Conjugation of double bonds results in a molecular configuration that allows an excited species to be longer lived, permitting time for fluorescence or phosphorescence to occur¹¹⁹.

Unless a molecule has the proper structure an electron that is excited will return to the ground state too quickly to fluoresce or phosphoresce. The environment the species is within can alter the molecular configuration and thus the energy of the excitation. The solvent and temperature are examples of environmental factors affecting which excited state is occupied. Lower temperatures decrease the number of collisions between electrons, so that processes such as external conversion are not as common¹¹⁹. The amount of energy of the photon that the species absorbs determines which excited state is occupied. For example, two different species that are excited with the same energy will not have the same number of excited states occupied if they do not absorb the same amount of energy required to promote the electrons to a specific excited state, which is determined by the molecular configuration.

Experimentally, fluorescence can be differentiated from phosphorescence by its short radiative lifetime (10^{-9} - 10^{-7} seconds). Phosphorescence persists from 10^{-4} to several seconds after excitation. Due to its longer decay time, phosphorescence is more susceptible to collisional quenching process in solution and in the gas phase than is fluorescence. The radiationless deactivation for

most molecules in the triplet states is so efficient that phosphorescence is normally observed only when the molecule is frozen in a rigid glass at low temperature. Recently, intense phosphorescence from organic compounds adsorbed on solid substrates, such as paper, has been observed at room temperature¹¹⁹.

Luminescence Spectra

Measurement of luminescence intensity allows the characterization and quantitative determination of trace amounts of many inorganic and organic species.

Two basic types of luminescence spectra can be obtained. When a compound is excited at a constant energy (fixed excitation wavelength), an emission spectrum is produced by recording the luminescence intensity as a function of the emission wavelength, λ_{em} . The vibrational structure in the emission spectrum gives information about the ground state, S_0 . The bands in the emission spectrum correspond to the transition from S_1 vibrational energy levels of the ground state (Figure 3). The intensity of the fluorescence corresponds to the probability of the electron being excited to a certain excited state (energy level). The excitation

spectrum on Figure 3 shows peak c having the highest intensity for a fixed λ_{em} . Basically this means more electrons will be excited to the corresponding energy level (c) than to any other level. Energy is inversely proportional to frequency (wavelength) i.e. higher energy levels correspond to lower wavelengths.

An excitation spectrum is obtained by varying the wavelength, λ_{ex} (or energy) of the exciting radiation while the observation of the luminescence is performed at a fixed emission wavelength. As a consequence, the vibrational structure of an excitation spectrum corresponds to the vibrational nature of the excited state, e.g., S_1 (Figure 3). The conventional absorption spectrum of the compound can be obtained by scanning the excitation monochromator through the absorption wavelengths with the emission monochromator set at the maximum wavelength of the emission spectrum. Excitation spectroscopy can, therefore, provide absorption characteristics of a luminescing compound, but at concentrations much too low and/or under sampling conditions not suitable to absorption spectroscopy. In absorption spectroscopy, the differences between two signals (sample and reference blank) are being examined whereas in fluorescence spectroscopy the actual

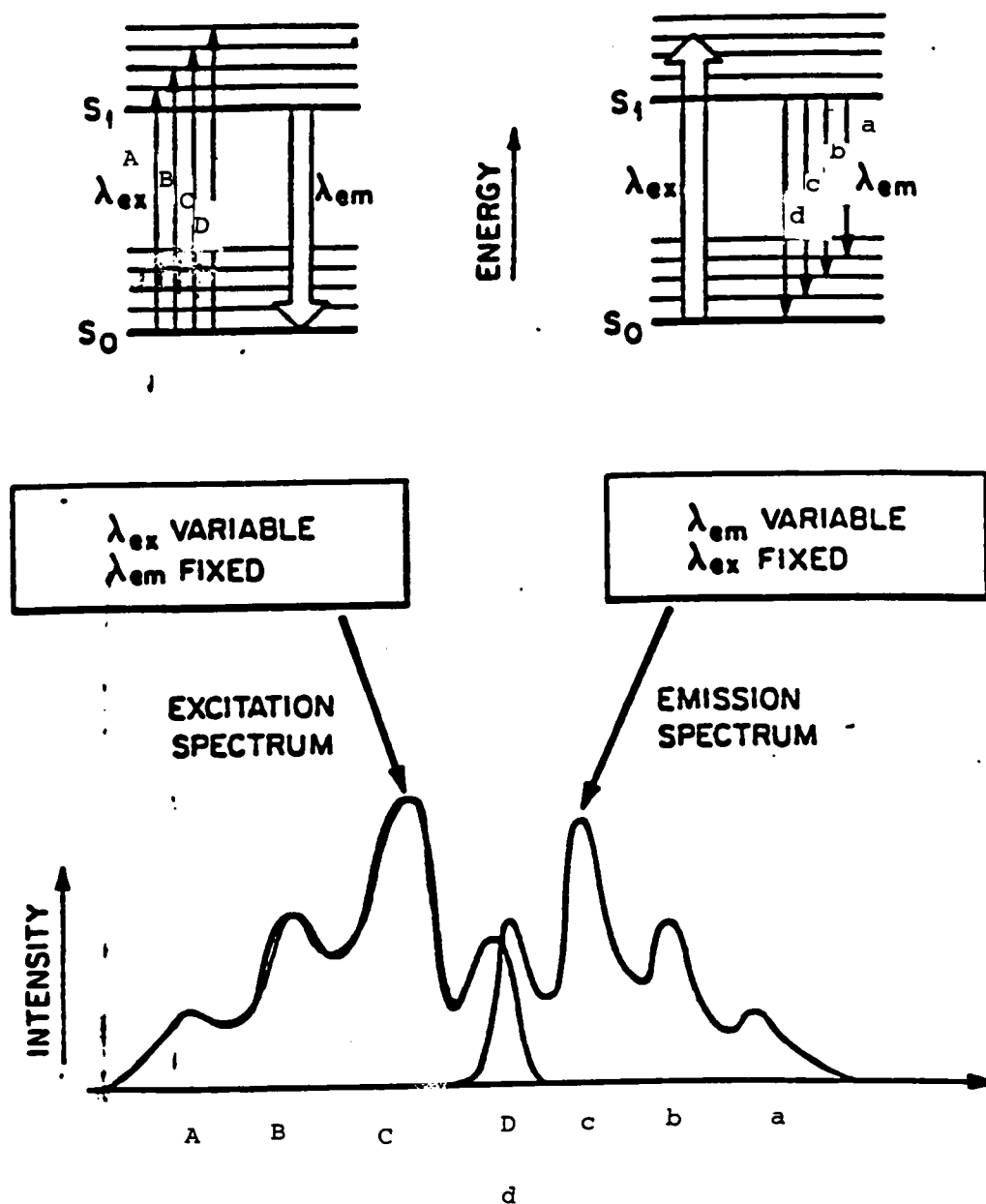


Figure 3. Excitation and Emission Spectrum Measurement

photon count of the sample is being taken. For example, excitation spectroscopy is necessary for the study of singlet-triplet absorptions and for the investigation of quantum efficiency in energy transfer between donor and acceptor molecules because it is the emission that is being monitored and absorption spectroscopy does not monitor emission.

A third possibility of luminescence measurement consists in scanning simultaneously both λ_{em} and λ_{ex} , while keeping a constant wavelength interval ($\Delta\lambda$) between them, reported as emission. This method, called synchronous luminescence, is generally used for obtaining fingerprints of real-life samples and for enhancing the selectivity in the assay of complex systems¹²⁰⁻¹²⁵. This procedure simplifies the emission spectrum and provides for greater selectivity when measuring the fluorescence or phosphorescence from mixtures of compounds.

The most commonly used excitation light sources are high-pressure arc lamps (mercury, xenon, or xenon-mercury lamps). Laser-induced fluorescence (LIF) uses a laser as the excitation source. Other principals remain the same. LIF can be more sensitive than normal fluorescence because the energy is more intense than

energy from conventional light sources. Laser stands for Light Amplification by Stimulating Emission of Radiation.

CHAPTER 2. MATERIALS AND METHODS

A. MATERIALS

The individual macromolecules tryptophan, NADH, FADH₂ and collagen were obtained from Sigma (Sigma, St. Louis, MO). The macromolecules were dissolved in phosphate buffered saline (PBS, pH = 7.4, Sigma, St. Louis, MO) and Richters medium (Sigma, St. Louis, MO). Swim's medium and agarose were also obtained from Sigma (St. Louis, MO).

The rat hepatoma cell line McA and U373 astrocytoma cells were obtained from American Type Culture Collection. The normal rat liver epithelial cell line (RLE) was kindly provided by Craig Dees (Oak Ridge National Laboratory) and originally isolated by J.E. Trosko, Michigan State University¹²⁶. Normal rat astrocytes were isolated in the laboratory of T.K. Rowles according to a previously described method¹²⁷ and kindly provided by these investigators.

For the protein extraction, a Tris-HCl, SDS PAGE denaturing buffer was prepared containing: 2.5 mL 0.5 M Tris-HCl, pH = 6.8 (Biorad, Richmond, CA), 4.0 mL 10% sodium dodecyl sulfate (SDS, Sigma, St. Louis, MO), 0.5 mL β -mercaptoethanol (Sigma, St. Louis,

MO) and 0.5 mL distilled H₂O. Dialysis of the protein extract was performed using a dialysis membrane (Spectrapor, Philadelphia, PA) against a Tris-HCl buffer consisting of 400 mL 10% SDS, 250 mL 0.5 M Tris-HCl and 350 mL distilled water.

Polyacrylamide gels (4-20 gradient) were purchased from Novex (San Diego, CA). The gel buffer solution consisted of 100 mL Tris-Glycine, 10 mL of 10% SDS and 890 mL distilled water. One μ L of a 0.10% bromophenol blue/glycerol (Sigma, St. Louis, MO) solution was mixed with 5 μ L of protein extract. Coomassie Blue was used for staining gels (Novex, San Diego, CA).

B. METHODS

Preparation of Macromolecules and Cells for Fluorescence Studies

Tryptophan (1×10^{-2} M), collagen (1×10^{-2} M), NADH (1×10^{-2} M), and FADH₂ (1×10^{-2} M) were dissolved in 1) PBS, pH 7.4, 2) doubly distilled water and 3) Richters media. Calibration curves were generated from stock solutions to determine if concentrations of these macromolecules were linearly correlated to fluorescence.

Cell Culture Initiation and Splitting

Frozen cells were initiated by removal of the ampule holding

the cells from liquid nitrogen and thawing in a water bath at 37-40°C. When the ice was melted, the ampule was removed from the water and immersed in 70% ethanol in H₂O at room temperature. In a sterile environment, the contents of the ampule were then transferred into a sterile culture flask (T-75cm²). The cell suspension was diluted with 12 mL of Swim's medium (Sigma, St. Louis, MO) for McA cells or Richters medium for RLE cells and placed in a CO₂ incubator. The first subculture was performed 24 hrs after initiation. The medium was removed, fresh trypsin (0.25%) added and the cells were incubated until the cells detached (3 minutes.) Fresh medium was added and the cells were split into 2 new flasks. The doubling times of the RLE and McA cell lines were about 4 days. Rat astrocyte cultures isolated in the laboratory of T.K. Rowles were shown to be 95% pure as identified by the intermediate filament marker protein, glial fibrillary acidic protein (GFAP). Subcultures were used after passage 2 or 3. These cells senesce after approximately 30 days in culture. Astrocytes were maintained in Waymouths media. Human astrocytoma U373 cells were maintained in DMEM media. The doubling time for U373 cells was approximately 24 hours.

Cells were harvested for fluorescence studies by trypsinizing, as described above. Media was added to quench the trypsin reaction and the cells were transferred to 10 ml centrifuge tubes under sterile conditions. The cells were then centrifuged at 200 x G for 5 min., the supernatant was removed by aspiration, and the cells were rinsed three times with PBS. A preparation of low melting agarose (0.5 g in 15 mL PBS) was prepared, heated in a microwave oven for 20 s, then allowed to cool for 2 min. Equal amounts of agarose and cells (v/v) were added to a cuvette for fluorescence analysis.

Protein Extraction and Dialysis

Protein was extracted from the trypsinized cells by adding 0.5 mL of Tris-glycine, SDS PAGE denaturing buffer and heating for 90 s in boiling water. The cell/buffer mixture was then centrifuged at 200 x G for 15 min and the supernatant was removed for fluorescence measurements. A solution consisting of the buffer solution treated in the same manner was used for background controls.

After fluorescence analysis, the protein extract was placed in dialysis membrane tubing and left overnight in 1000 mL buffer solution consisting of 400 mL of 10% SDS and 250 mL 0.5 M Tris-

HCl, pH 6.8 and 350 mL of distilled water. The dialysis membranes were then removed from the buffer, and fluorescence of the samples within were analyzed.

Polyacrylamide Gel Electrophoresis

After analysis of the dialyzed extracts, the samples and a kaleidoscope marker were heated for 90 seconds in boiling water, then the protein extracts were centrifuged for 15 min at 200XG.

One μL of a 0.10% bromophenol blue/glycerol solution was mixed with every 5 μL of protein extract. The polyacrylamide gels (4-20 gradient) had 10 columns, two of which were reserved for the kaleidoscope marker. The remaining eight columns were used for the protein extracts with each line having 4 columns for 2 different volumes. The gel wells were washed, filled with the Tris-glycine buffer described on page 27, and the marker and extracts were loaded (20 μL) underneath the buffer. The gel was run using a voltage of 150 V for 1.5 hrs. The gels were then either stained with Coomassie Blue, or placed in destain solution (40% methanol, 10% acetic acid, 60% distilled water) for 30 min, then placed in distilled water until analysis.

Fluorescence Detection

Fluorescence was determined using a Perkin-Elmer Model LS-50 B luminescence spectrophotometer (Atlanta, GA). Fluorescence excitation and emission scans of tryptophan, collagen, NADH and FAD were performed using the following parameters: tryptophan: $\lambda_{ex}=287$, $\lambda_{em}=355$; collagen: $\lambda_{ex}=280$, $\lambda_{em}=350$; NADH: $\lambda_{ex}=340$, $\lambda_{em}=455$; FAD: $\lambda_{ex}=360$, $\lambda_{em}=520$. Slit widths for all measurements were 2.5/2.5. Excitation and emission wavelengths were chosen to correspond with previously cited research and were used for measurements of components, cells and protein extracts. Synchronous scans were performed using wavelength differences or delta lamdas ($\Delta\lambda$) of 10 nm to 100 nm.

CCD Cellular Imaging

The fluorescence of McA and RLE cells were examined using a CCD camera (Photometrics, San Diego, CA) attached to a Nikon Microphot-SA (Nikon, Garden City, NY). 2.0 μ l of harvested, suspended cells were spotted onto quartz microscope slides and covered with quartz microscope cover slides. Images were first recorded using excitation by white light, then fluorescence exposures were taken by exciting the cells using a He-Cd laser (325

nm, 8 mW, Omnicrome, Chino, CA). Images were saved using PMIS software (Photometrics, San Diego, CA).

CCD Fluorescence Imaging of Gel

A charge coupled device (CCD) (Photometrics, San Diego, CA) was used to image fluorescence from the gels. Figure 4 shows a schematic of the CCD detection system. A HeCd laser (325 nm, 8 mW, Omnicrome, Chino, CA) was used as the excitation source. The CCD was focused on the entire gel so that protein bands of both tumor and nontumor cell lines could be examined at the same time. Exposure times up to 1 hour were used.

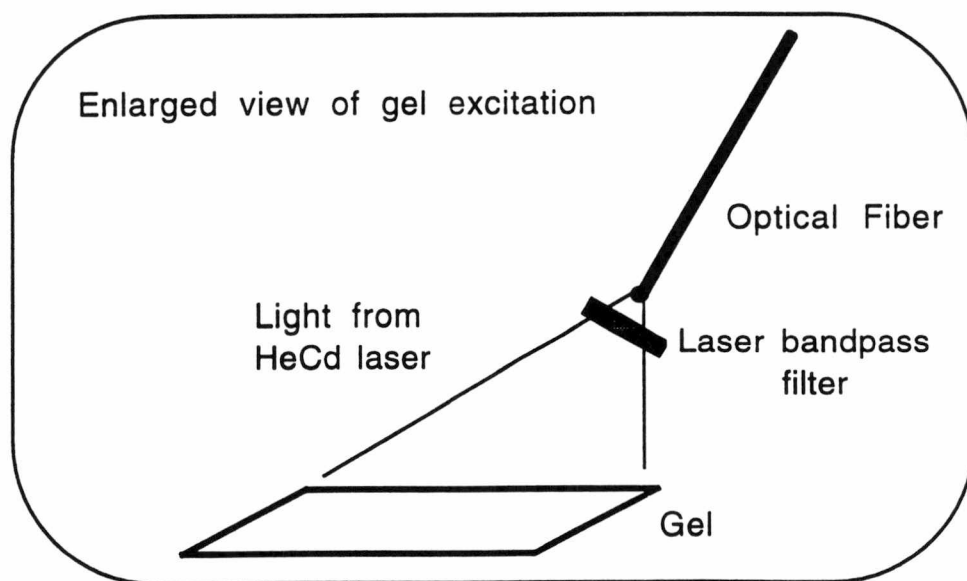
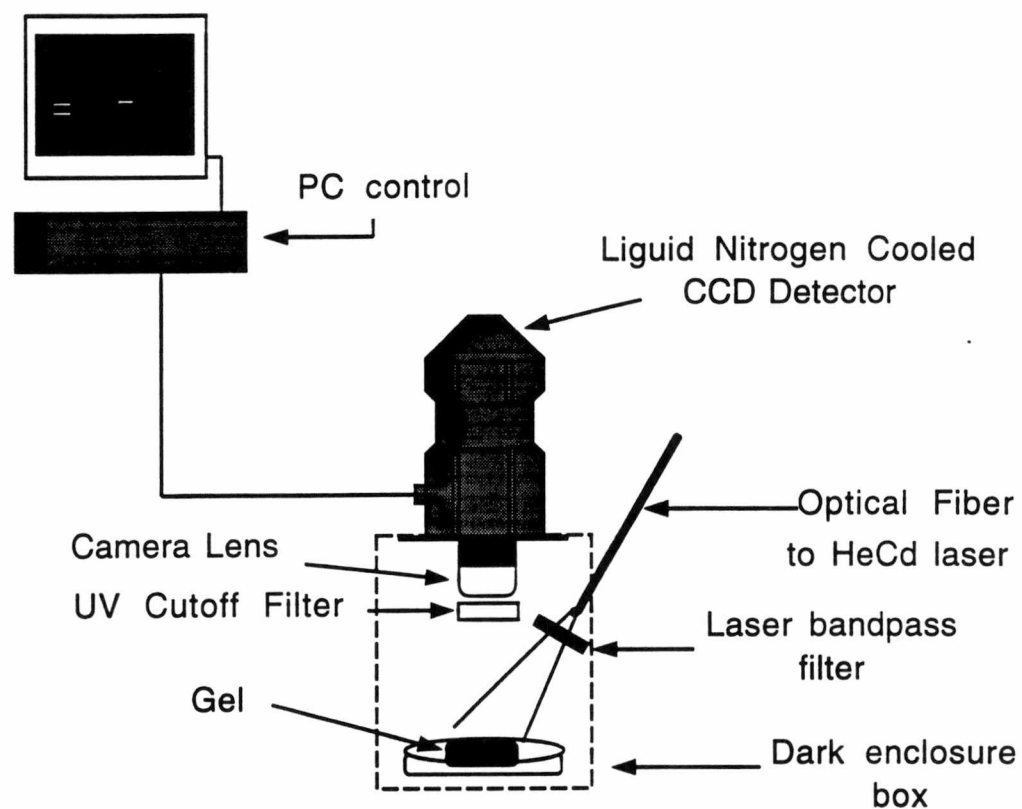


Figure 4. Schematic Diagram of CCD Detection System

CHAPTER 3. RESULTS

Excitation and emission scans for tryptophan, collagen, NADH and FADH₂ in PBS are shown in Figure 5. Synchronous measurements were performed using $\Delta \lambda$ s of 10nm to 100nm, and $\Delta \lambda = 20$ nm and slit widths 2.5/2.5 were chosen for further studies on the basis of separation of components and well defined peaks. Synchronous fluorescence spectra of the individual compounds are shown in Figure 6. Peak fluorescence of tryptophan, collagen, NADH and FADH₂ using this technique were 280nm, 290nm, 380nm and 480nm, respectively. Mixtures of tryptophan, NADH and FADH₂ were analyzed using synchronous scans, ($\Delta \lambda = 20$ nm) to ensure detection was possible with varying concentrations (Figures 7,8). In the mixture, the tryptophan peak shifted 10 nm to 290 nm whereas NADH and FADH₂ peaks remained at 380 nm and 480 nm. Equimolar concentrations were used in Figure 7 whereas Figure 8 shows the same spectrum for a molar ratio of 20:1:1 (tryptophan: NADH: FADH₂).

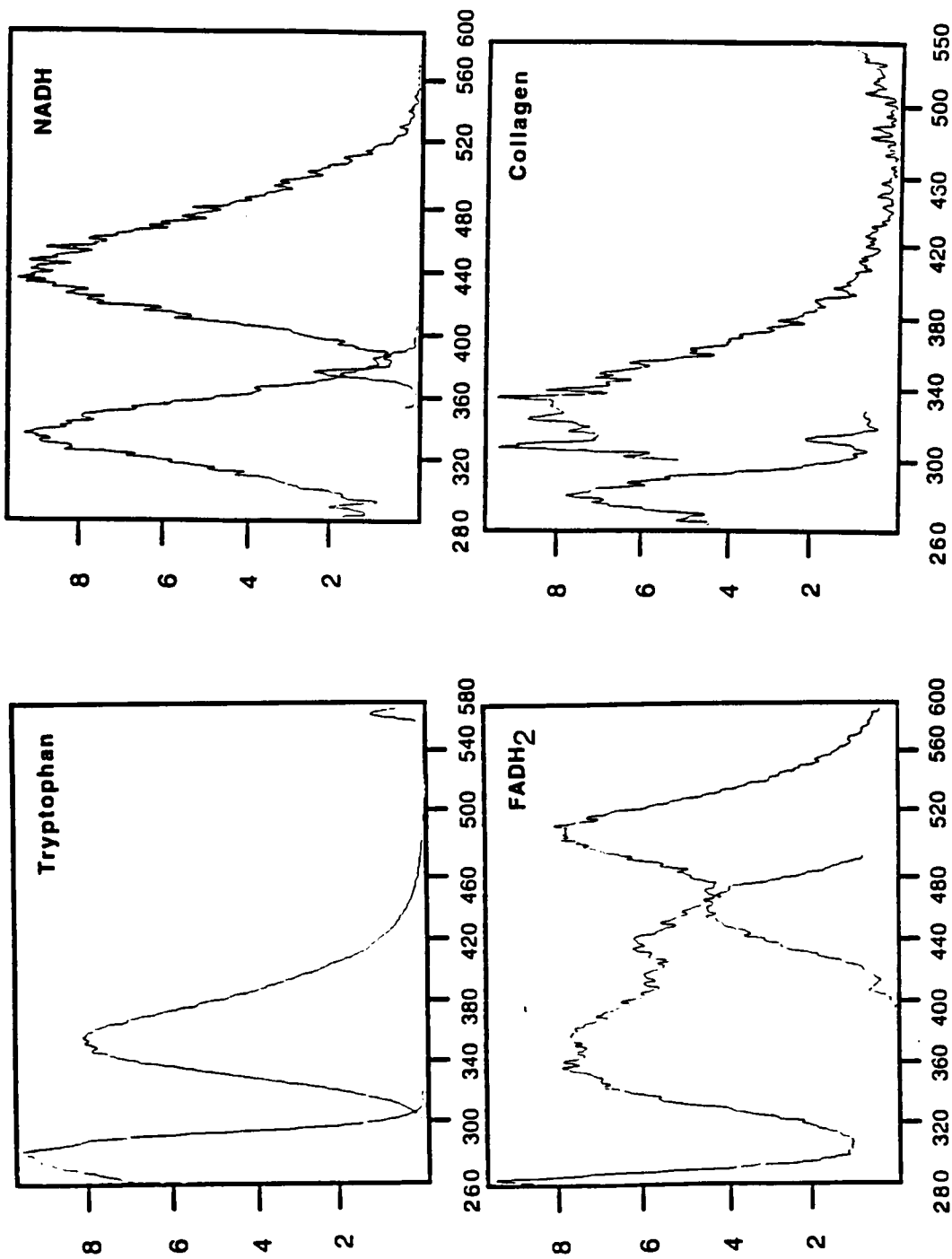


Figure 5. Excitation and Emission Fluorescence of Tryptophan, Collagen, NADH and FADH₂

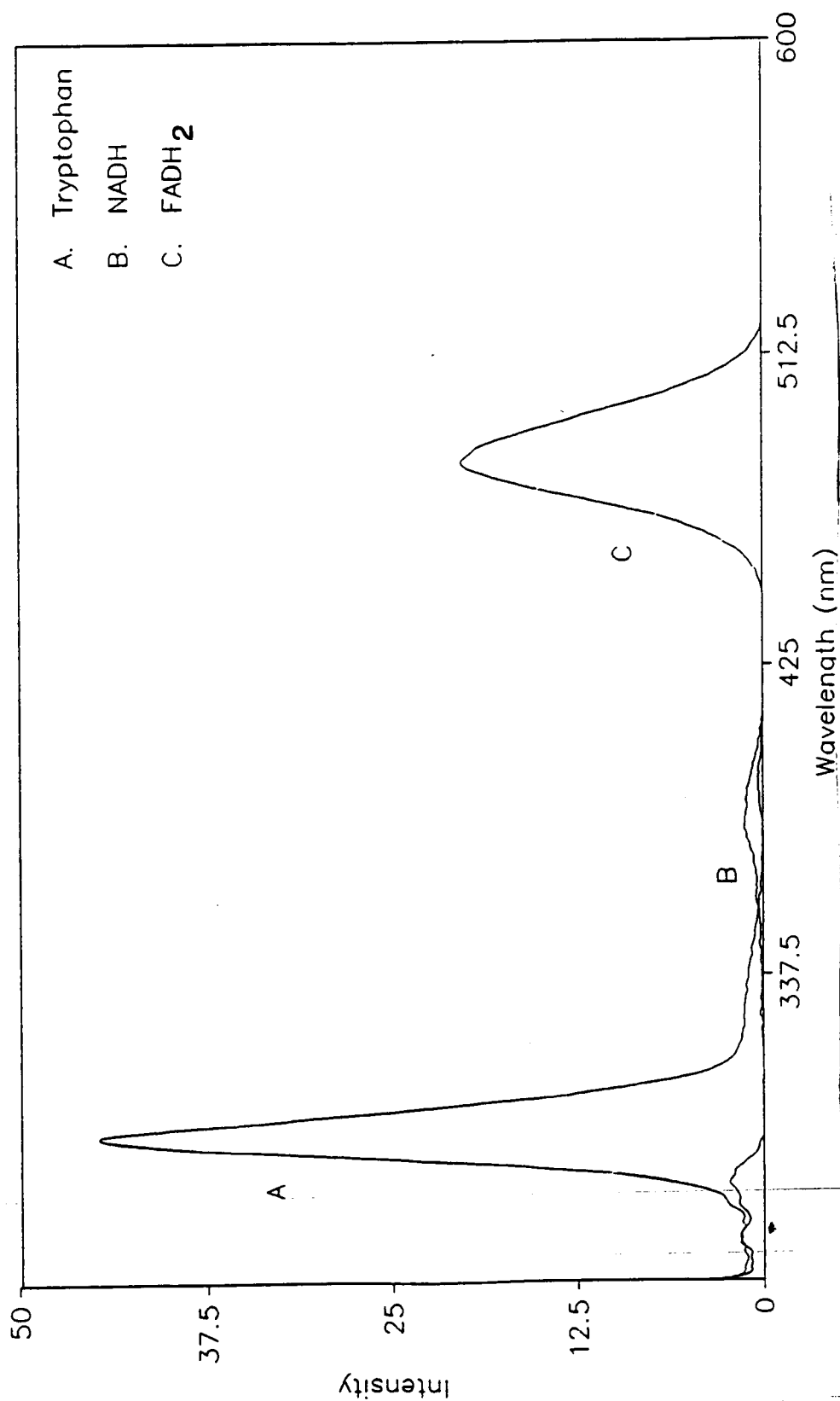


Figure 6. Synchronous Fluorescence of Tryptophan, Collagen, NADH and FADH₂,

$\Delta\lambda = 20 \text{ nm}$

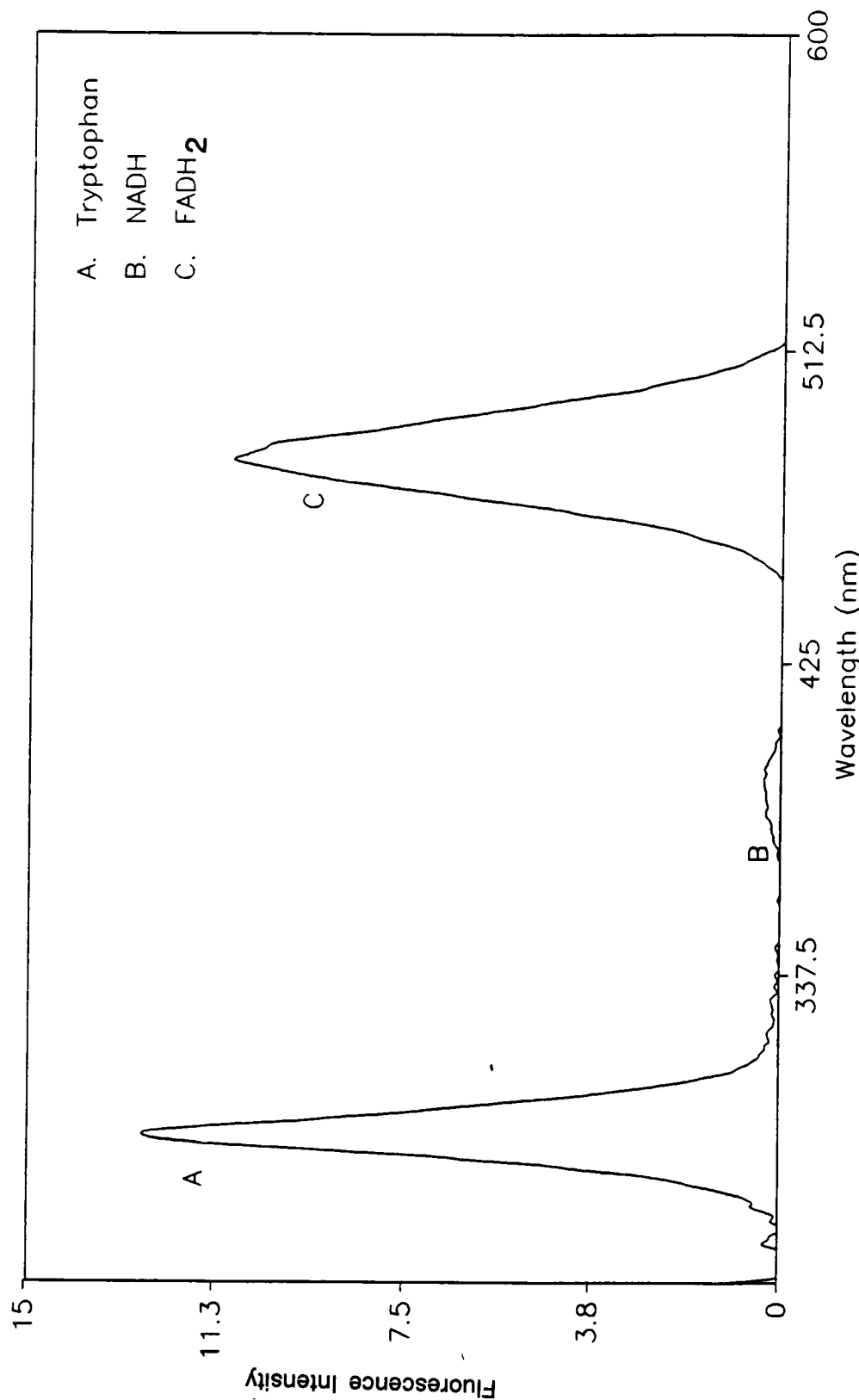


Figure 7. Synchronous Fluorescence of Tryptophan, Collagen, NADH and FADH, 1:1:1 Mixture, $\Delta\lambda = 20$ nm

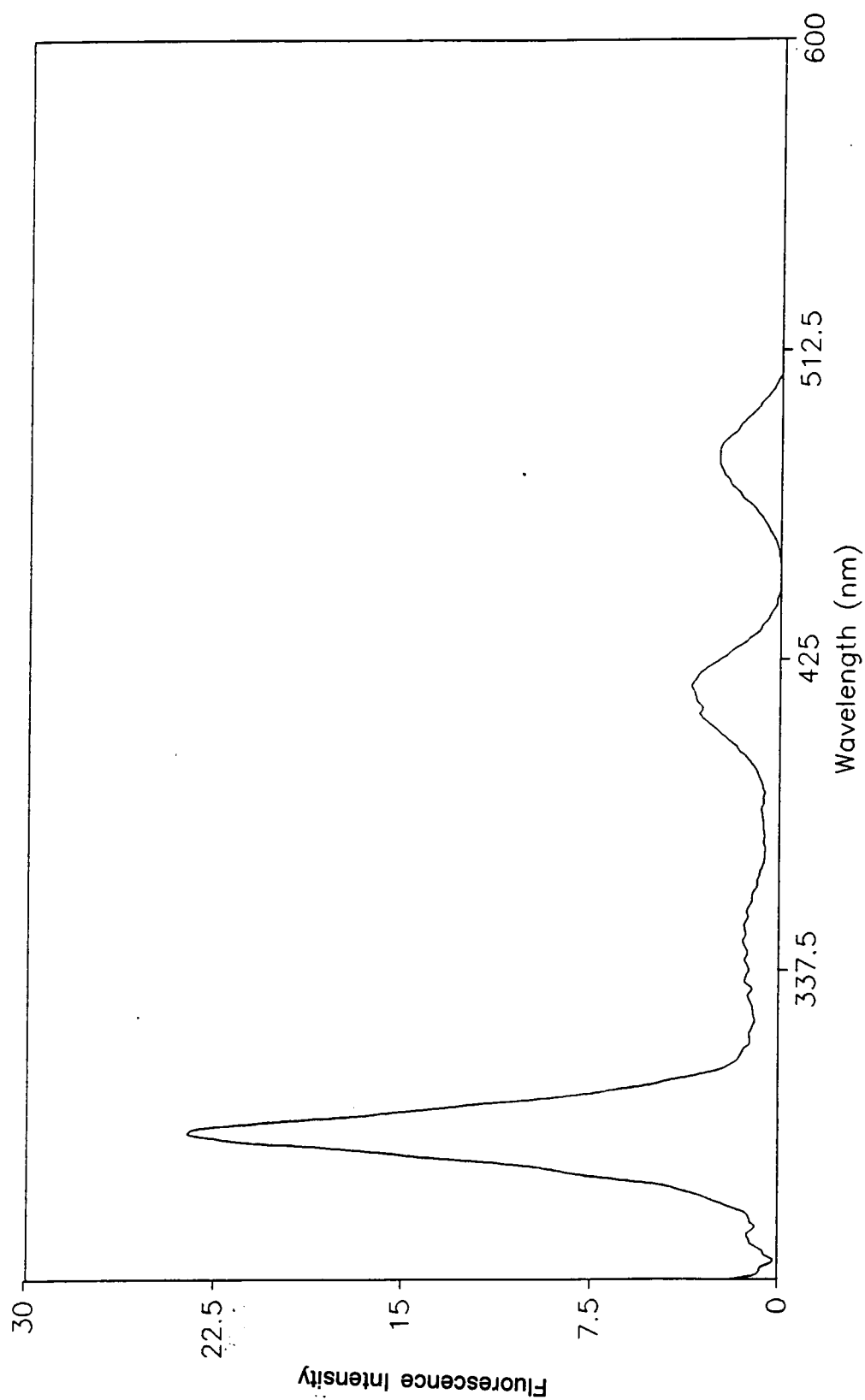


Figure 8. Synchronous Fluorescence of Tryptophan, Collagen, NADH, and FADH₂, 20:1:1:1 Mixture, $\Delta\lambda = 20$ nm

The synchronous fluorescence spectrum ($\Delta \lambda = 20\text{nm}$) of a mixture of tryptophan, NADH and FADH_2 was also analyzed in porcine serum (Figure 9). Peaks occurred at the same positions in the serum as before. Figure 10 shows the synchronous spectrum ($\Delta \lambda = 20 \text{ nm}$) of porcine serum alone.

Fixed excitation spectra of cells suspended in agarose are shown using excitations as follows: 280nm, 287nm, 310nm, 325 nm, 340nm and 360nm (Figures 11-16). The spectrum of agarose did not interfere with any cellular signal. The emission spectra of the two cell lines did not differ with respect to emission at any of the wavelengths. Synchronous measurements using $\Delta \lambda$'s from 10 nm to 100 nm indicated $\Delta \lambda = 20 \text{ nm}$ and $\Delta \lambda = 60 \text{ nm}$ resulted in sharper and better separated peaks compared to other wavelength differences (Figure 17,18).

When cells excited by the He-CD laser were imaged using the CCD camera attached to a microscope, fluorescence could be seen. (Figure 19, 20)

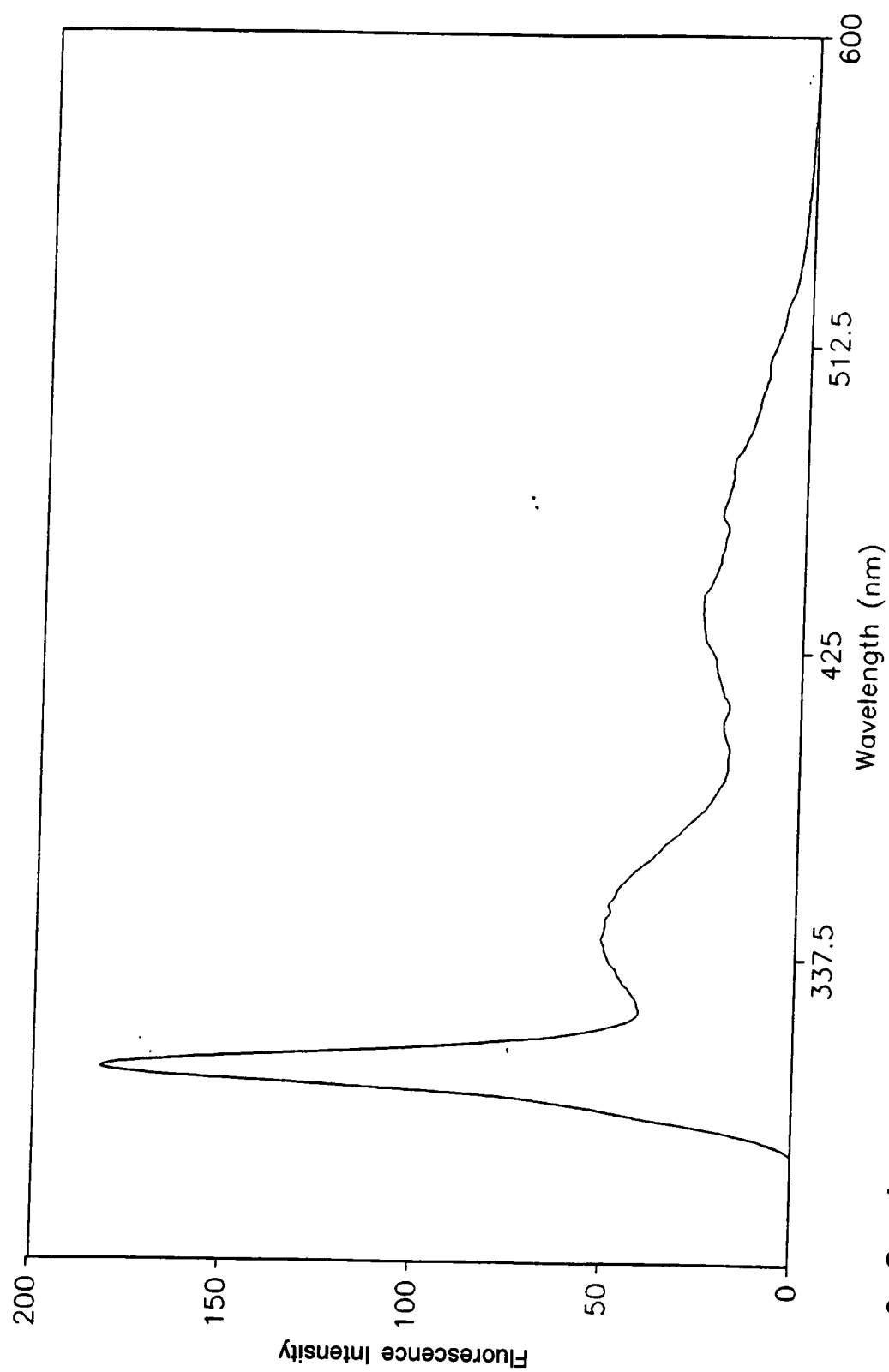


Figure 9. Synchronous Fluorescence of a 1:1:1 Mixture of Tryptophan, NADH and FADH₂ in Porcine Serum, $\Delta\lambda = 20$ nm

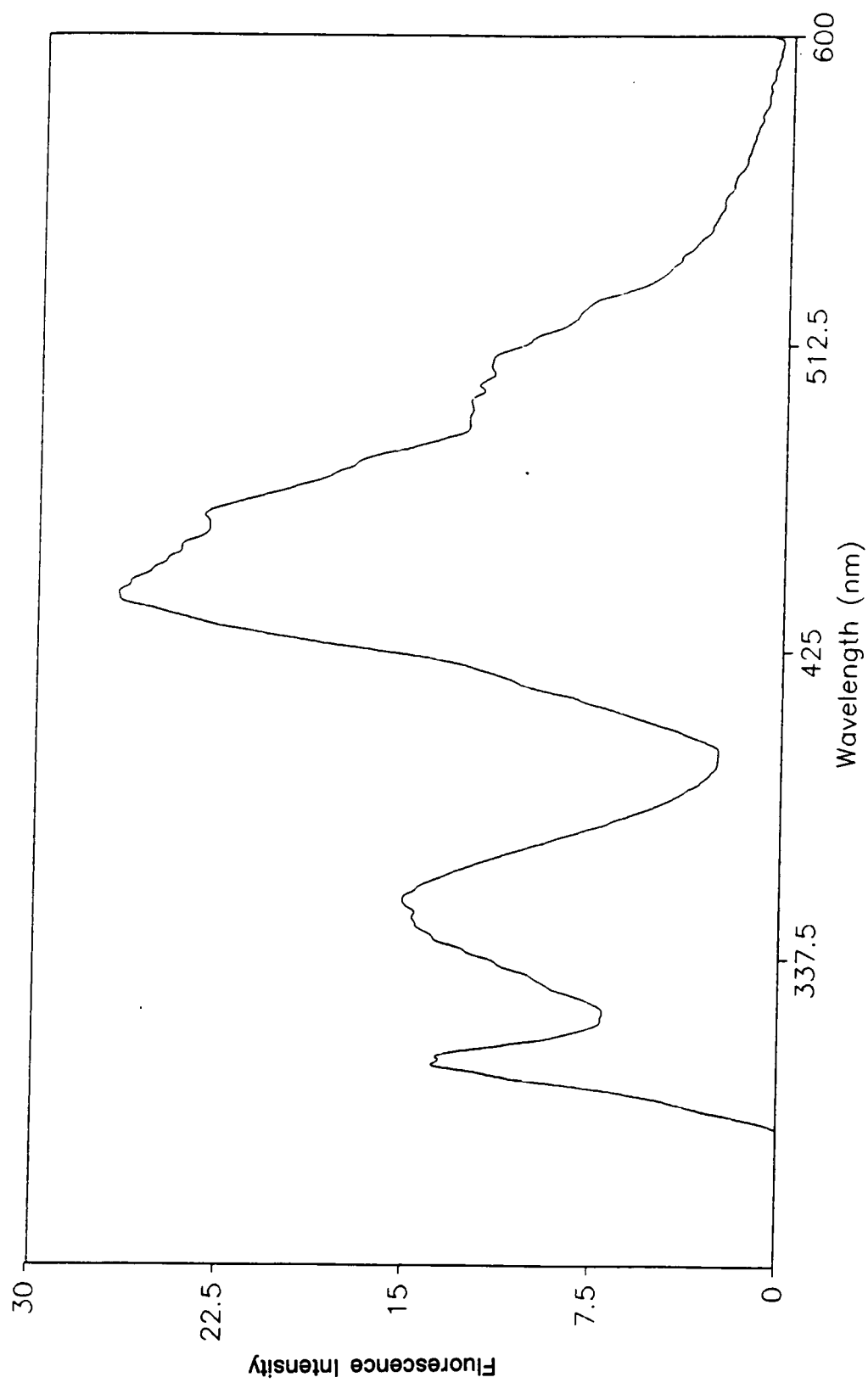


Figure 10. Synchronous Fluorescence of Porcine Serum, $\Delta\lambda = 20$ nm

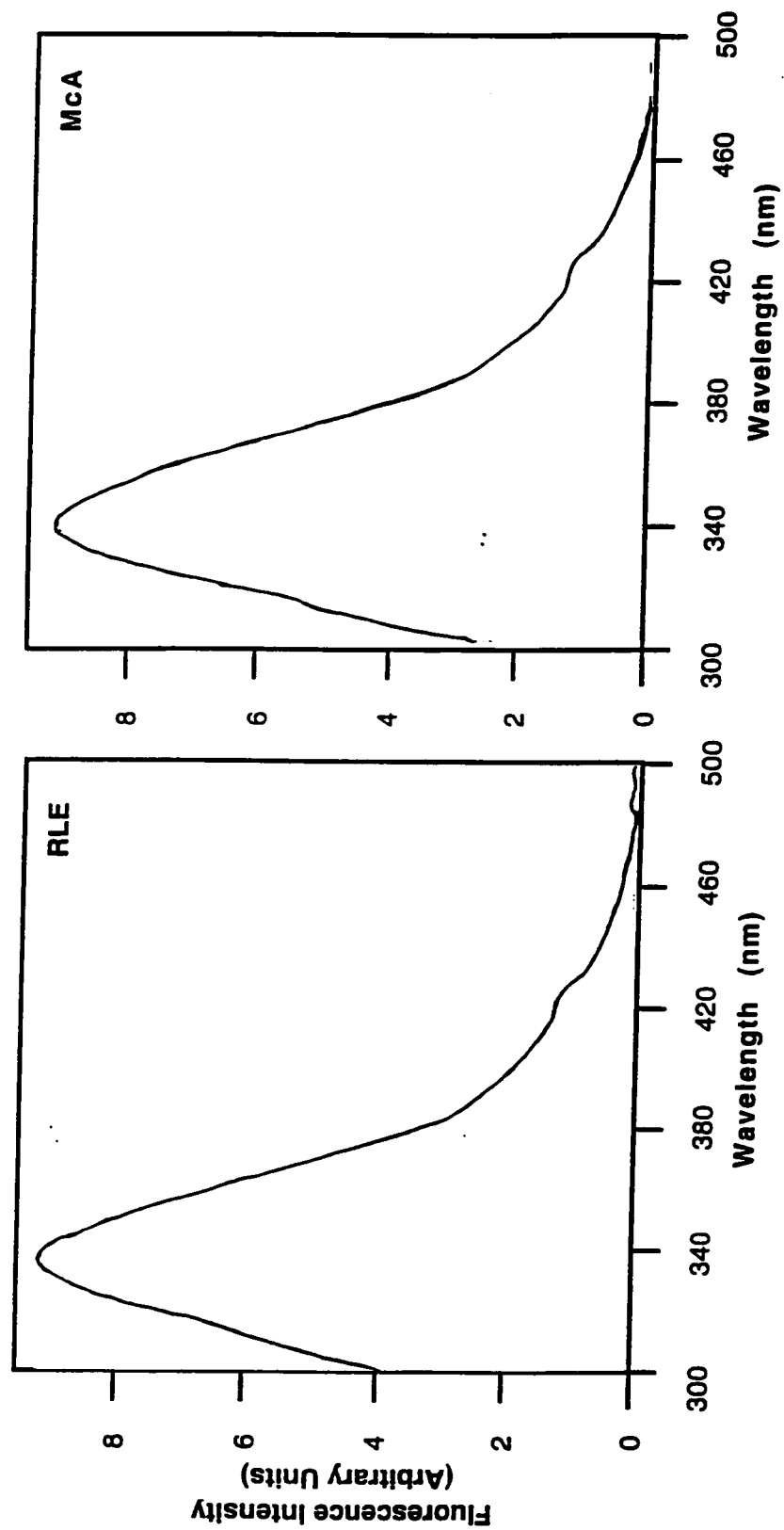


Figure 11. Emission Fluorescence of RLE and McA Cells Suspended in Agarose,
 $\lambda_{ex} = 280 \text{ nm}$

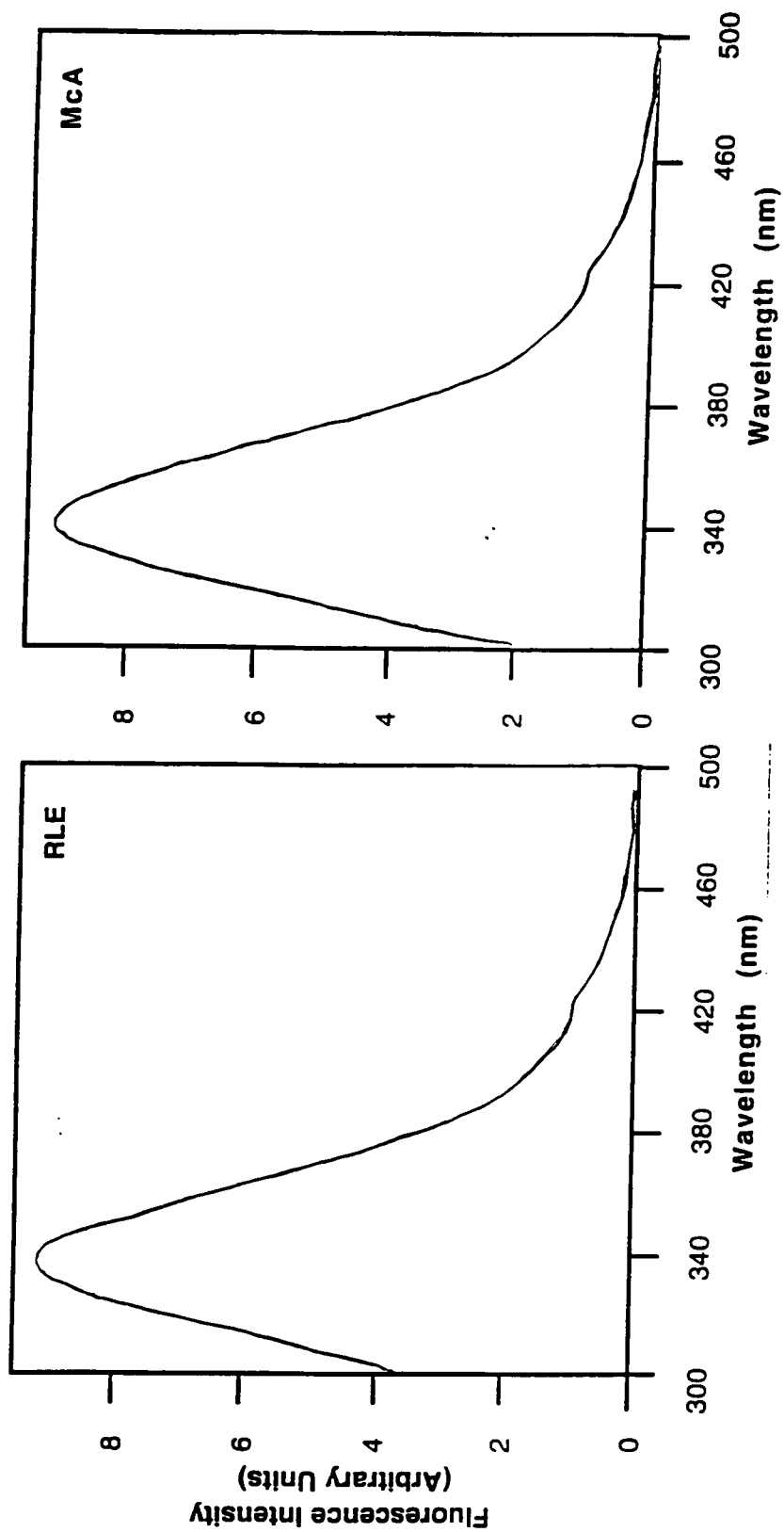


Figure 12. Emission Fluorescence of RLE and McA Cells Suspended in Agarose,
 $\lambda_{ex} = 287 \text{ nm}$

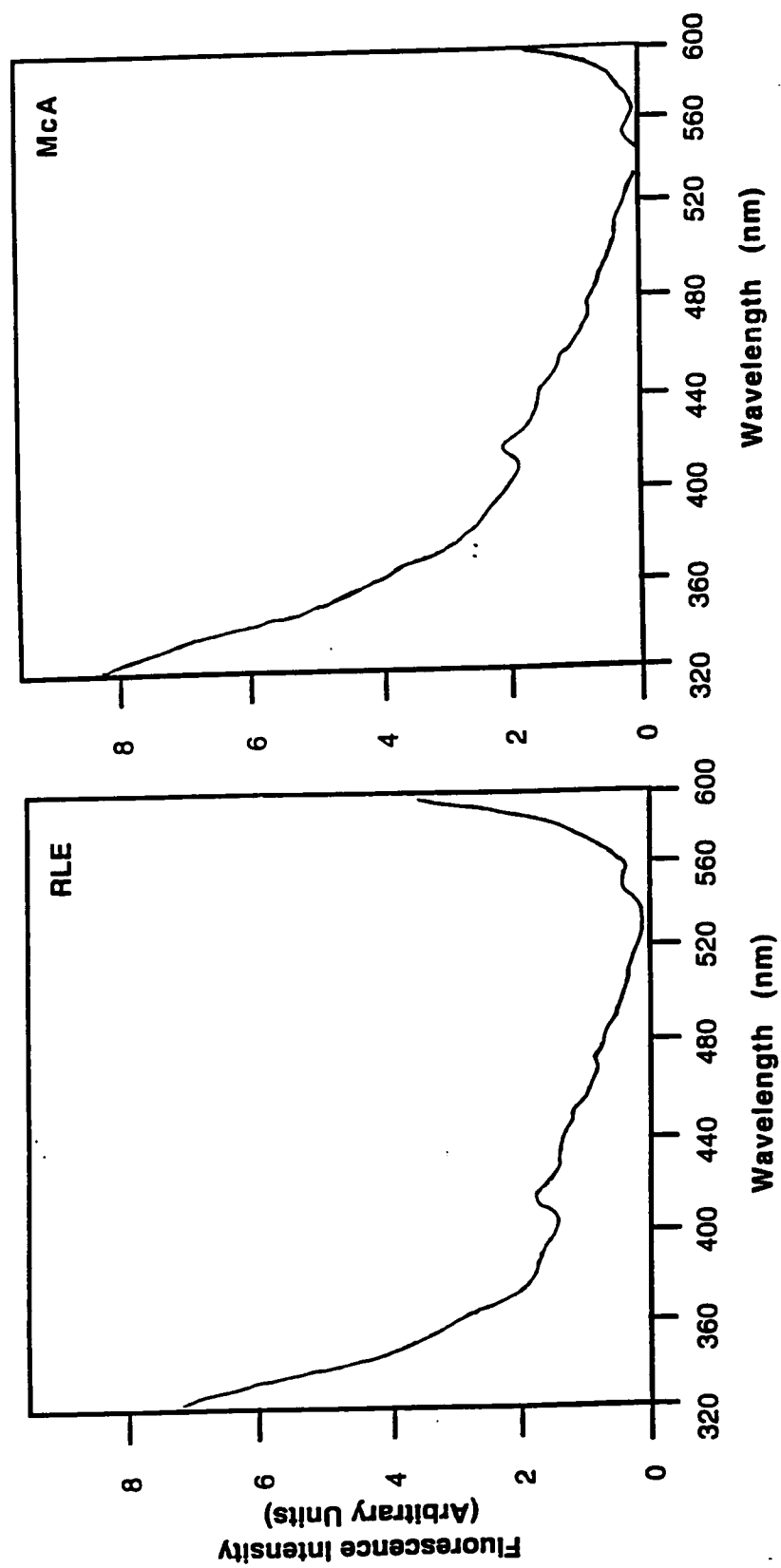


Figure 13. Emission Fluorescence of RLE and MCA Cells Suspended in Agarose,
 $\lambda_{ex} = 310 \text{ nm}$

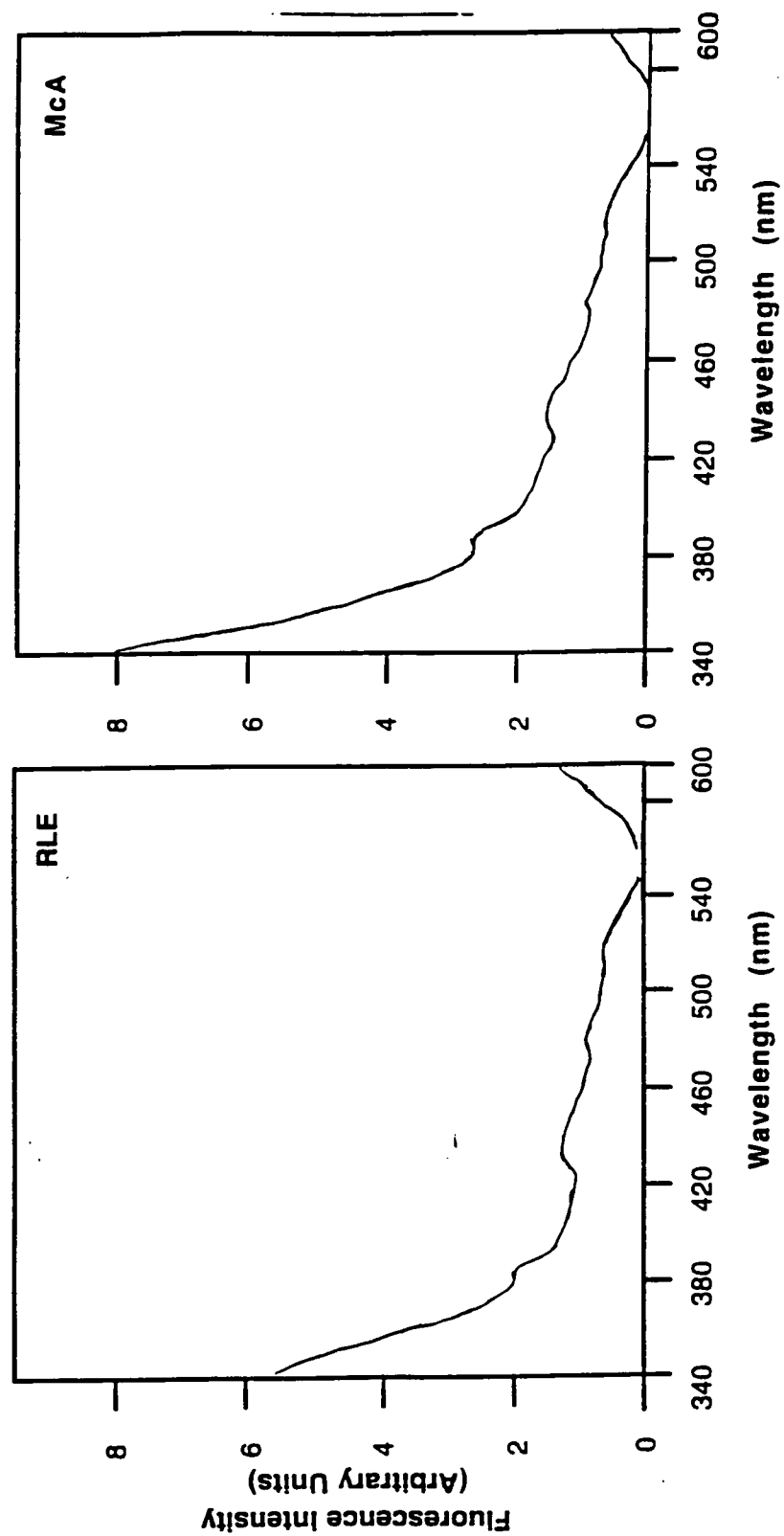


Figure 14. Emission Fluorescence of RLE and McA Cells Suspended in Agarose,
 $\lambda_{\text{ex}} = 325 \text{ nm}$

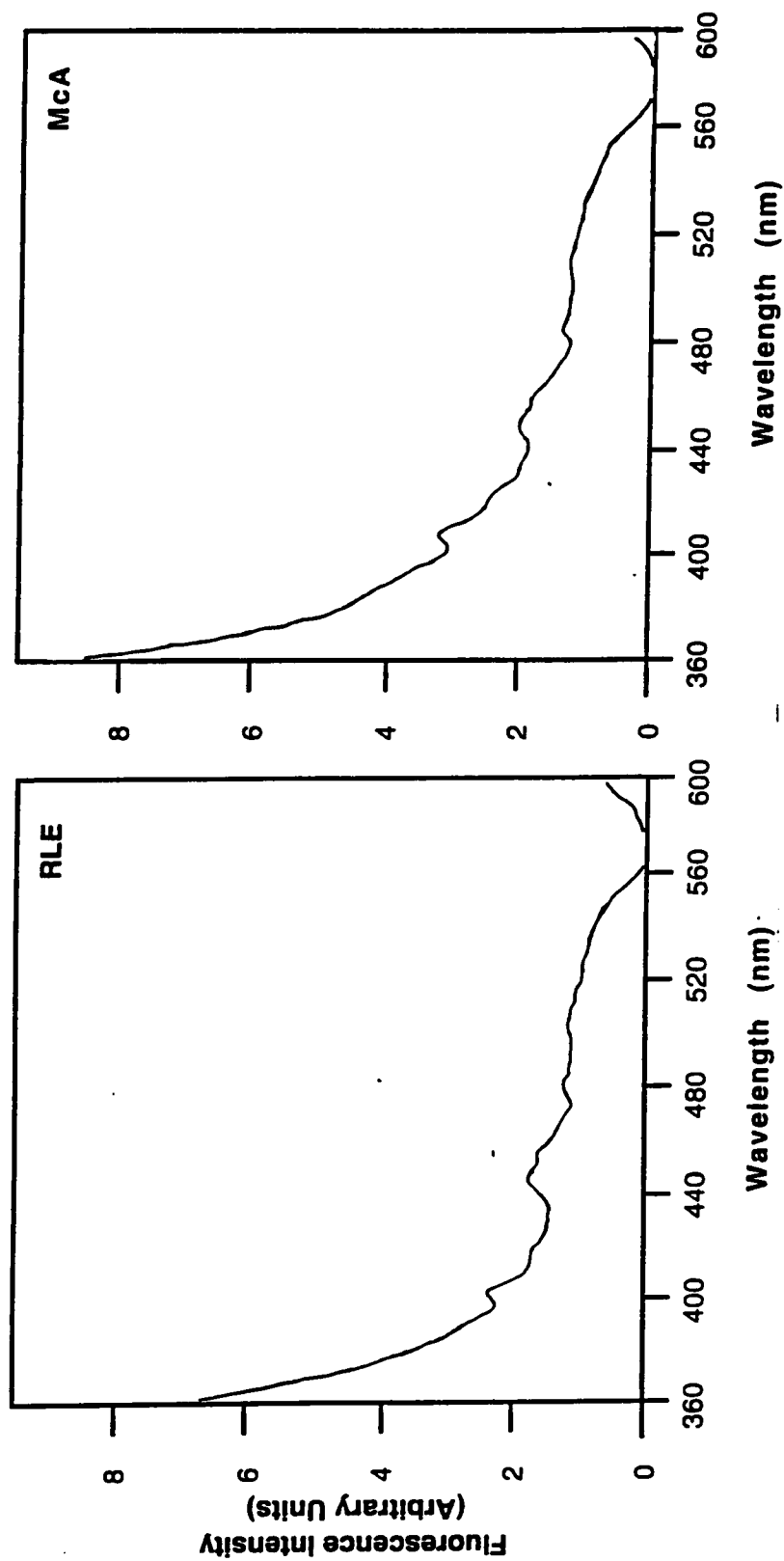


Figure 15. Emission Fluorescence of RLE and McA Cells Suspended in Agarose,
 $\lambda_{ex} = 340 \text{ nm}$

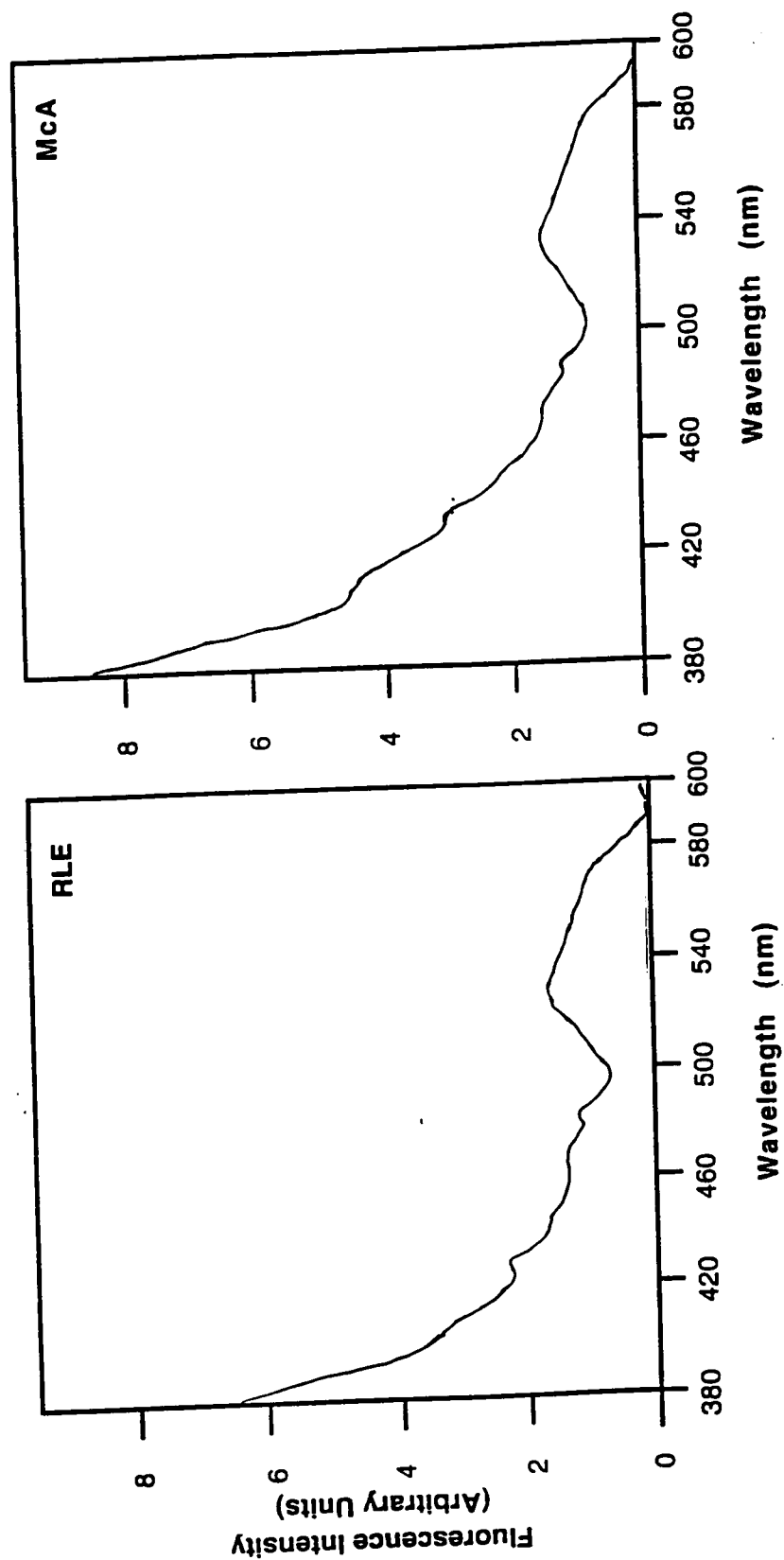


Figure 16. Emission Fluorescence of RLE and McA Cells Suspended in Agarose,
 $\lambda_{ex} = 360 \text{ nm}$

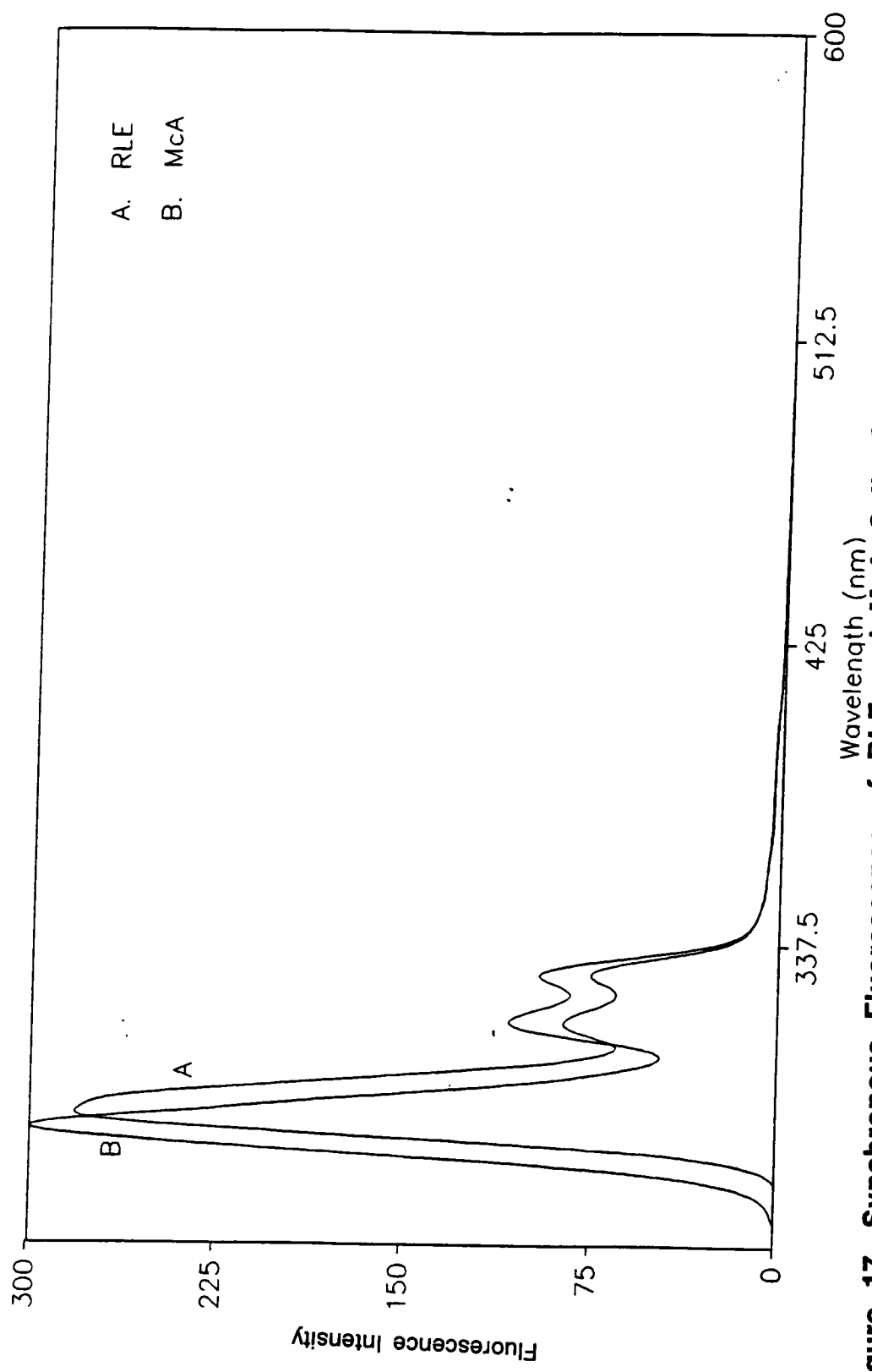


Figure 17. Synchronous Fluorescence of RLE and MCA Cells Suspended in Agarose,
 $\Delta\lambda = 20 \text{ nm}$

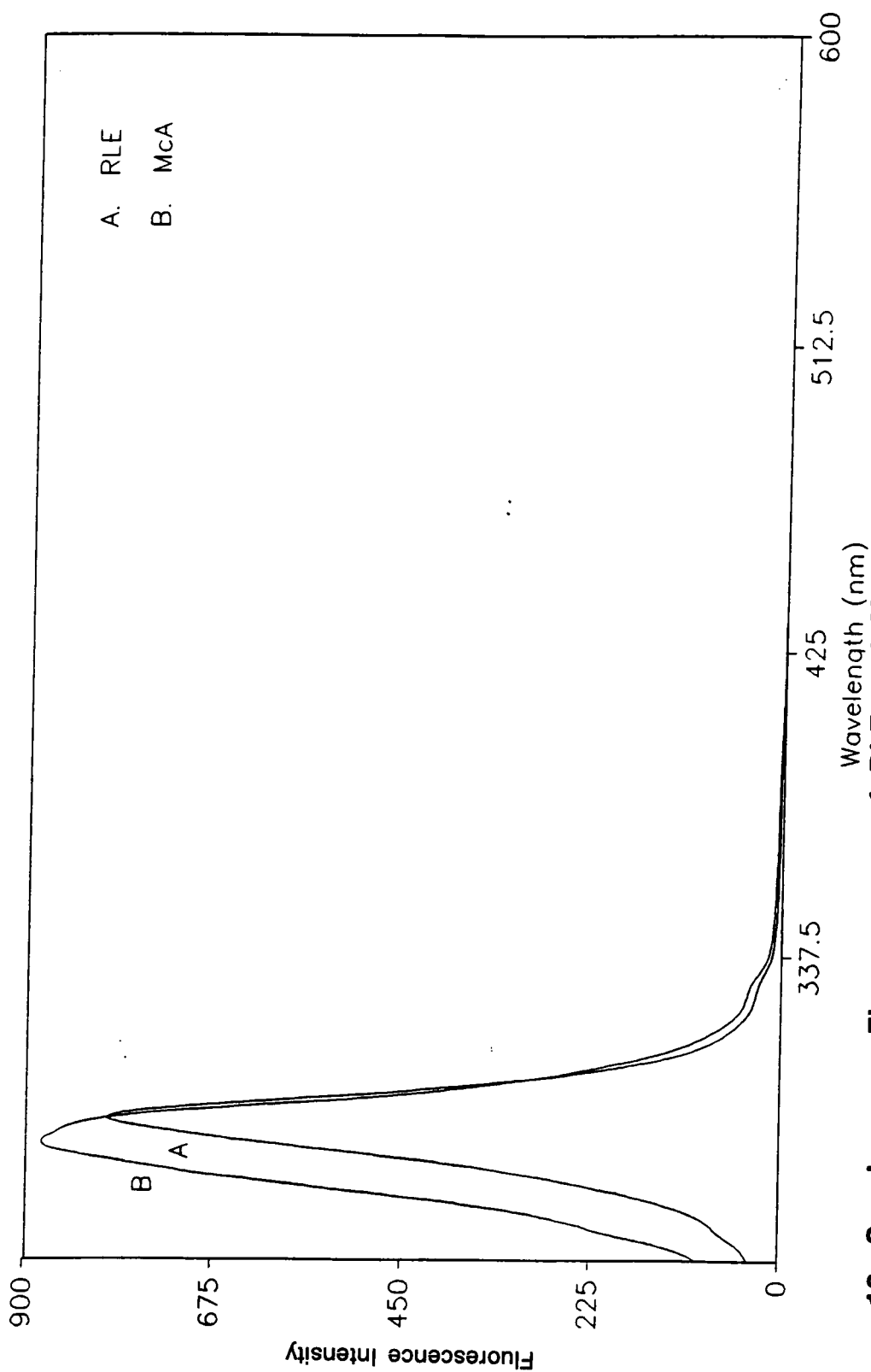


Figure 18. Synchronous Fluorescence of RLE and McA Cells Suspended in Agarose,
 $\Delta\lambda = 60 \text{ nm}$

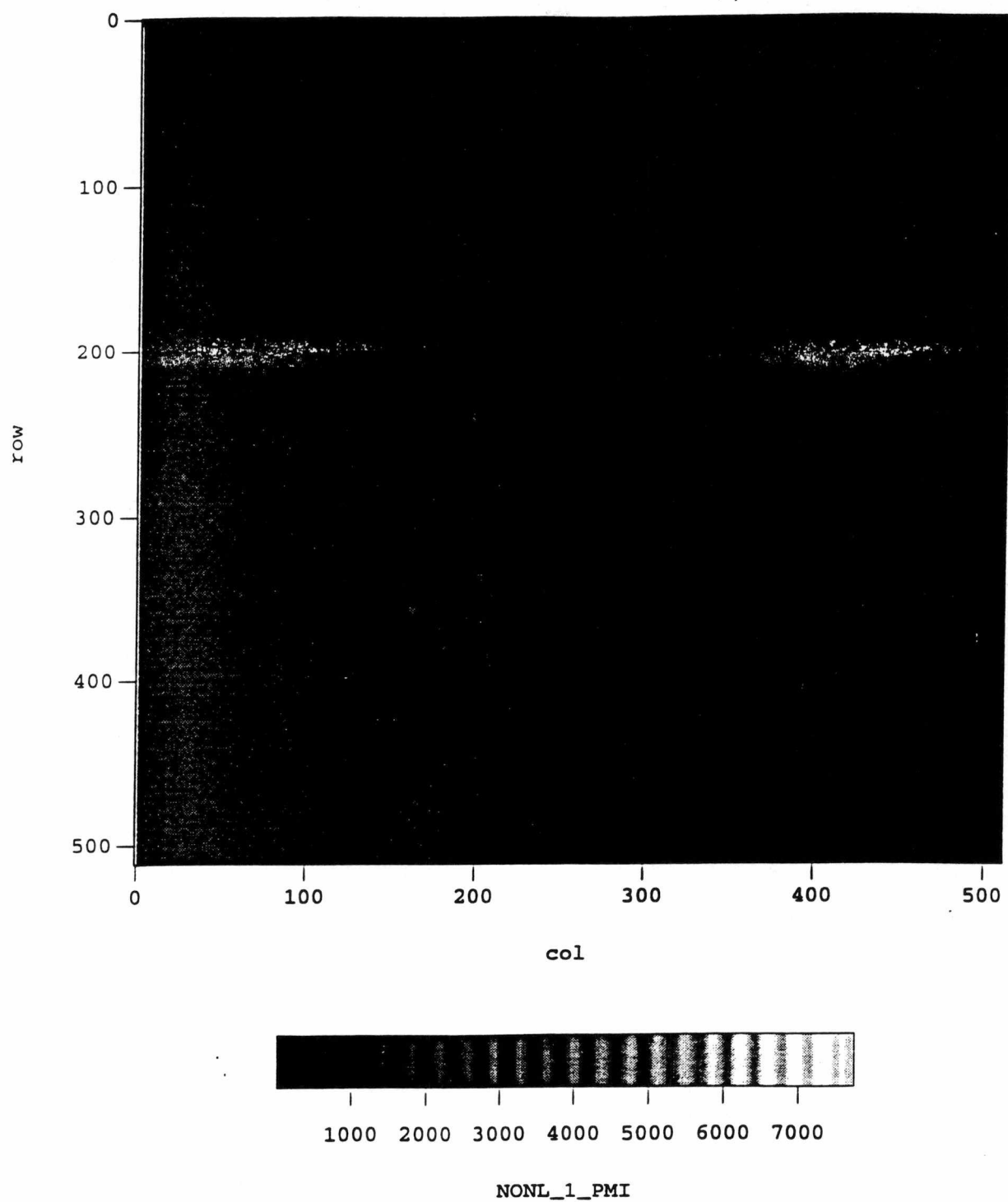


**Figure 19. CCD Image of Normal Rat Liver Epithelial (RLE)
Cell Fluorescence**

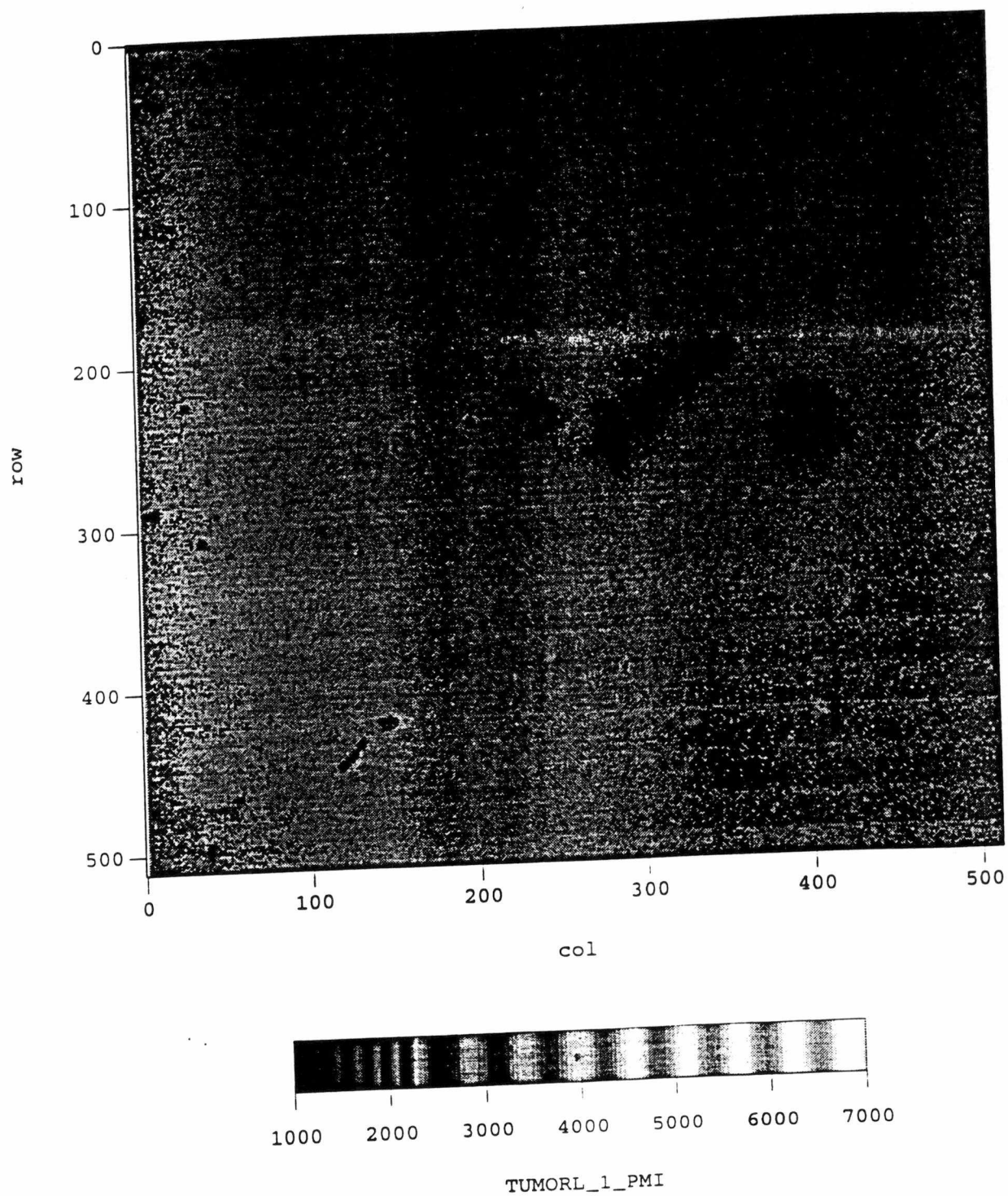


**Figure 20. CCD Image of Neoplastic McA Cell
Fluorescence**

The denatured proteins (prior to dialysis) from the two cell lines were excited at 280nm, 287nm, 292nm, 310nm, 325nm in the luminescence spectrophotometer (Perkin-Elmer LS50B, Atlanta, GA). Excitations were based on those used for the macromolecules and cells suspended in agarose; and on experimental exploration of varying excitation wavelengths. Excitation and emission spectra are shown (Figures 21-25). At $\lambda_{ex}=280, 287$ and 292nm, McA cell extracts exhibited strong emission peaks at 340 nm and 350 nm whereas the peaks were not as predominant in the RLE cell line extracts. The maximum emission for the McA cell extract was 410nm whereas the maximum emission for the RLE cell extract was 390nm when both were excited at 325nm.

Dialysis of the protein extracts were performed in order to remove small molecules that might quench fluorescence. The excitation/emission spectra at $\lambda_{ex}= 287\text{nm}, 310\text{nm}, 325\text{nm},$ and 360nm were recorded in Figures 26, 27, 28 and 29. Synchronous spectra at $\Delta \lambda = 20\text{nm}$ and $\Delta \lambda = 60\text{nm}$ are shown in Figures 30 and 31. As before the conventional scans showed no differences in the two cell lines. The McA cells were shifted to the blue about 10nm in the synchronous scans.

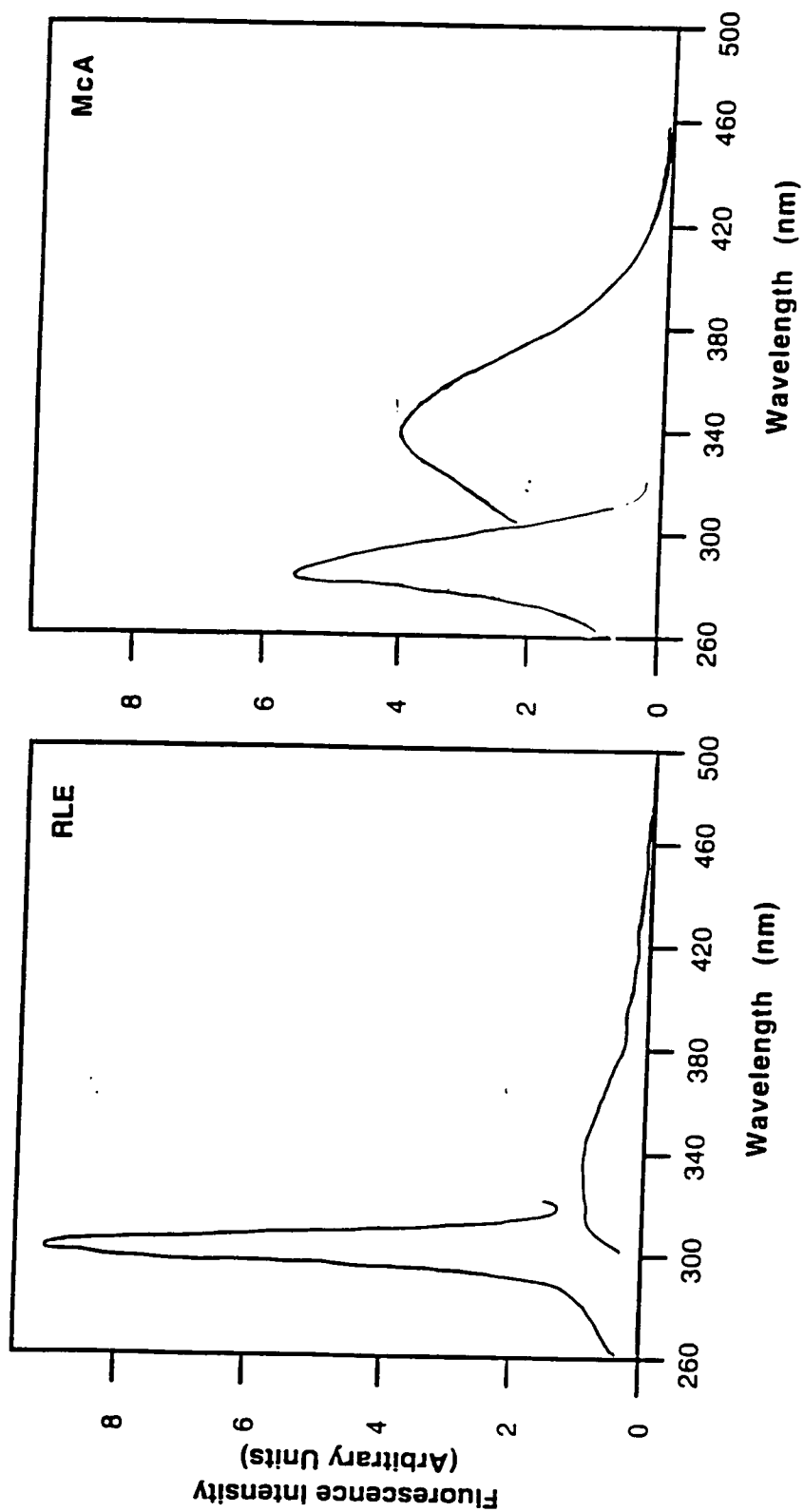


Figure 21. Excitation/Emission Fluorescence of Protein Extracts from RLE and MCA Cells, $\lambda_{\text{ex}} = 280 \text{ nm}$

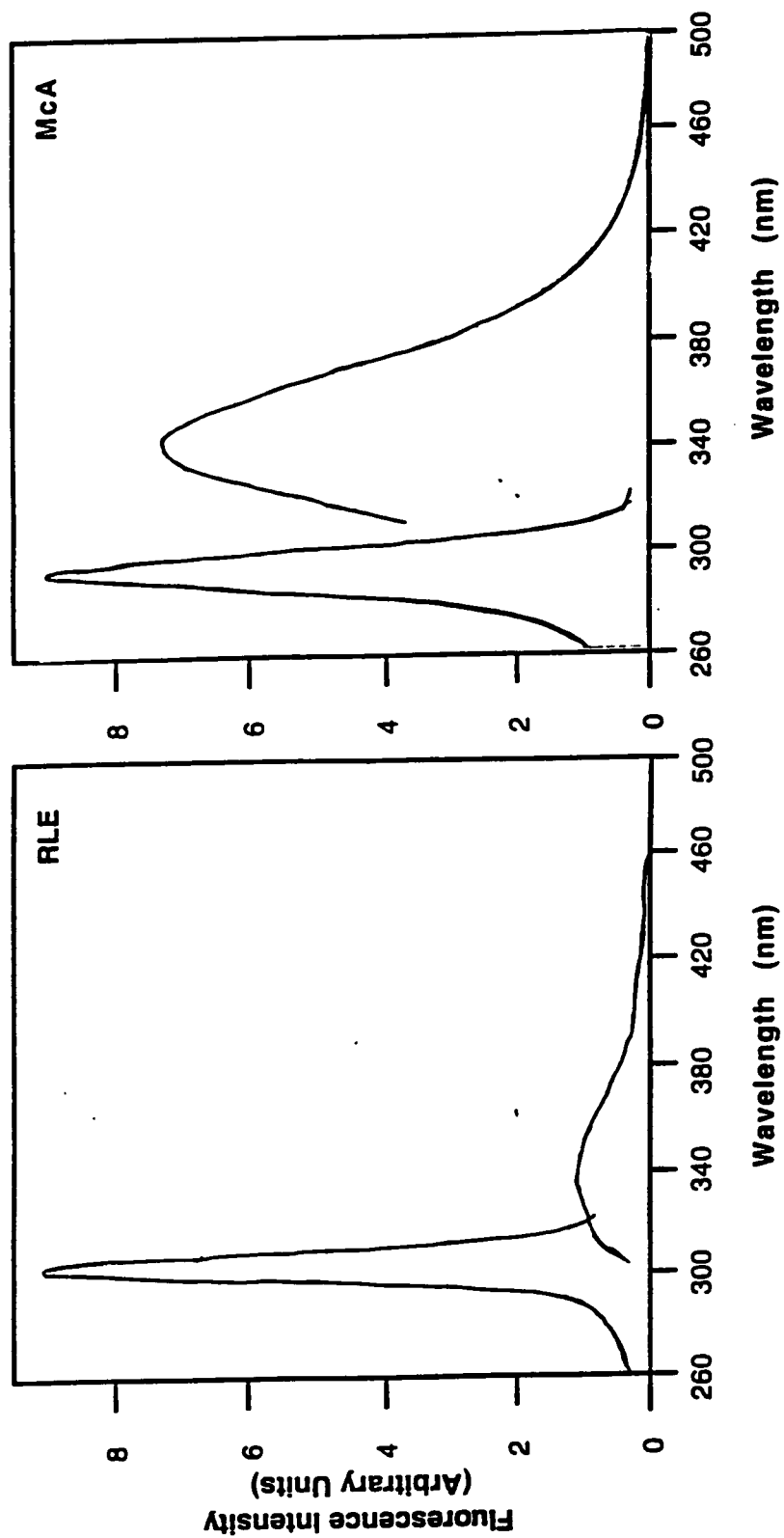


Figure 22. Excitation/Emission Fluorescence of Protein Extracts from RLE and McA Cells, $\lambda_{ex} = 287 \text{ nm}$

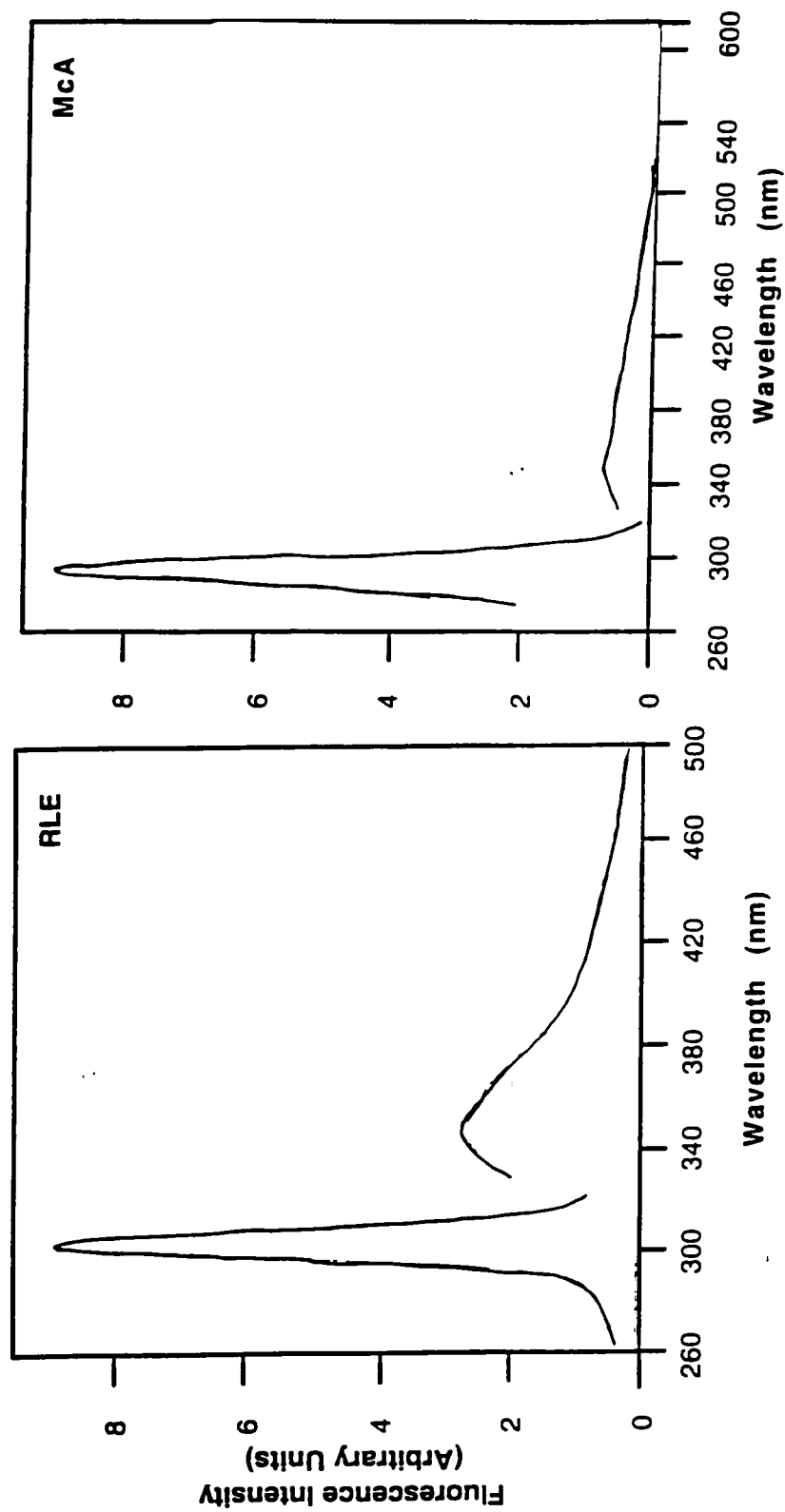


Figure 23. Excitation/Emission Fluorescence of Protein Extracts from RLE and McA Cells, $\lambda_{ex} = 310$ nm

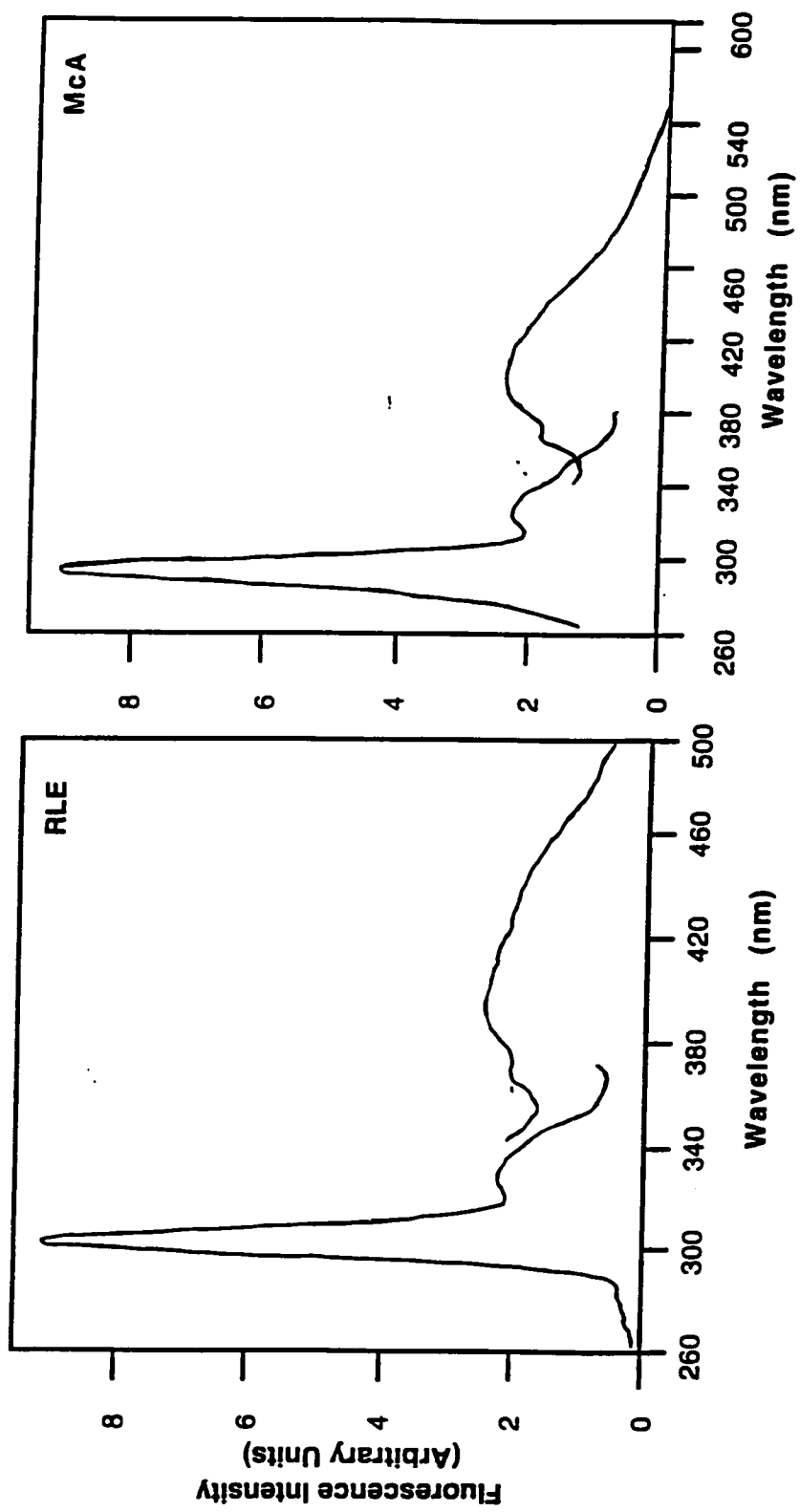


Figure 24. Excitation/Emission Fluorescence of Protein Extracts from RLE and MCA Cells, $\lambda_{ex} = 325$ nm

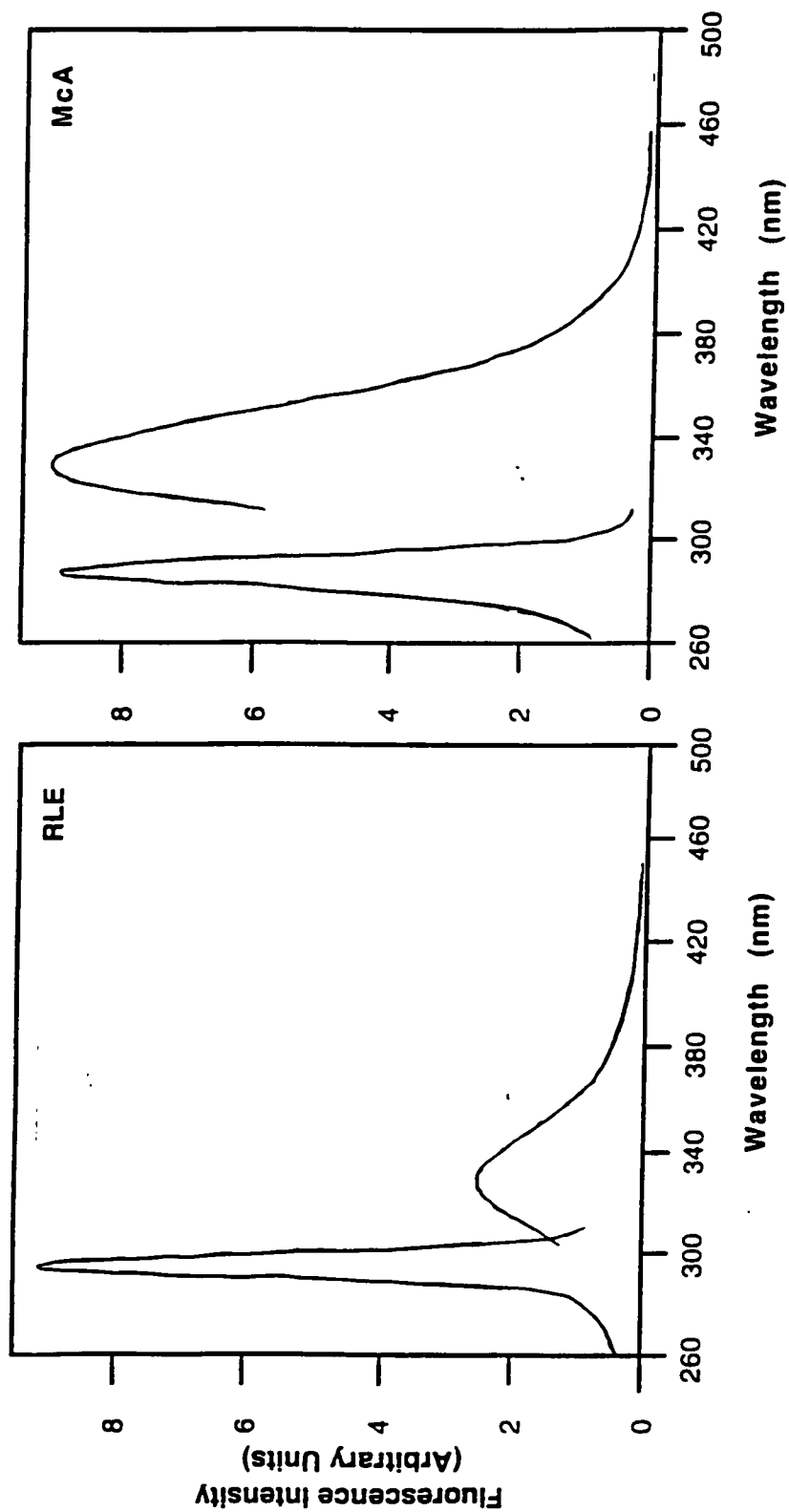


Figure 25. Excitation/Emission Fluorescence of Protein Extracts from RLE and McA Cells, $\lambda_{\text{ex}} = 292 \text{ nm}$

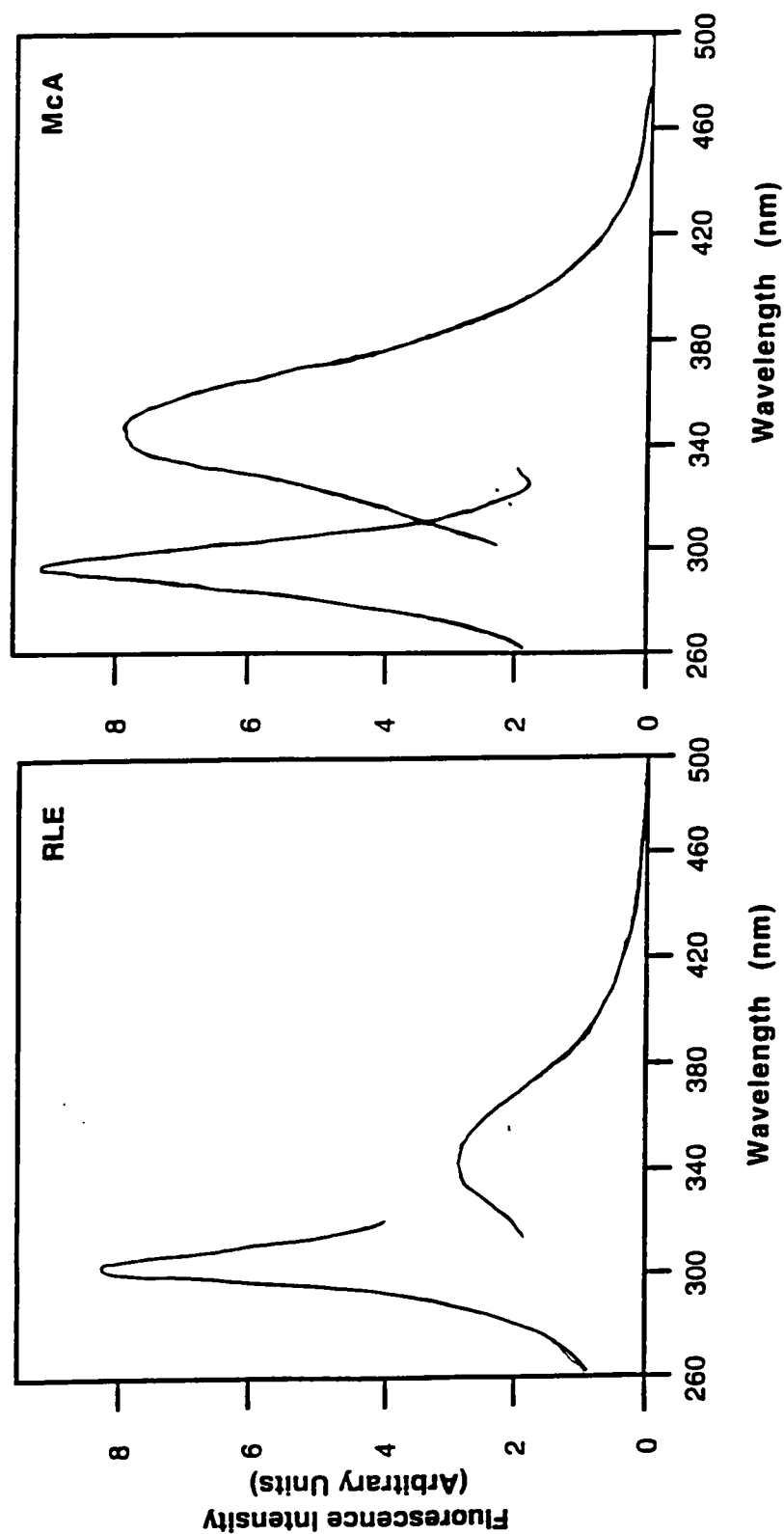


Figure 26. Excitation/Emission Fluorescence of Protein Extracts from RLE and McA Cells after Dialysis, $\lambda_{ex} = 287 \text{ nm}$

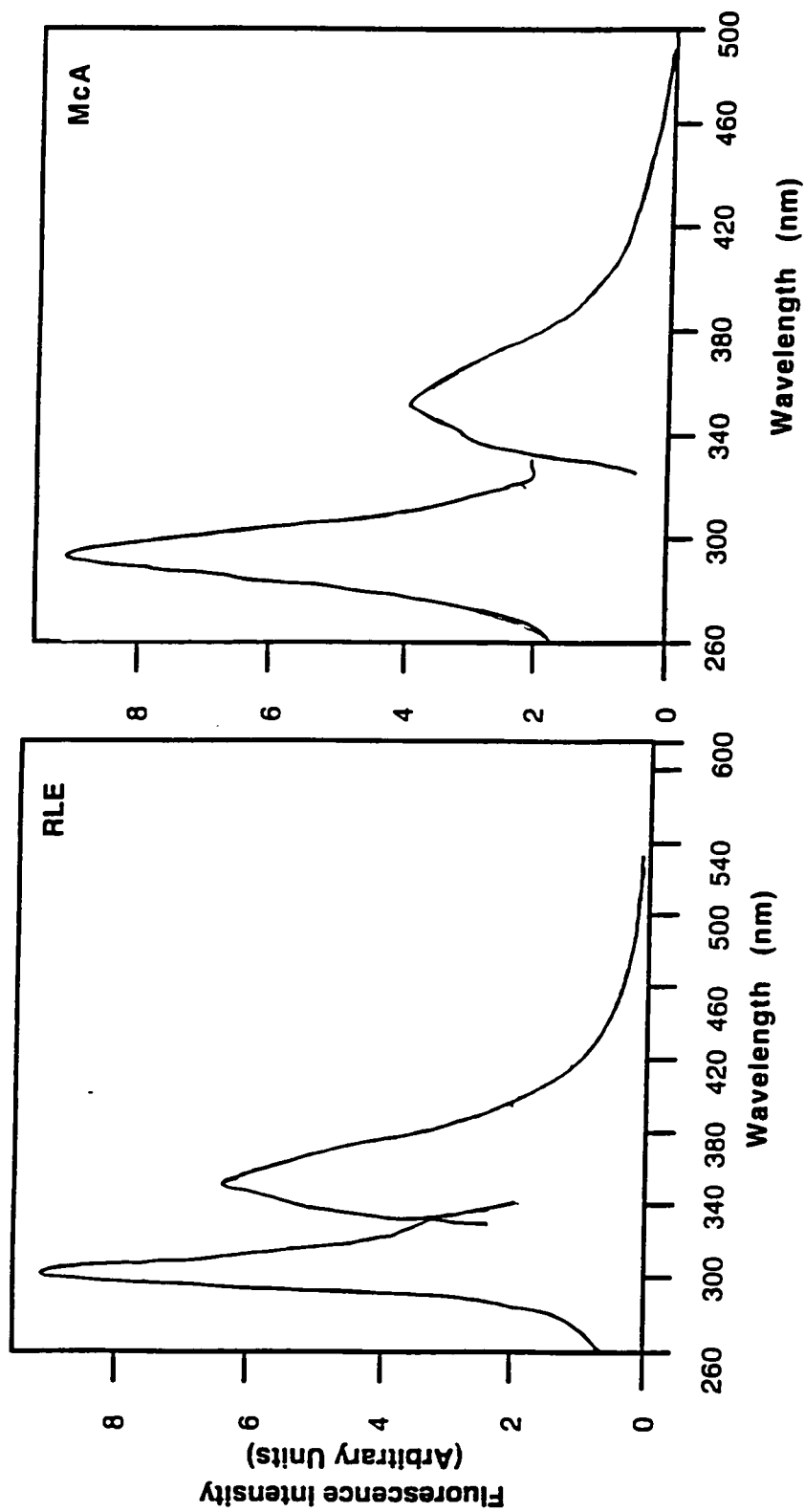


Figure 27. Excitation/Emission Fluorescence of Protein Extracts from RLE and McA Cells after Dialysis, $\lambda_{ex} = 310$ nm

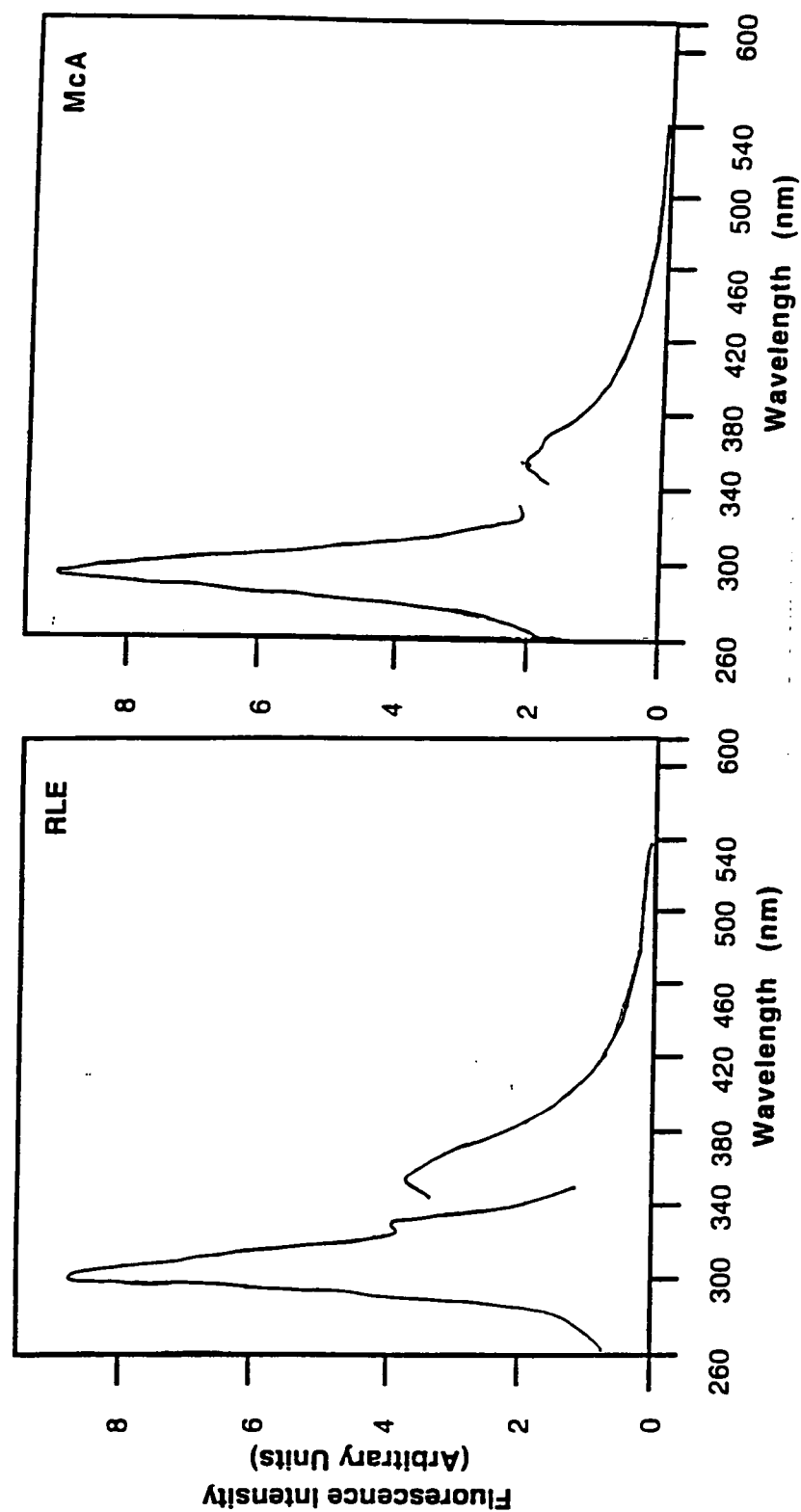


Figure 28. Excitation/Emission Fluorescence of Protein Extracts from RLE and MCA Cells after Dialysis, $\lambda_{\text{ex}} = 325 \text{ nm}$

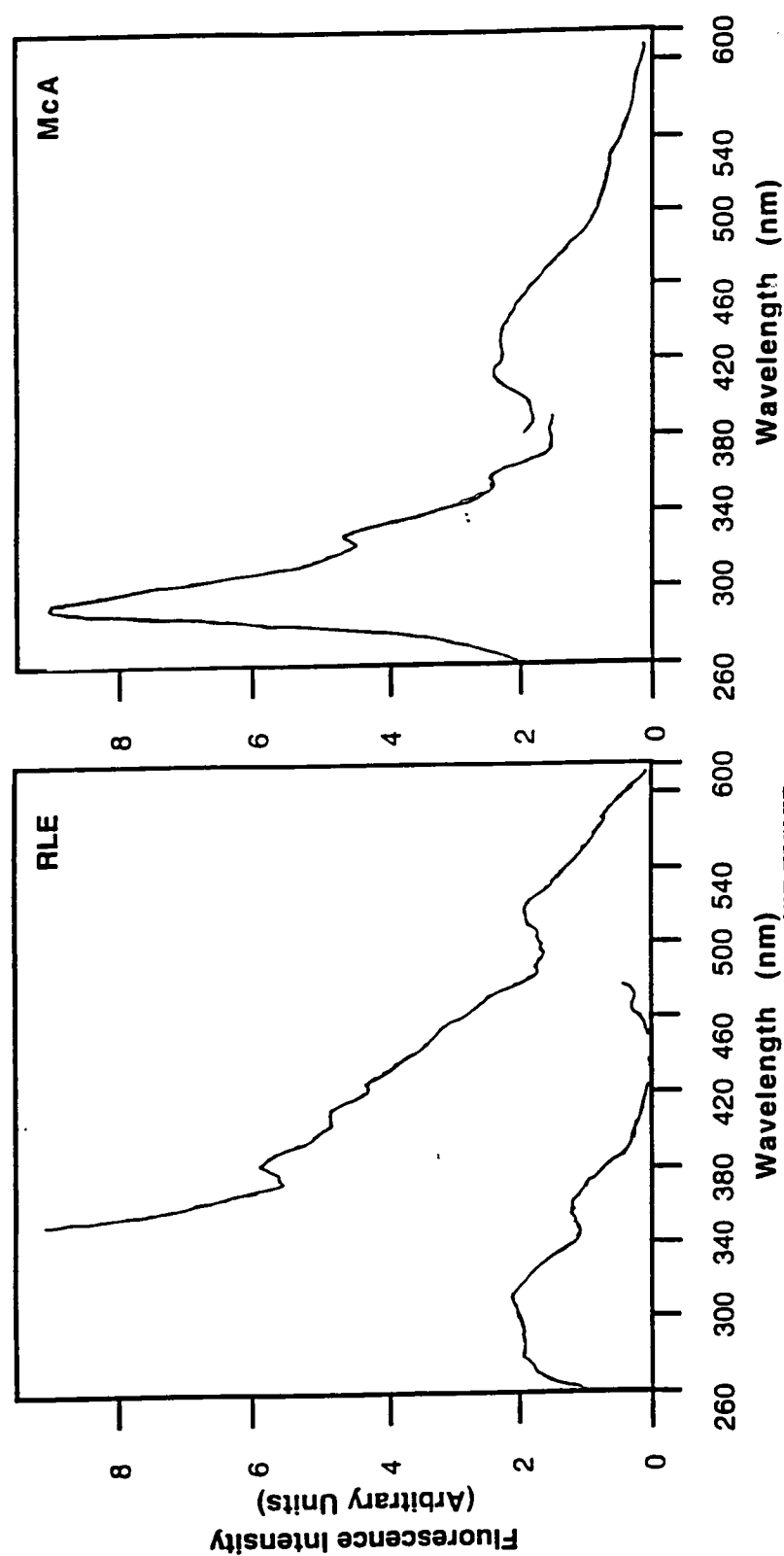


Figure 29. Excitation/Emission Fluorescence of Protein Extracts from RLE and MCA Cells after Dialysis, $\lambda_{ex} = 360$ nm

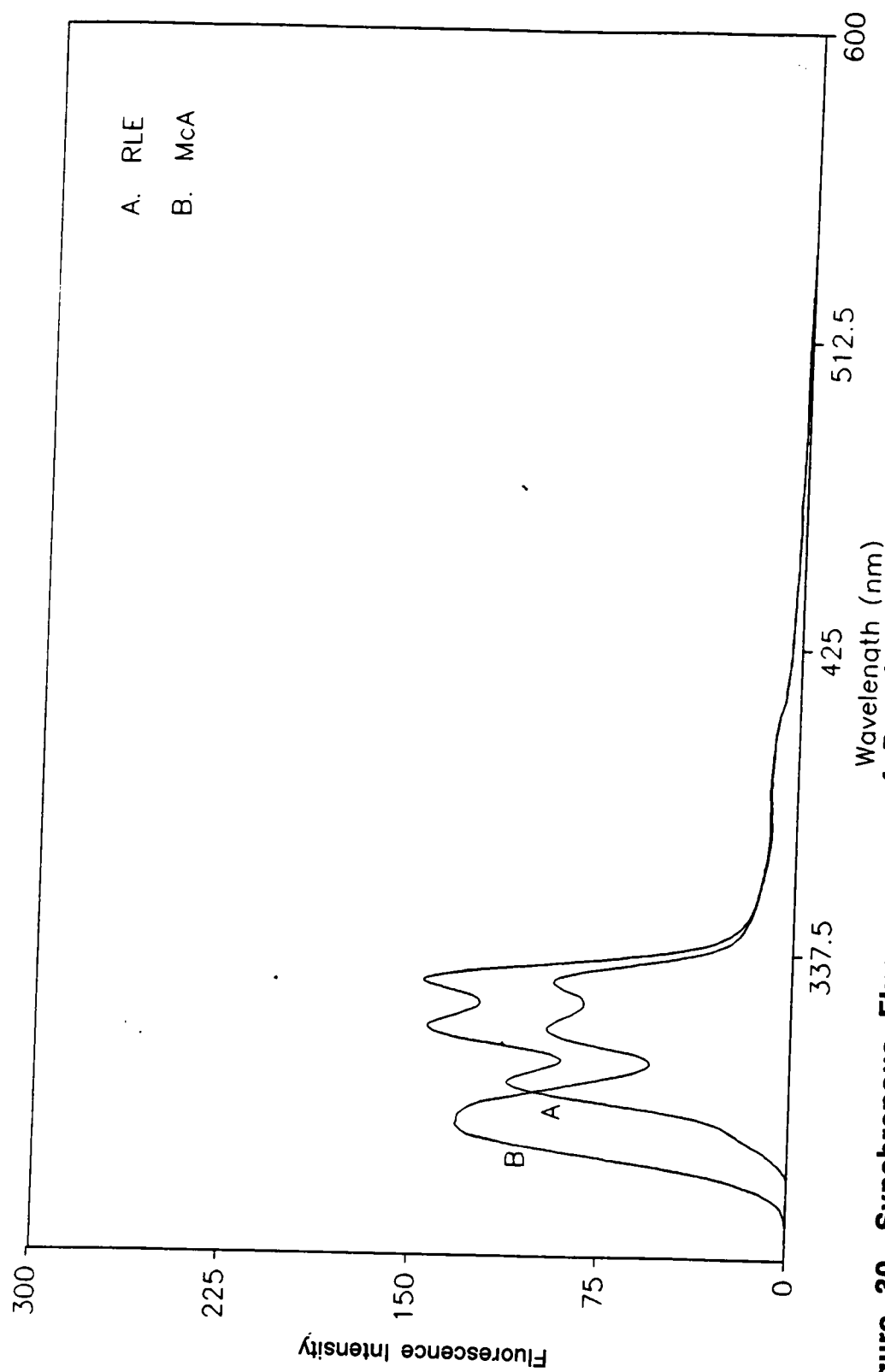


Figure 30. Synchronous Fluorescence of Protein Extracts from RLE and McA Cells after Dialysis, $\Delta\lambda = 20$ nm

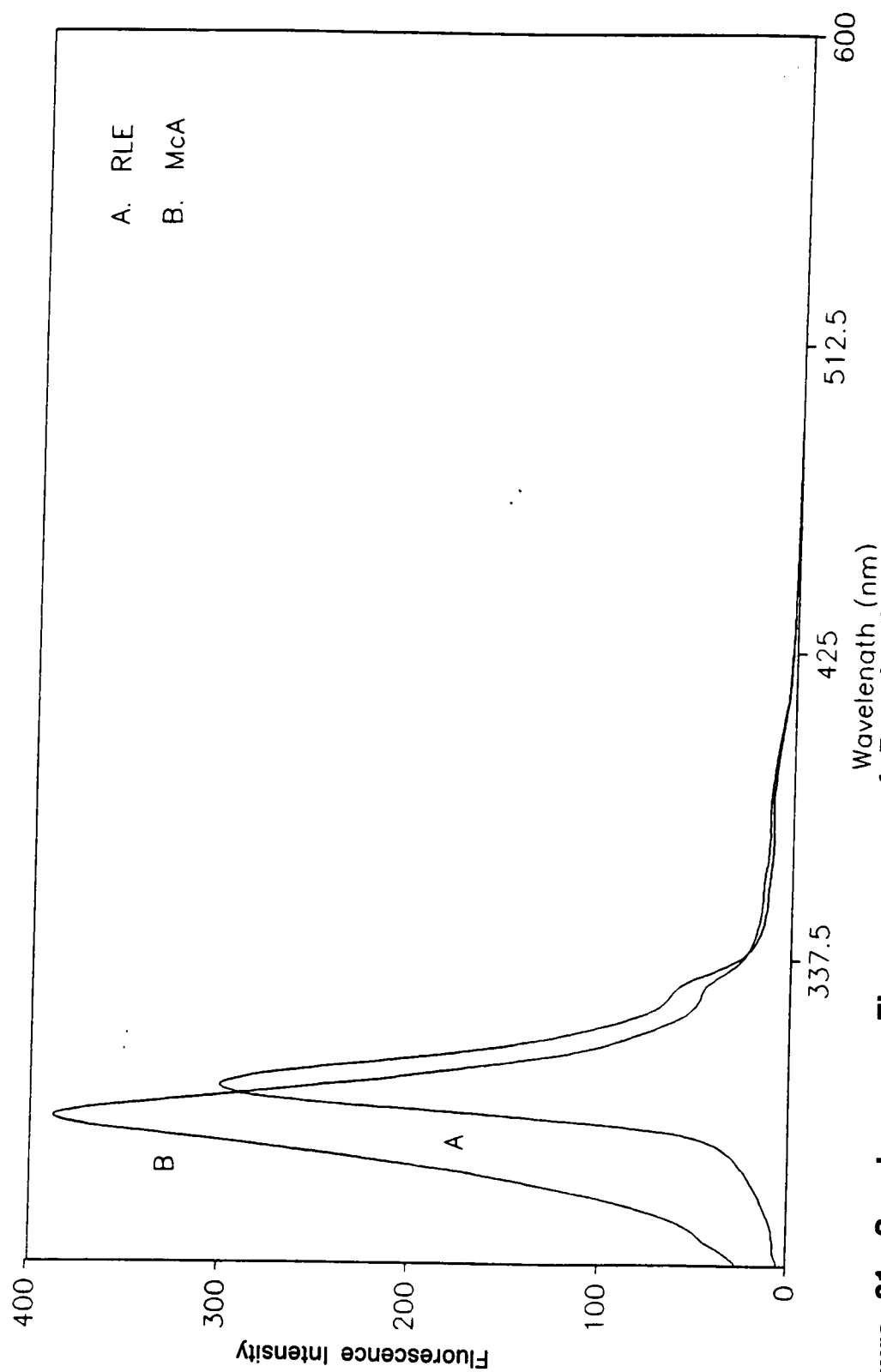


Figure 31. Synchronous Fluorescence of Protein Extracts from RLE and MCA Cells after Dialysis, $\Delta\lambda = 60$ nm

Synchronous $\Delta \lambda$'s of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 nm were performed of dialyzed protein extracts. Figure 32 shows all synchronous spectra of the McA tumor line. Figure 33 shows the synchronous spectra of the normal RLE cell line.

Reproducibility of the synchronous technique was tested using 5 different flasks each of the RLE and McA cell line dialyzed extracts. Synchronous fluorescence measurements using a $\Delta \lambda = 20\text{nm}$ showed the intensity of the fluorescence was not statistically different between flasks and peaks appeared consistently at the same wavelengths in the five replicates. (Figure 34, 35)

To determine the statistical significance of the reproducibility study, a t-test was performed analyzing the differences in the peak positions in the 2 cell lines. Using a confidence level of 8, it was found that the probability that the 2 cell lines peak positions are different is 99.9%.

Synchronous fluorescence spectra of the extracts of the rat primary astrocytes and U-373 astrocytoma cells showed that the peak position differed from spectra of the extracts from McA line and RLE line. The astrocytes and U-373 cell lines did not differ with regards to peak position from each other. (Figure 36)

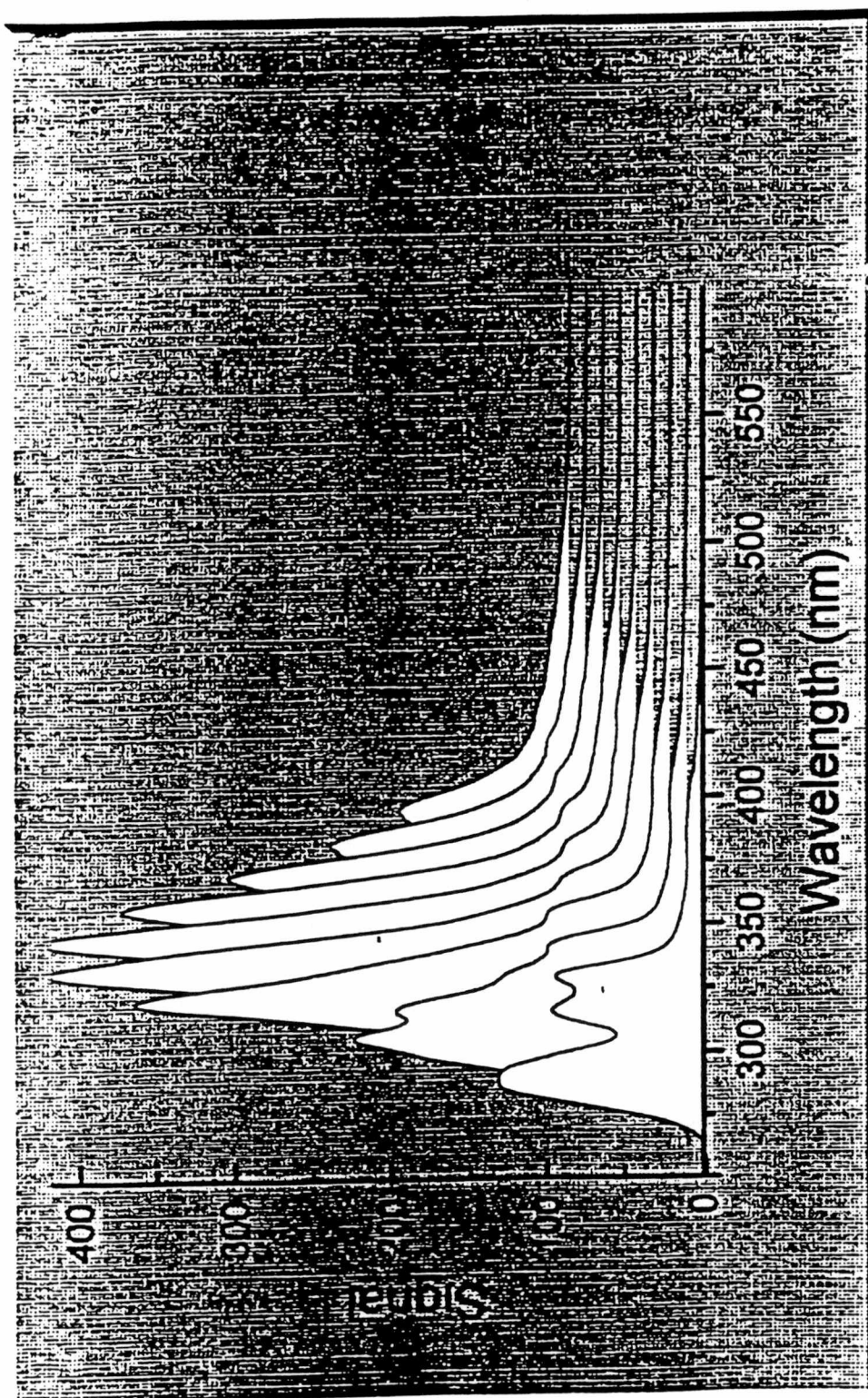


Figure 32. Synchronous Fluorescence of Protein Extracts from McA Cells,
 $\Delta\lambda_s = 10-100 \text{ nms}$

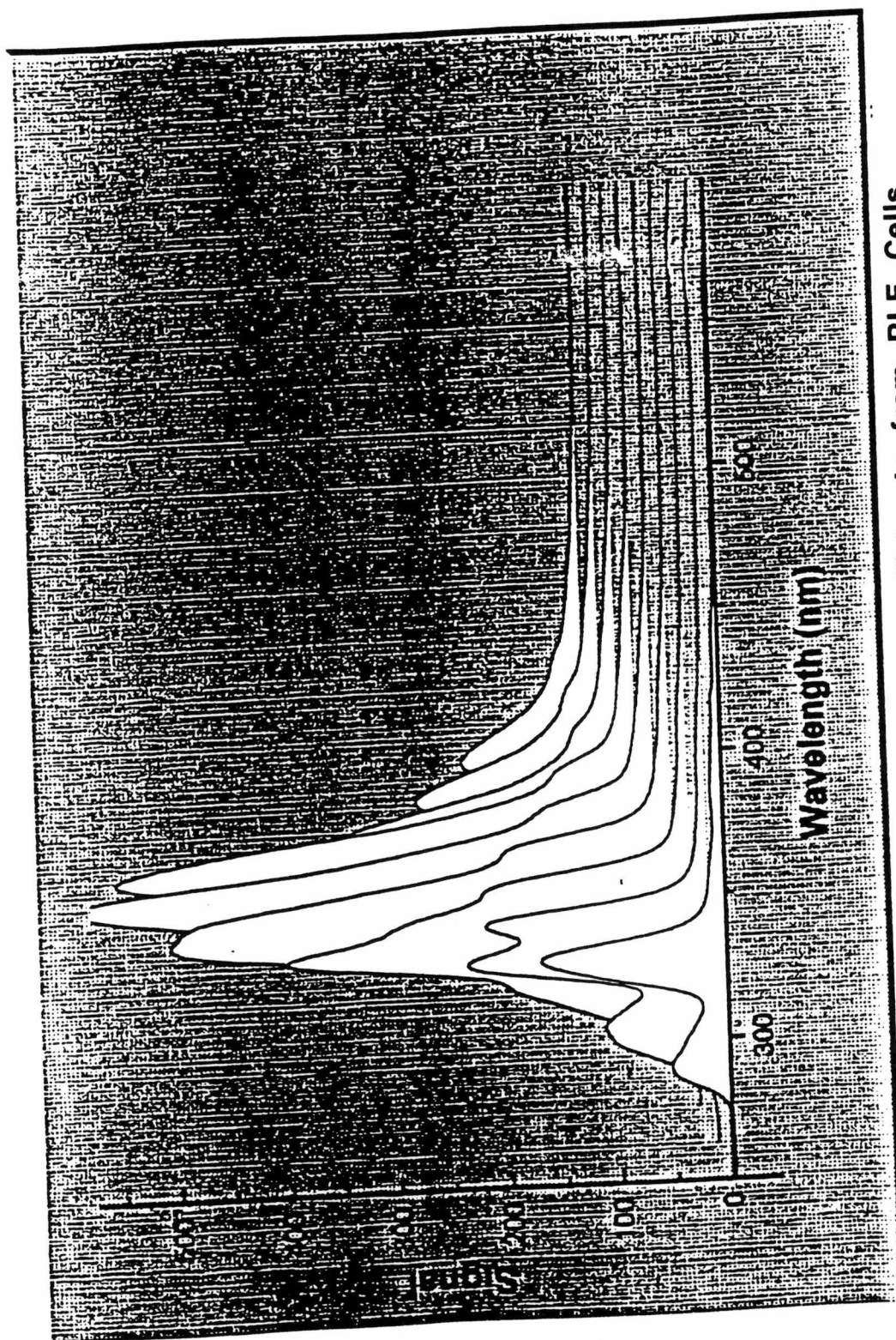


Figure 33. Synchronous Fluorescence of Protein Extracts from RLE Cells,
 $\Delta\lambda_s = 10-100$ nms

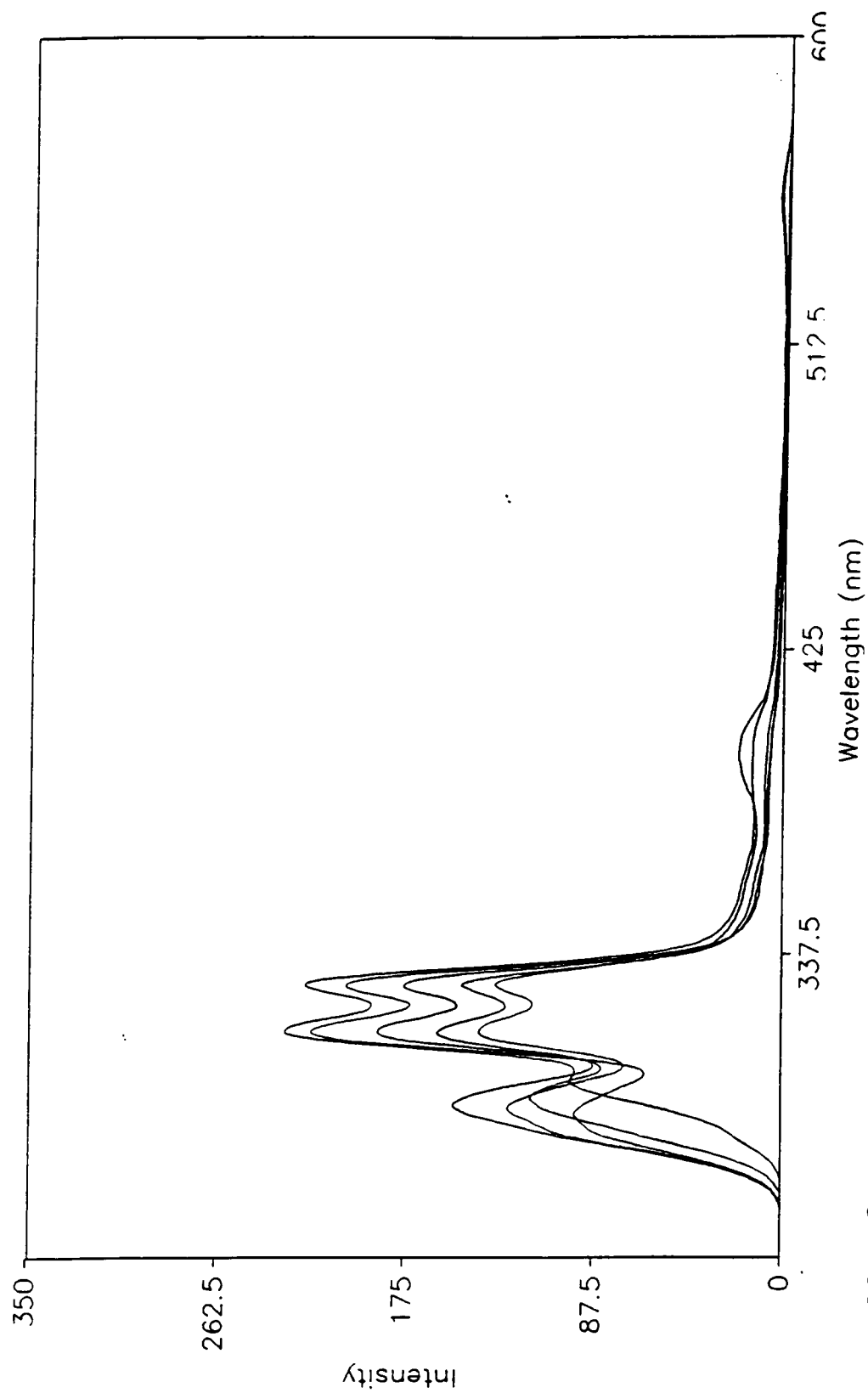


Figure 34. Synchronous Fluorescence of Protein Extracts for 5 Different Flasks of RLE Cells, $\Delta\lambda = 20$ nm

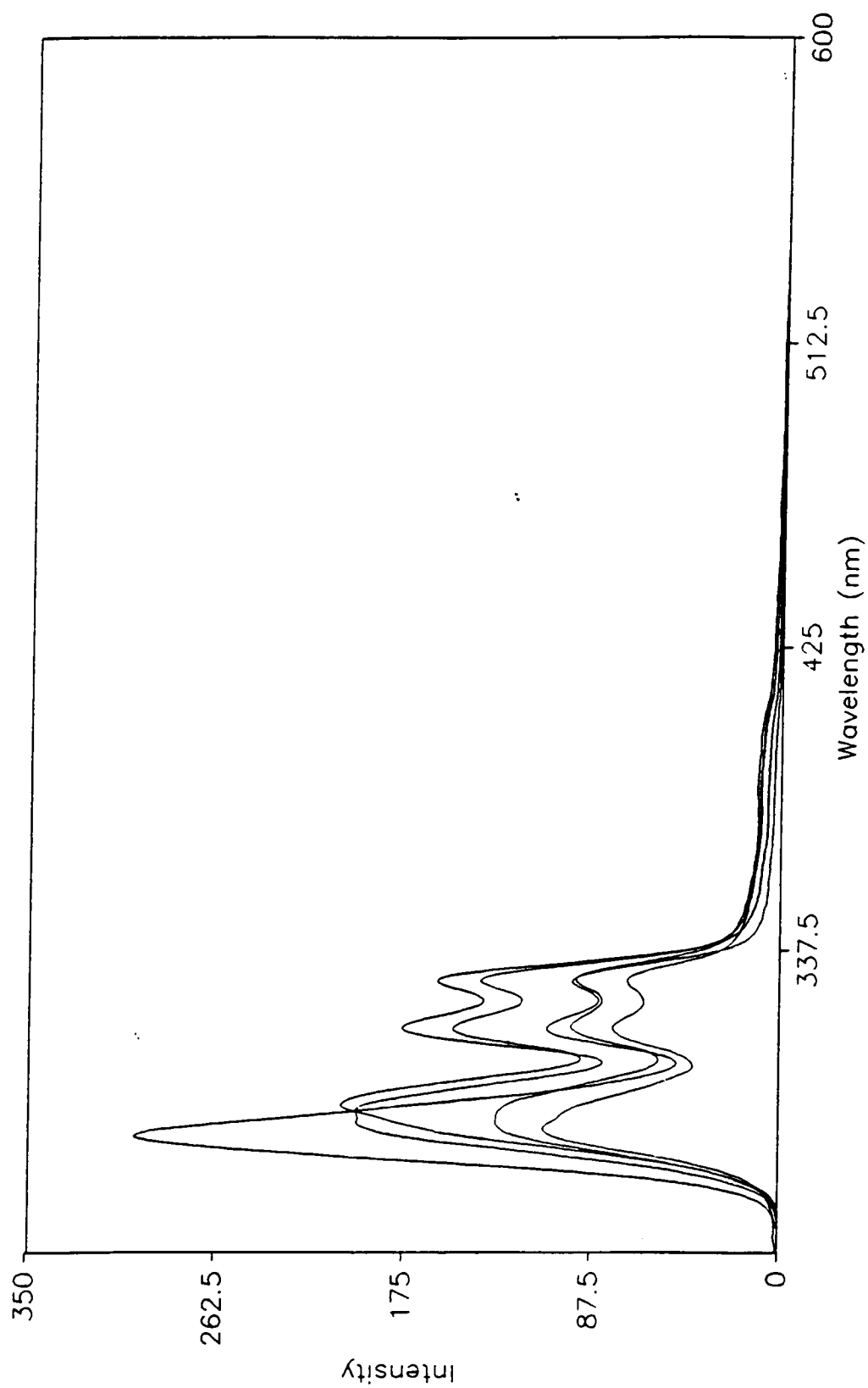


Figure 35. Synchronous Fluorescence of Protein Extracts from 5 Different Flasks of McA Cells, $\Delta\lambda = 20$ nm

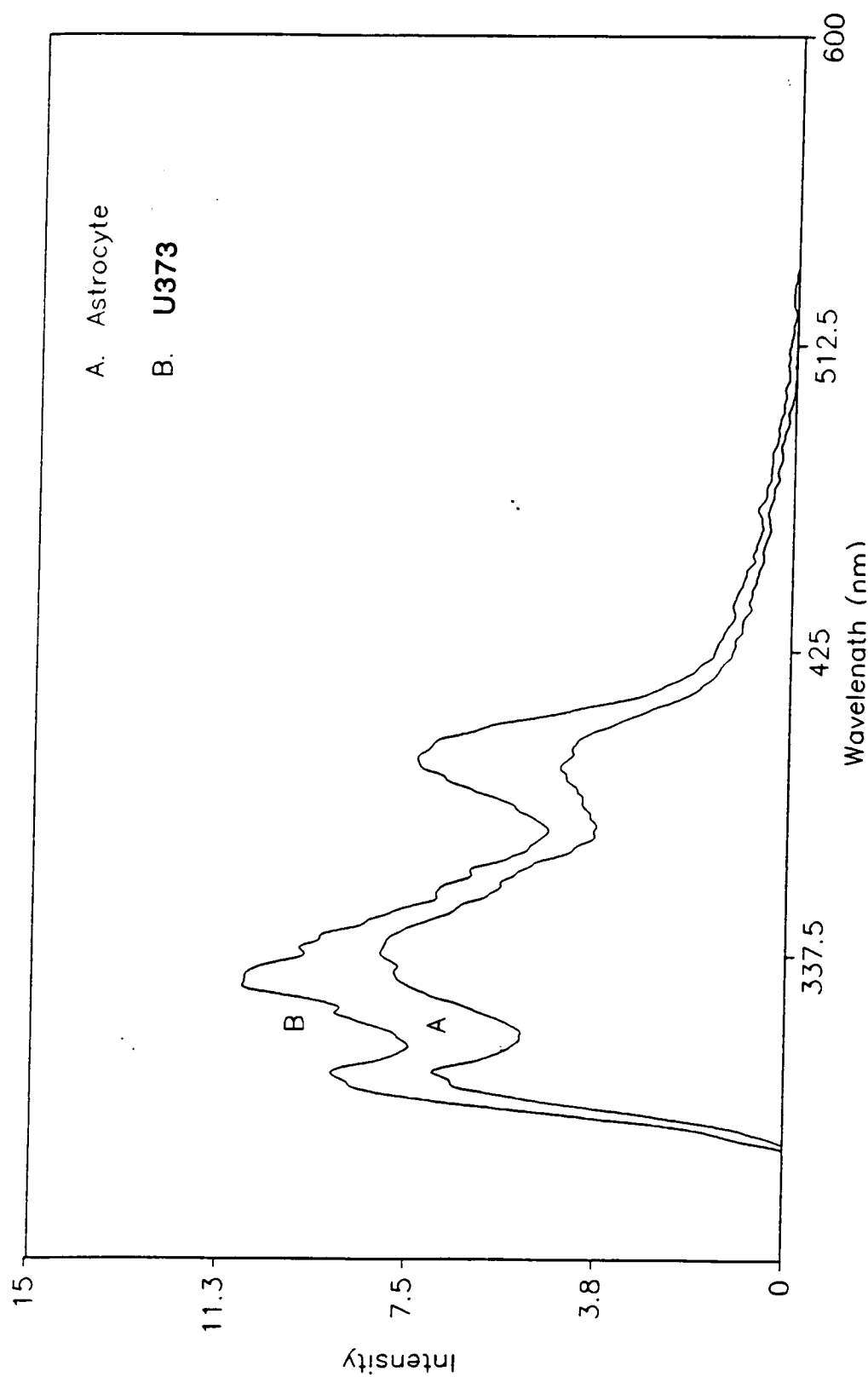


Figure 36. Synchronous Fluorescence of Protein Extracts from Primary Rat Astrocytes and U-373 astrocytoma cells, $\Delta\lambda = 20$ nm

CCD fluorescence measurements of polyacrylamide gels show consistently showed bands in the 70-75 kD range of the McA cell line that did not appear in the normal RLE cell line. (Figure 37)

Neither the astrocytes nor the U373 cells showed any fluorescent bands. (Figure 38)

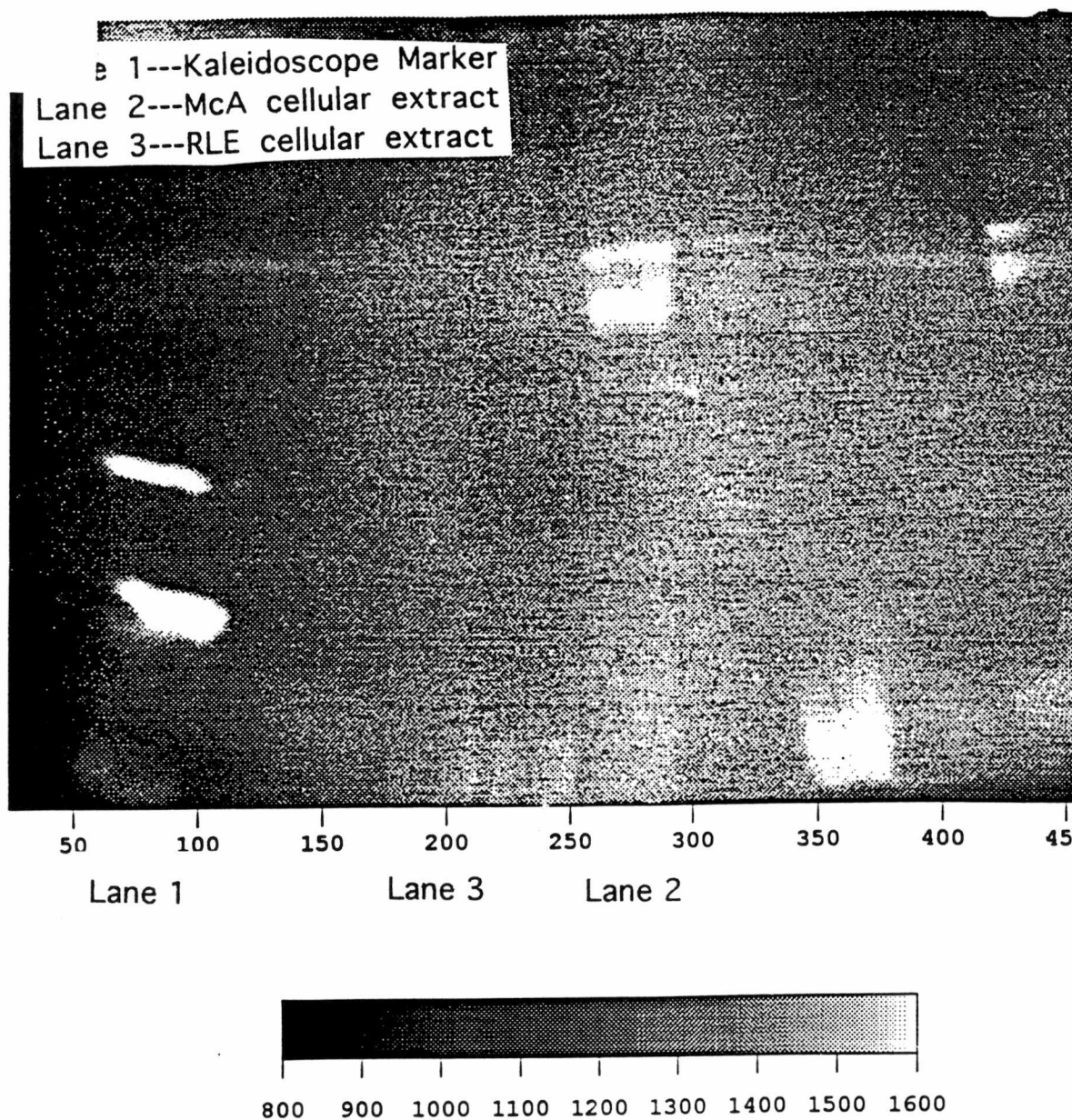
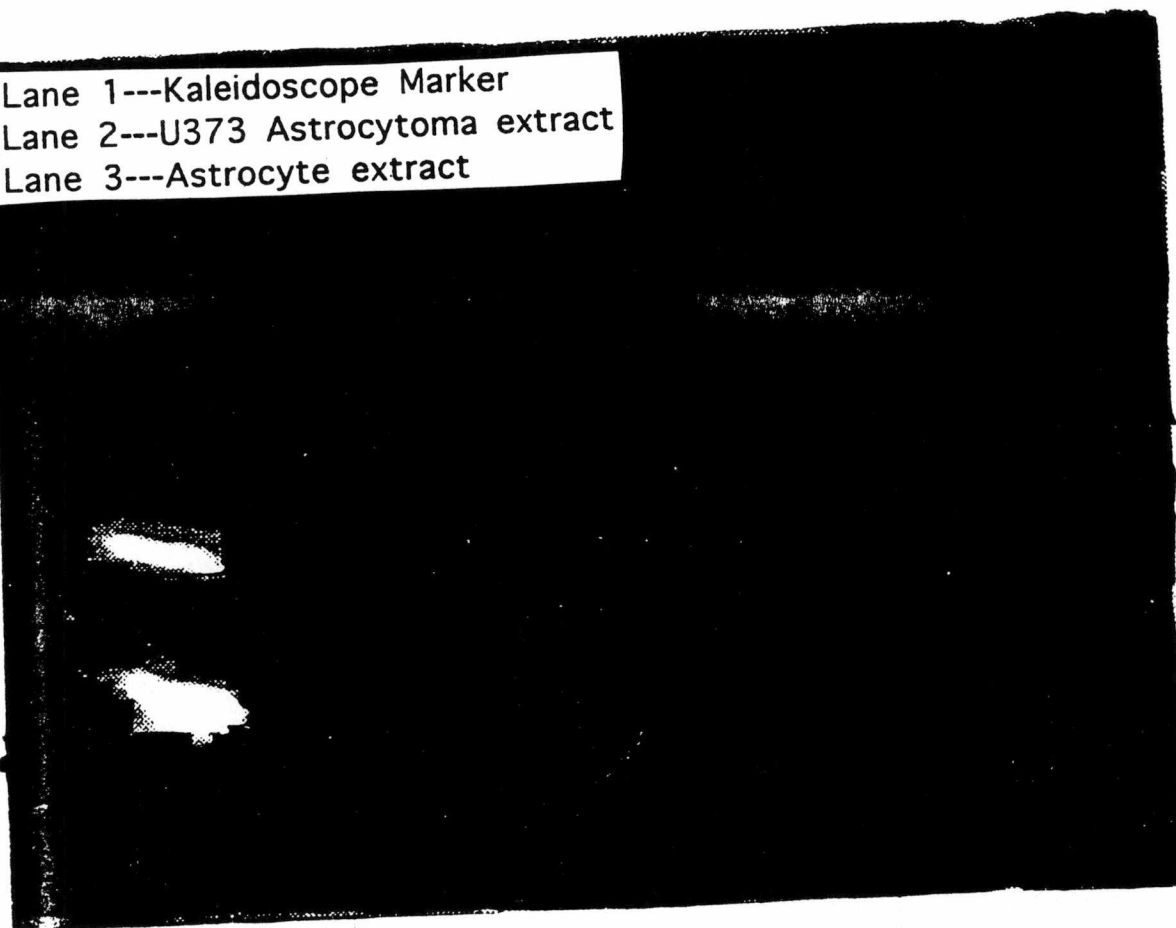


Figure 37. CCD Image of Fluorescence from Polypeptides in a Polyacrylamide Gel (McA/RLE)

Lane 1---Kaleidoscope Marker
Lane 2---U373 Astrocytoma extract
Lane 3---Astrocyte extract



Lane 1

Lane 2

Lane 3

Figure 38. CCD Image of Fluorescence from Polypeptides in a Polyacrylamide Gel (Astrocyte/U373 Astrocytoma)

CHAPTER 4. DISCUSSION

Clinical diagnosis for detection of neoplasia generally relies on histological examination. Because histological examination relies on the ability of a highly trained individual to distinguish between various cell types according to appearance, the technique is somewhat subjective. Development of a reliable technique that can distinguish biochemical differences between neoplastic cells and normal cells may be a more objective means of detection.

Fluorescent properties of cells may be attributed to tryptophan⁶⁹, NADH²⁴⁻²⁷, FADH₂^{46,47} and collagen⁶⁹. The conventional and synchronous fluorescence spectra of these compounds were compared to the fluorescence spectra of the neoplastic and normal cells. Conventional fluorescence showed no differences in peak positions for the RLE and McA cell lines. In this study fluorescence intensities could not be compared because the concentrations of the cells were not known; therefore, quantitation of individual compounds was not possible. No spectral features which could be attributable to any of the four compounds could be readily identified in the whole cell spectra when using synchronous

fluorescence. This was probably due to the weakly fluorescent nature of these compounds. It is also possible that NAD and FAD were present in the oxidized state in the cells rather than in the reduced state. The fluorescence for oxidized and reduced forms of the cofactors is different and the wavelengths chosen for the reduced forms would not allow for measurement of the oxidized form in cells.

Comparison of the normal RLE and neoplastic McA cells by conventional fluorescence spectra showed, no measurable differences in the excitation nor emission spectra. Both spectra were broad and featureless, making potential identification of the two cell types difficult. Quantitative measurements by comparison of fluorescence intensities were not possible because cell concentrations were not known. In the synchronous spectra ($\Delta\lambda = 20$ nm), a 10nm blue shift in the tumor cell spectra was observed when compared to the normal cell. This suggested the potential for rapid discrimination between tumor and normal cells.

Tryptophan, NADH, FADH₂ and collagen did not appear to contribute to the differences in fluorescence spectra of the normal and neoplastic cells by synchronous fluorescence. Because no peaks

in the cells corresponded with synchronous peaks of the components, it was suspected that a protein was the cause of the fluorescence difference. Numerous studies have shown that aberrant proteins may occur in tumors that are not present in corresponding normal tissue⁷⁰⁻¹⁰³. When the fluorescence profiles of denatured tumor and normal cells were examined, the denatured tumor cells showed a pronounced blue shift from the whole cells spectra, probably due to a difference in the composition of the two cell lines. A second peak appeared which may have been the result of conformational changes due to the denaturing process.

Dialysis was performed to remove non-protein material that may have quenched fluorescence in previous cell preparations. The appearance of the third peak after dialysis of RLE and McA extracts suggests a possible quenching effect from some molecule was removed during the dialysis.

PAGE was used to isolate proteins that may have caused differences in the fluorescence spectra of the McA and RLE cells. Illumination of the gels by a He-Cd laser and images taken by a CCD camera showed fluorescence bands in the lanes containing the extract from the tumor cell line (70-75 kD range), but not in the

lanes containing the normal cell line extract when excited at 325nm. Further studies are required to find out whether this fluorescent band is unique to the tumor cell line or not.

The fluorescence spectroscopic technique may not be applicable to all tumor types. In this study, normal rat astrocytes and the U373 astrocytoma cells did not display the same fluorescence as the RLE and McA cells. The fluorescence spectra of the astrocytes and astrocytomas did not differ. No fluorescent bands were detected in gels from either astrocytes or the astrocytoma cells when examined with CCD imaging using an excitation at 325nm.

The fact that no fluorescent bands other than in the cellular extract of McA cells were detected does not necessarily mean that the other cells do not contain fluorescent proteins. The excitation for the imaging of all gels was at 325nm. This excitation might not have been at the appropriate wavelength for other fluorescent polypeptides that were in the other cells. It may be possible to detect other spectral differences in polypeptides of the other cells by using other excitation wavelengths.

Two techniques have been evaluated in this study for use in

screening for cancer. A difference between the fluorescent spectra of the normal RLE and neoplastic McA cell lines were detected using synchronous fluorescence. Further research is needed to determine if the fluorescence differences detected are universal to all tumor cell lines. Cellular protein extraction, gel electrophoresis, and CCD fluorescence imaging identified one potential source of cellular fluorescence that differed in the RLE and McA cells. In a separate study, this unique protein, 70-75kD in size, will be sequenced for determination of the specific cause of the fluorescent differences in the cells. Identification of protein tumor markers using fluorescence spectroscopy techniques may lead to a promising avenue of research in the differentiation between normal and neoplastic cells.

CONCLUSION

In conclusion, spectral differences in the RLE and McA cells were seen by using synchronous fluorescence, whereas no distinction could be made between the astrocytes and astrocytomas. Quantitative differences could not be determined by studying intensity differences because cell concentrations were not determined. However, shifts in peak positions were seen.

Also, a fluorescent polypeptide (70-75 kD) was detected in the McA cells by CCD imaging of gels when excited at 325nm, while no fluorescent polypeptides were detected in the RLE cells when excited at 325nm. No fluorescent polypeptides were detected in astrocytes or astrocytomas by CCD imaging of gels when excited at 325nm.

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