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The Development of an Optical PH Sensor Based on Fluorescence

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THE DEVELOPMENT OF AN OPTICAL PH SENSOR BASED ON FLUORESCENCE

by

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ABSTRACT

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We are interested in developing optical pH sensors based on the fluorescence of an immobilized fluorophor confined on the end of a bifurcated fiber optic. Such optical pH sensors have several potential advantages: they should be inherently less sensitive to electrical noise than conventional pH electrodes, they require no reference electrode, and they make possible remote measurements. The fluorophors evaluated are bound electrostatically to an ion exchange membrane which is then attached to the common end of a bifurcated fiber optic. The fluorophor is excited through one arm and the fluorescence emission is passed through the other arm of the fiber optic to a photomultiplier tube for detection. Before a potential fluorophor is evaluated with this instrument, the pH dependence of the absorption and fluorescence spectra of the dissolved and bound fluorophor are studied. The parameter measured in the sensor is a ratio measurement of the fluorescence emission of one form of the fluorophor when excited at two different wavelengths. The use of the ratio measurement offers additional advantages over electrodes. Any given fluorophor is limited to sensing a range of +/-1 pH unit on either side of the pKa. Thus, it is necessary to find fluorophors suitable for various pH ranges.

ACKNOWLEDGEMENTS

I wish to thank the members of the Union College Chemistry Department for the best undergraduate education I could have hoped for. I am especially thankful to Professor Thomas C. Werner for his scientific, as well as general advice. I will always admire his superb ability to teach and to maintain his patience. I must also thank the General Electric Company for the support they granted through the Expanded Horizons Program. I am very grateful I was able to be involved in the program, and work with Professor W. Rudolf Seitz, whose guidance and advice I am also thankful for. I also wish to thank Peter Schulam for supporting me during the sometimes long hours of lab, and for sharing his knowledge to help me solve some of the problems of research. My deepest thanks go to my parents for their care and support. Without them, my experiences would not have been possible. I am very grateful to all who gave me the incentive to continue, even when I discovered that "something always goes wrong with research -- usually."

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

INTRODUCTION

Fluorescence is a process in which radiation is emitted by molecules or atoms that have been excited by the absorption of radiation (1). Before excitation, a molecule exists in the singlet ground state, S_o. When a photon of appropriate energy is absorbed by a molecule, an electron is promoted into a higher energy orbital and the molecules enter a higher energy state, S_1. The energy of the photon required to produce a particular excited state is the difference in the energy between that state and the original ground state, S_o. The actual fluorescence is the radiant emission which occurs as the excited species returns to its more stable ground state. Thus, fluorescence simply appears to be the reverse of absorption. However, while fluorescence is directly related to the amount of radiation absorbed, the energy of the absorbed radiation is usually greater than that of the emitted radiation (1). Therefore, the emitted radiation would have a lower frequency, or longer wavelength, than the radiation that was absorbed. This shift to lower frequencies, often called Stokes shift, is illustrated by the diagram in Figure 1 (2).

The Franck-Condon principle states that molecular

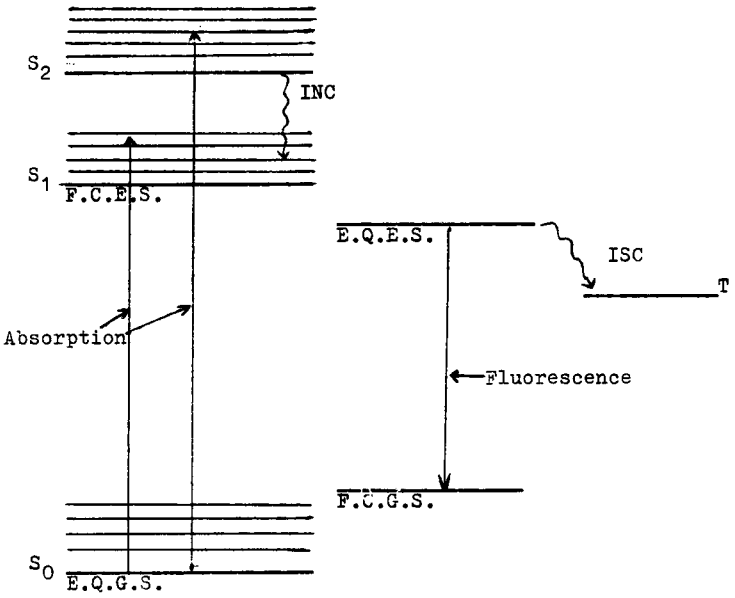


Figure 1 Franck-Condon Energy Diagram

electronic transistions occur in a time interval of about 10^{-15} second, while nuclear configurational transistions take relatively longer (10^{-11} to 10^{-13} second). Therefore, absorption initially produces an excited state possessing an altered electron distribution but the ground state geometry and solvent cage. This is termed the Franck-Condon Excited State (FCES). Quickly the molecule loses excess vibrational energy by thermal relaxtion to acquire a structure which approaches an equilibrium excited-state (EQES) geometry. Simultaneously, the solvent cage relaxes to a new configuration as required by the changed electron distribution and geometry of the excited molecule. The resulting EQES is lower in energy than the FCES (1).

All fluorescence originates in the relaxed EQES. The emission of fluorescence results in the formation of the Franck-Condon ground-state (FCGS), which has the same geometry and solvent cage as the EQES. By means of thermal relaxation, structural and solvent reorientation results in the lower energy equilibrium ground state (EQGS). The energy relation for these four states is shown in Figure 1. The smaller energy difference that exists between the EQES and the FCES, which are the two levels involved in fluorescence, accounts for the observation that fluorescence frequencies are usually lower than those of absorption.

In addition to the radiative process of fluorescence,

a molecule can return to its ground state through radiationless processes. One such process, internal conversion (INC), is the result of conversion of electronic energy into vibrational energy and then into solvent and thermal energy. Since fluorescence occurs only from the lowest vibrational level of the first excited singlet state, excitation to any excited singlet state above the first is normally followed by internal conversion. Although internal conversion from the first excited singlet state can occur, it is less likely than among the higher excited singlets (3). In aromatic hydrocarbons, internal conversion between the lowest excited singlet state and the ground state is often nonexistant (2). Another non-radiative decay mode, called intersystem crossing (ISC), occurs between the first excited singlet and the lowest triplet. Such singlet-triplet processes are less probable than singlet-singlet processes; however, they can occur within the lifetime of an excited singlet state (10^{-8} second) (2).

The route to the stable ground state which minimizes the lifetime of the excited state is the one that will be followed. Therefore, only systems that cause the rate of radiationless relaxation to be slowed to a point where the emission reaction can compete kinetically will produce fluorescence.

Molecular structure and chemical environment both are

influential in determining whether a substance will fluoresce or not. The intensity of the emission that may occur is also determined by these two factors. It has been experimentally determined that fluorescence is favored in molecules that are rigid in structure. Lack of rigidity would increase the likelihood for radiationless relaxation, thereby enhancing the internal conversion rate. This explains why enhanced emission usually results when fluorescent dyes are adsorbed on a solid surface (4).

The most intense and most useful fluorescence behavior is found in aromatic compounds and those containing highly conjugated double-bond structures. An unsubstituted aromatic hydrocarbon will fluoresce in solution, and the quantum efficiency usually increases with the number of rings (4). Therefore, naphthalene, a double ring structure, will fluoresce more efficiently than benzene.

Substitution on the aromatic ring causes shifts in wavelength of maximum absorption and affects the fluorescence efficiency. Thus, the position of fluorescence bands of aromatic compounds with exocyclic substituents depends on the differences between the electronic interactions of the substituents and the aromatic rings in the ground and excited states (5).

When the ring substituents are acidic or basic, the fluorescence of the aromatic compound is usually pH

dependent. The ionized and nonionized forms of the compound are likely to have different emission wavelengths and intensities. For example, fluorescence of the protonated form of 1-naphthol-4-sulfonic acid occurs in the ultraviolet region. With addition of base, the compound is converted to the naphtholate ion, and the emission peak shifts to visible wavelengths. This change occurs at a different pH than would be predicted from the acid dissociation constant (pKa) for the naphthol because the acid dissociation constant for the excited molecule (pKa^*) differs from that of the same species in the ground state. In the case of the naphthol, the acidity is increased greatly upon excitation. As a result, anion fluorescence is observed in solutions of lower pH than the ground state pKa. Such changes in acid or base dissociation constants with excitation are common and can be as large as four or five orders of magnitude (1).

Thiols and aromatic amines, similar to the naphthols, also become much stronger acids upon excitation. Other compound classes, such as carboxylic acids and aldehydes, become much more basic (1). It is believed that acidity is a function of molecular electron distribution in both the ground and excited states. Therefore, the excited state acidities may be explained in terms of the electron charge densities in the excited molecules.

With excitation, electron redistribution occurs. It is because of this redistribution that the pKa^* differs

from the pKa. Electron donor groups, such as -OH, NH_2, -OR, and -NH, have lone electron pairs which are partially conjugated with the aromatic ring system in the ground state. These lone pairs can be promoted to the lowest vacant π^* orbitals of the aromatic system upon excitation producing an intramolecular charge-transfer lowest excited singlet state. In this singlet state the functional groups are more extensively conjugated with the aromatic ring and are more charge deficient, thereby facilitating loss of a proton from these groups. As a result, molecules that contain electron donor groups are usually stronger acids in the excited state than in the ground state (6).

A good example which illustrates this is naphthol. Upon excitation, the oxygen of the hydroxy group loses electron density and therefore attains a slight positive charge. The electrons forming the bond with the hydrogen are pulled closer to the oxygen. This causes weakening of the O-H bond, which allows the hydrogen ion to be donated much more easily.

When the substituent is electron withdrawing, such as a carboxylate group, the acid form is more resonance stabilized thereby increasing the pKa. Therefore, upon excitation a decrease in acidity in the first excited singlet state is observed (1).

The fact that the fluorescence intensity of certain molecules is dependent on pH makes the development of a

pH sensor based on fluorescence possible. We are interested in developing optical pH sensors based on fluorescence. These sensors involve an immobilized fluorophor confined on the end of a bifurcated fiber optic. The term "optrode", formed by combining "optical" and "electrode", is used to describe such sensors. This term emphasizes that optical sensors are very similar to electrodes; however, in operating principles they are quite different. As a result of the differences, optrodes offer new possibilities relative to electrodes; however, at the same time, they are subject to limitations and problems that are not observed with electrodes (7).

Anyone who has used a pH electrode knows that they are characteristically subject to an electrically noisy environment, and it is often difficult to obtain stable readings. More accurate readings could be obtained with an optical sensor, which would not be subject to electrical interference because the signal is optical. Also, the sensor would not require a reference electrode. Because the reagent phase does not have to physically contact the fiber optic, the optrodes offer additional advantages over electrodes. With optrodes the changing of the reagent phase is a simple matter, and, also, the reagent phase could be in a different environment from the fiber optic making remote measurements possible. The cost of the fibers is quite reasonable, and several

different fiber optics may be used with one centralized spectrometer, which would reduce instrumental needs.

Optical sensors also offer the possibility of using multiwavelength and temporal information. Such multiwavelength measurements may be used to relate analyte concentrations to intensity ratios at two wavelengths or to monitor reagent phase stability (7). The procedure used for ratio measurements will be explained.

Optrodes are subject to several limitations which must be considered. Ambient light will interfere with the sensor. Therefore, unless the optrode is used in a dark environment, the optical signal must be encoded, for instance, by modulation, so that it may be resolved from the ambient background. Another disadvantage of optical sensors is the lack of long-term stability of the reagent system. This problem may be somewhat alleviated by multiwavelength detection and the use of a ratio measurement, and also by the ease of changing reagent phase. The response time of optical sensors is limited by the necessary mass transfer step which results because the reagent and the analyte are in different phases. The observed intensity of the optical measurement is proportional to the amount of reagent phase. Detected intensities can be increased when the amount of reagent phase is small by using more intense probe radiation. However, any reagent photodegradation processes will then

be accelerated. Thus, the sensors involve a three-way trade off between the intensity of the probe radiation, the amount of reagent phase, and stability. Additionally, compared to electrodes, optical sensors will have limited dynamic ranges of approximately +/-1 pH unit about the pKa of the probe molecule used (7).

Because of the advantages optrodes have to offer, interest in their development is increasing. Previous work by Peterson, Goldstein, and Fitzgerald lead to the development of a pH probe based on the absorption of phenol red, suitable for tissue and blood pH measurements over the range of pH 7.0-7.4, with an accuracy of 0.01 pH unit (8). Another practical pH sensor has been developed by Seitz and is based on the fluorescence of the trisodium salt of 8-hydroxypyrene-1,3,6-trisulfonic acid (HOPSA), immobilized electrostatically on an anion exchange membrane. This sensor is suitable over the physiological pH range (7).

To search for other suitable pH probes we used the approach of Seitz involving electrostatic immobilization of the fluorophor on ion-exchange membranes. This requires any potential probe molecule have two characteristics. The molecule must contain a functional group whose state of ionization affects the fluorescence properties of the aromatic system in a measurable way and must have a pKa in a pH range commonly measured. A hydroxy group and an amino group are two examples of such

functional groups. Secondly, the probe must contain a charged functional group which will permit electrostatic immobilization on an ion-exchange membrane. For example, a sulfonate group allows for immobilization on anion-exchange membrane, while a quartenized amino allows for immobilization on cation-exchange membrane. As seen in Table 1, HOPSA, the molecule used by Seitz, fulfills both probe requirements. The goals of our search are to extend the range of a probe beyond a single pH unit and to allow measurements above and below the physiological pH range.

EXPERIMENTAL

Reagents: All compounds evaluated were purchased from Aldrich and were used without further purification. A list of the specific compounds evaluated is given below, and their respective structures are shown in Table 1. All other chemicals were reagent grade or better.

LIST OF COMPOUNDS EVALUATED

Pyrogallol Red

Quinaldine Red

Sulfonazo III

Alizarin Blue Black B

Alizarin Red S

4,4'-Diamino-2,2'-Disulfonic Stilbene (DADS)

5-Dimethylamino-1-Naphthalene Sulfonic Acid (DANS)

Safranine O

8-Anilino-1-Naphthalene Sulfonic Acid

Rhodamine B

Nuclear Fast Red

4,5-Dihydroxynaphthalene-2,7-Disulfonic Acid

2,7-Dihydroxynaphthalene-3,6-Disulfonic Acid

6,7-Dihydroxynaphthalene-2-Sulfonic Acid

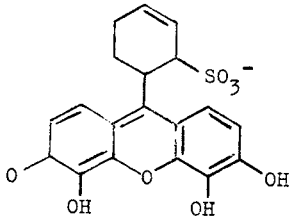
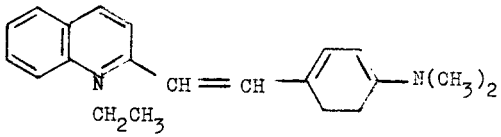
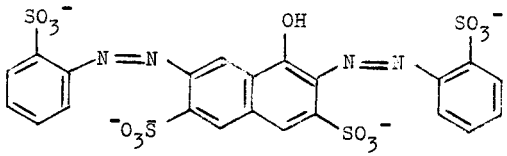
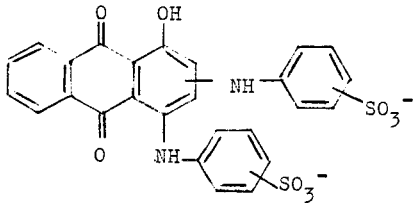
1-Naphthol-2-Sulfonic Acid

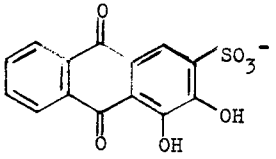
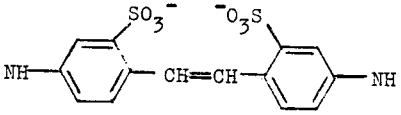
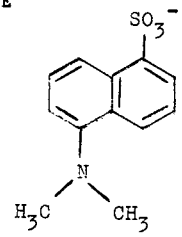
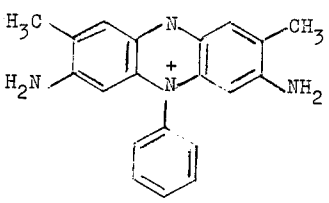
2-Naphthol-7-Sulfonic Acid

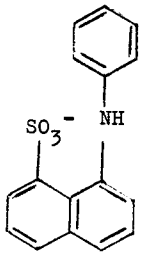
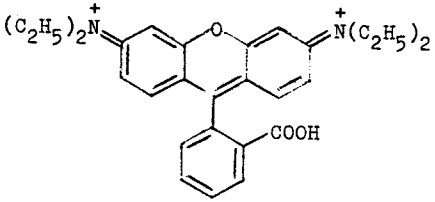
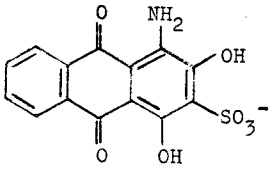
8-Hydroxypyrene-1,3,6-Trisulfonic Acid (HOPSA)

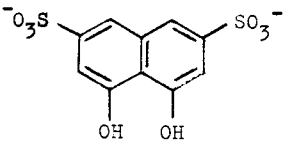
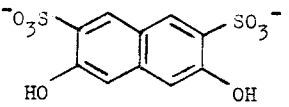
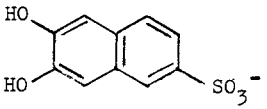
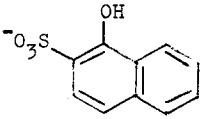
TABLE 1

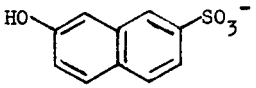
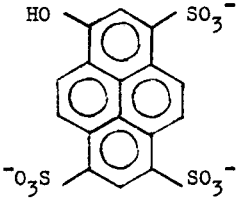
STRUCTURES OF COMPOUNDS EVALUATED

Compound	Structure
PYROGALLOL RED (anion-exchange)	
QUINALDINE RED (cation-exchange)	
SULFONAZO III (anion-exchange)	
ALIZARIN BLUE BLACK B (anion-exchange)	

Compound	Structure
ALIZARIN RED S (anion-exchange)	
4,4'-DIAMINO-2,2'-DISULFONIC STILBENE (DADS) (anion-exchange)	
5-DIMETHYLAMINO-1-NAPHTHALENE SULFONIC ACID (DANS) (anion-exchange)	
SAFRANINE O (cation-exchange)	

Compound	Structure
8-ANILINO-1-NAPHTHALENE SULFONIC ACID (anion-exchange)	
RHODAMINE B (cation-exchange)	
NUCLEAR FAST RED (anion-exchange)	

Compound	Structure
4,5-DIHYDROXYNAPHTHALENE-2,7-DISULFONIC ACID (anion-exchange)	
2,7-DIHYDROXYNAPHTHALENE-3,6-DISULFONIC ACID (anion-exchange)	
6,7-DIHYDROXYNAPHTHALENE-2-SULFONIC ACID (anion-exchange)	
1-NAPHTHOL-2-SULFONIC ACID (anion-exchange)	

Compound	Structure
2-NAPHTHOL-7-SULFONIC ACID (anion-exchange)	
8-HYDROXYPYRENE-1,3,6-TRISULFONIC ACID (HOPSA) (anion-exchange)	

Buffer Systems:

For pH's less than 6, 2.0 M hydrochloric acid was added to 200 milliliter aliquots of 1.0 M sodium acetate to obtain the desired pH.

For the pH range of 6 to 8, concentrated sodium hydroxide was added to 75 milliter aliquots of tris(hydroxymethyl) aminomethane hydrochloride (Trizma HCl).

For the pH range of 8 to 10, concentrated sodium hydroxide was added to 100 milliter aliquots of 1.0 M ammonium chloride.

For pH values greater than 10, concentrated sodium hydroxide was added to aliquots of 1.0 M sodium carbonate.

Instrumentation:

The pH dependence of the absorption and fluorescence spectra of the dissolved and membrane-bound potential probe was studied using conventional spectral instrumentation. Absorption measurements were recorded on a Cary Model 118 Spectrophotometer. The autogain mode was used with a period of 1 second and a slit width of 0.05 mm. A Perkin-Elmer Hitac PF-2A Spectrofluorometer interfaced with an Apple II+ computer was used to record fluorescence spectra and intensities.

An Orion Research model 701A/digital Ionalyzer was used to measure pH.

Figure 2 contains the diagram of the optical sensor and its associated instrumentation. Other than the fiber optic itself, the instrumentation required is not too specific. The source is a tungsten lamp. The detector is a photomultiplier tube (RCA 1P21 operated at 700 V) which is contained in a housing with a variable slit width and the capacity to hold the interference filters used for selection of excitation and emission wavelengths (1 inch X 1/2 inch,Edmund Scientific). A digital photometer/power supply is used (SPEX DPC-2). A bifurcated fiber optic is threaded to fit the other components (diameter 4.5 mm). Fluorescence is excited through one arm of the fiber optic and the emission is observed through the other. The common end of the optic is immersed in solutions of various pH's contained in a 15 ml beaker and covered with a lighttight aluminum casing. There is a shutter in front of the detector which excludes ambient light when this casing is removed. A piece of ion exchange membrane containing the immobilized probe molecule is held on to the common end of the bifurcated fiber optic by a piece of tygon tubing. When placed in the solution the optical properties of the immobilized fluorophor are affected. The sensor involves an equilibrium between analyte and immobilized reagent. Since the reagent phase is not consumed by its

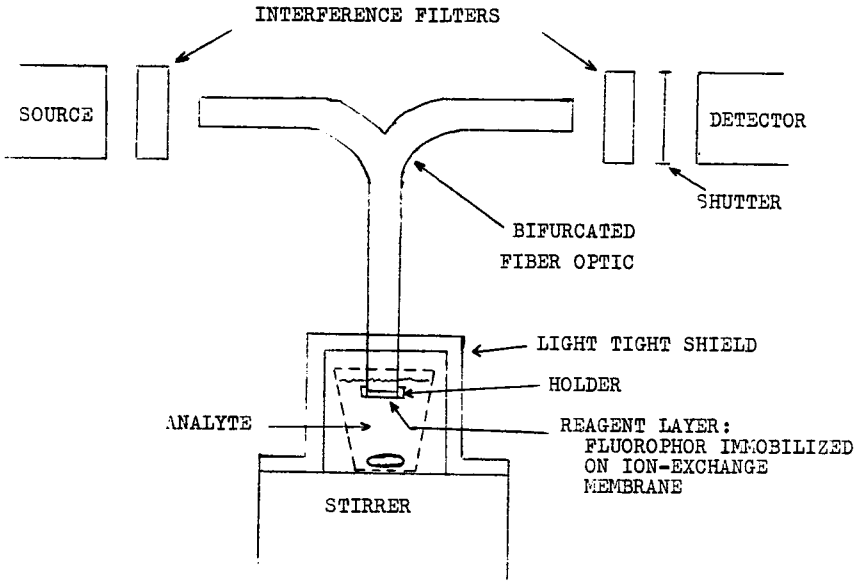


Figure 2 Optical Sensor and its Associated Instrumentation.

interaction with the analyte this sensor is classified as reversible (7).

Procedures:

Immobilization Procedure: The ion exchange membranes were purchased from Raipore Research Corporation (R-1010 and R-1035). The immobilization procedure involves placing a 10 centimeter squared piece of membrane in 10 ml of a 1X10E-4 M fluorophor solution and stirring for several hours. All compounds evaluated were completely bound to the membrane, resulting in 1X10E-6 moles of fluorophor immobilized on the membrane. Despite the fact that there are no covalent bonds, the electrostatic attraction is strong enough to make the adsorption process irreversible. From the micromoles of saturation (0.7-1.0 micromole/cm) we estimate that for most of the probes typically no more than 10-20% of the available binding sites are used in the immobilization process.

Absorption and Fluorescence Measurements:

The absorption and fluorescence properties of a potential probe molecule were studied both in solution and immobilized on an ion exchange membrane.

For absorption measurements in solution a 1X10E-4 M solution of the fluorophor was made in deionized water.

A 20 ml aliquot of this solution was then adjusted to a particular pH by dropwise addition of the appropriate buffer while monitoring with the pH meter. The solution was then brought up to a total volume of 25 ml with deionized water. For the fluorescence measurements in solution the 1X10E-4 M solution was diluted to 1X10E-5 M. The fluorescence was measured as a function of pH by following the above procedure.

Next, the absorption and fluorescence of the immobilized fluorophor were studied. From the prepared piece of membrane, a portion was cut so as to fit flatly against an inner side of the absorption cell. Once the membrane has been placed in the cell, absorption measurements at various pH's can be obtained by filling the cell with the appropriate buffers. A piece of untreated ion exchange membrane must be similarly placed in the reference cell containing deionized water.

The fluorescence of the immobilized fluorophor was studied as a function of pH using the same piece of membrane and the same procedure for adjusting the pH. However, for each fluorescence measurement the membrane must be carefully replaced in the cell at an angle slightly off of 45° to the excitation beam.

Once a suitable probe was found, fluorescence intensities of the immobilized fluorophor were measured using the fiber optic and its associated instrumentation described previously.

RESULTS

Absorption and fluorescence spectra were measured as a function of pH for membrane-bound and dissolved fluorophor. The spectral data obtained were used to determine the pKa values for the bound and dissolved fluorophors. The results of the evaluation of 4,5-dihydroxynaphthalene-2,7-disulfonic acid (4,5-DHN-2,7-DSA) are given in Table 2. The maximum absorbance of the basic form of 4,5-DHN-2,7-DSA is at 360 nm. The absorbance values at that wavelength, as a function of pH, are given in the table. Cb represents the concentration of the basic form of the molecule as calculated from Beer's Law. The concentration of the acid form is represented by Ca, and is equal to the difference between the total concentration of 4,5-DHN-2,7-DSA and Cb. For the solution of highest pH it is assumed that all of the molecules exist in the unprotonated form. A plot of the Henderson-Hasselbach equation (equation 1) was made using the results in Table 2, except for those for the two most extreme pH's (Figure 3). The value of the y-intercept of the straight line obtained is equal to the pKa of the compound evaluated. From least squares plot, the pKa of the 4,5-DHN-2,7-DSA

solution is 5.62.

$$pH = pKa + \log (Cb/Ca) \qquad (1)$$

TABLE 2

ABSORBANCE OF 6.17X10E-4 M 4,5-DHN-2,7-DSA
AS A FUNCTION OF PH

pH	Abs(360)	Cb	Ca	log Cb/Ca
3.33	0.015	0	6.17x10E-4	0
4.76	0.100	7.69x10E-5	5.40x10E-4	-0.848
4.94	0.145	1.12x10E-4	5.06x10E-4	-0.656
5.26	0.237	1.82x10E-4	4.35x10E-4	-0.378
5.44	0.340	2.62x10E-4	3.56x10E-4	-0.134
5.48	0.355	2.73x10E-4	3.44x10E-4	-0.100
5.72	0.457	3.52x10E-4	2.66x10E-4	0.122
6.19	0.640	4.92x10E-4	1.25x10E-4	0.596
6.42	0.700	5.39x10E-4	7.86x10E-5	0.836
6.76	0.723	5.56x10E-4	6.10x10E-5	0.960
7.05	0.764	6.17x10E-4	0	-

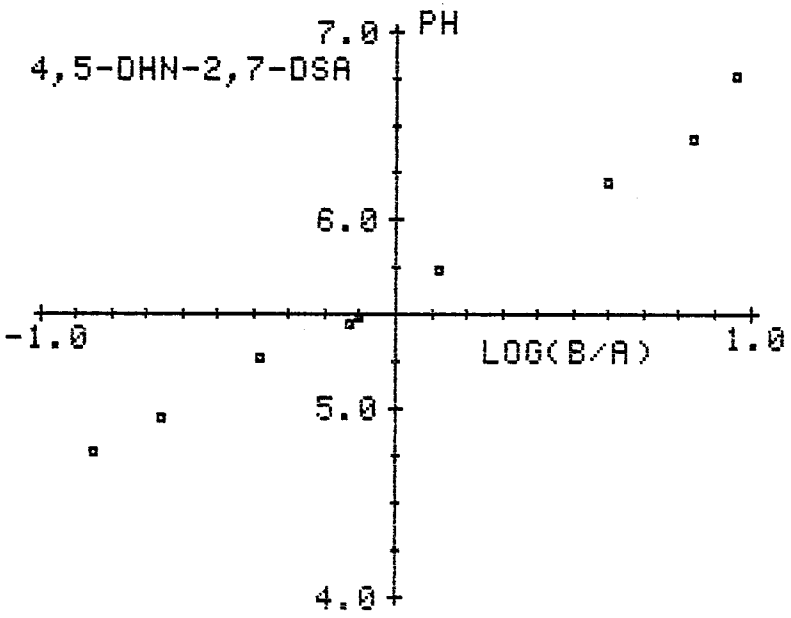


Figure 3 Henderson-Hasselbach Plot of results in Table 2. The y-intercept= pKa = 5.62.

A value for the pKa of the compound can also be determined from the variation in fluorescence intensity at a particular wavelength as a function of pH. The choice of the excitation and emission wavelengths is based on the absorption and fluorescence spectra of the compound and is explained in the discussion. Figure 4 shows a plot of pH versus the intensity of fluorescence emission of the anion form when excited at two different wavelengths, using the results of 4,5-DHN-2,7-DSA given in Table 3.

TABLE 3

FLUORESCENCE INTENSITY OF 6.17X10E-5 M 4,5-DHN-2,7DSA AS A FUNCTION OF PH

pH	Exc at 360nm	Exc at 300nm	I(360)/I(300)
7.45	282	31	9.0
7.08	253	28	8.9
6.82	254	34	7.5
6.46	232	34	6.9
5.66	176	39	4.5
5.48	147	41	3.6
5.44	143	41	3.5
5.20	114	42	2.7
4.88	70	46	1.5
4.68	52	46	1.1
3.84	20	46	0.4

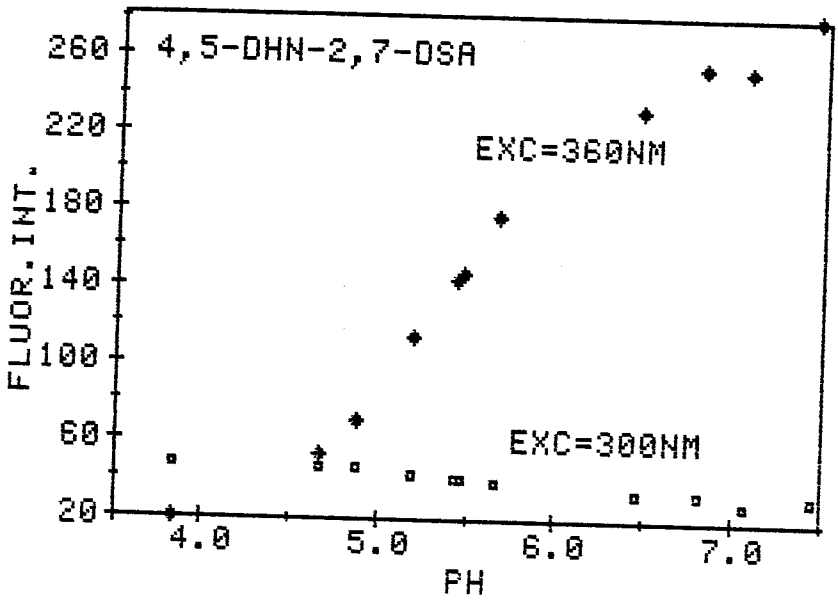


Figure 4 Fluorescence Emission of the Anion Form as a function of pH, excited at two wavelengths.

The ratio of the fluorescence intensity at one excitation wavelength to the fluorescence intensity at the second excitation wavelength was found to vary as expected over the pH range about the pKa of the fluorophor evaluated. Figure 5 shows the change in the ratio of intensity with pH for the 4,5-DHN-2,7-DSA about the range of its pKa.

All other potential probe molecules were similarly evaluated. A summary of the results of these evaluations is given here and the data are presented in Tables 4 through 7.

Four molecules we considered as possibilities for use in the pH probe, Pyrogallol Red, Alizarin Blue Black B, Sulfonazo III, and Quinaldine Red, are not measurably fluorescent on the membrane.

Alizarin Red S is stable on the membrane; however, the fluorescence intensity of this compound upon immobilization is too weak for use in the sensor as presently designed.

In the evaluations of the two fluorophors DANS and DADS it was discovered that these compounds came off of the anion-exchange at very acidic pH's.

Rhodamine B, Safranine O, 8-Anilino-1-Naphthalene Sulfonic Acid, and Nuclear Fast Red all have a pKa less than 2, which is too low to be a practical choice as a pH

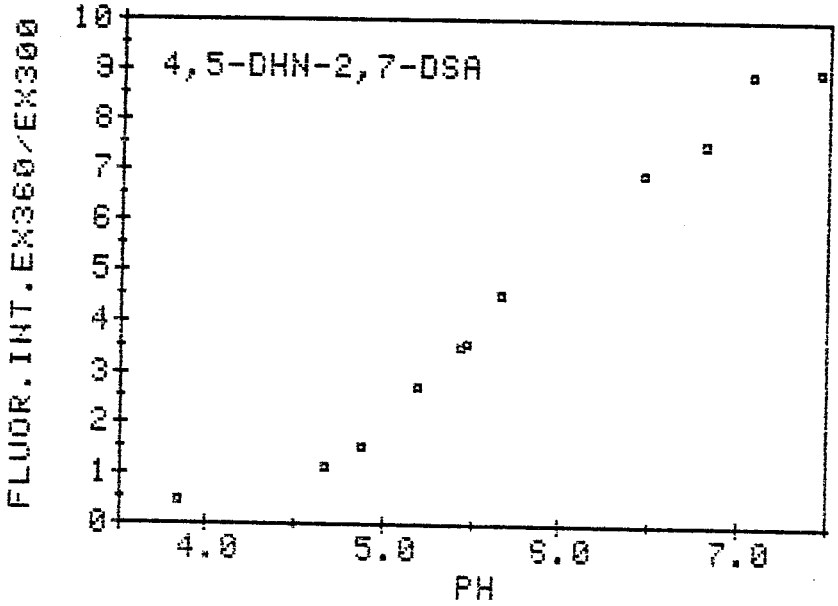


Figure 5 Ratio of the Fluorescence Intensity of the anion form verses pH.

probe.

The hydroxy naphthalene compounds, which include 4,5-Dihydroxynaphthalene-2,7-disulfonic acid, 2,7-Dihydroxynaphthalene-3,6-disulfonic acid, 6,7-Dihydroxynaphthalene-2-sulfonic acid, and 1-Naphthol-2-sulfonic acid, photodecompose much too readily to be a practical choice in a fiber optic pH probe.

TABLE 4

UNSUITABLE DUE TO PHOTODECOMPOSITION

Compound	Abs.Max (nm)		Fluor.Max (nm)		pKa
	base	acid	base	acid	
4,5-DHN-2,7-DSA					
solution	360	347	440	364	5.6
membrane	374	350	440	370	4.1
2,7-DHN-3,6-DSA					
solution	357	337			9.4
membrane	360	340	440	440	10.0
6,7-DHN-2-SA					
solution	340	330			
1-N-2-SA					
membrane	345	300		450	10.0

TABLE 5

FLUORESCENCE TOO WEAK

Compound	Abs.Max (nm) base	Abs.Max (nm) acid	Fluor.Max(nm) base	pKa_1	pKa_2
ALIZARIN RED S					
solution	558	420	620	5.6	10.9
membrane	576	428	660	5.3	10.7

TABLE 6

COME OFF ANION EXCHANGE MEMBRANE AT LOW PH

Compound	Abs.Max (nm) base	Abs.Max (nm) acid	Fluor.Max (nm) base	Fluor.Max (nm) acid	pKa
DADS					
solution	324	292	440	360	1.6
membrane	340		440		3.5
DANS					
solution	316	284	500	335	4.3
membrane	320				2.5

TABLE 7

pKa TOO ACIDIC

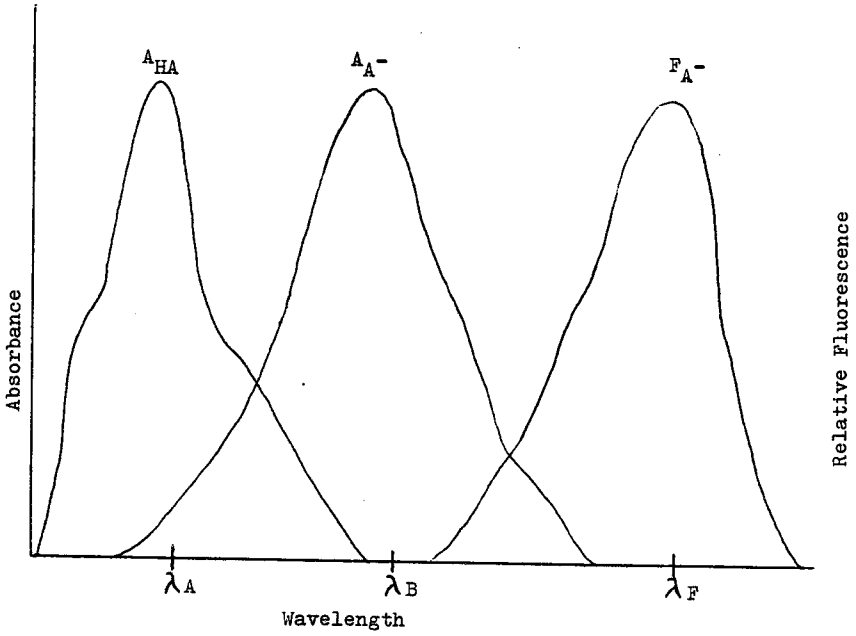
Compound	Abs.Max(nm)	Fluor.Max (nm)
NUCLEAR FAST RED		
solution	518	587
membrane	534	575
SAFRANINE O		
solution	520	
membrane	533	
8-ANILINO-1-NSA		
solution	350	
RHODAMINE B		
membrane	553-556	

DISCUSSION

The absorption spectra for the acid and base forms of the probe molecule must be significantly different in order to be useful in the pH sensor. The required probe spectral properties for the experimental approach we have chosen are shown in Figure 6. The absorption spectrum of the acidic form is labled A_{HA} while that of the basic form is labled A_A^-. The latter spectrum is at longer wavelengths, which is characteristic of aromatic systems. The fluorescence emission spectrum of the basic form, which is usually more intense than the emission of the acidic form, is labled F.

Before immobilization on the membrane, the probes are evaluated in solution to determine if they have a suitable pKa for the pH region we wish to measure, that is, above and below physiological pH's.

Upon immobilization, absorbance increases and there is a shift in the maximum absorbance of the forms to slightly longer wavelengths. For all but one of the fluorophors evaluated on the anion-exchange membrane, an acid shift in the pKa was observed. This shift may be due to the membrane itself. The anion-exchange membrane is a quarternized benzylamine grafted



Parameter Measured: $\dfrac{I_{F(\lambda_B)}}{I_{F(\lambda_A)}}$

Figure 6 Required Spectral Properties for Probe Molecule.

polytetrafluoroethylene one mil film. It can be visualized as being a piece of film containing positive charges. We calculated that there is one positive charge per ten angstroms, which is about the size of the molecules being immobilized. If the positive charges are viewed as being set up in an array, it is possible that these positive charges of the membrane may "get in the way". Thus, it is more difficult to protonate the fluorophor once it is immobilized and surrounded by positive charges than when it is dissolved in solution. How drastic the shift is depends on the fluorophor itself. Perhaps some fluorophors undergo a conformational change so as not to be as close to as many positive charges, in which case a less dramatic shift in pKa would be observed.

The spectral requirements shown previously must be met by the immobilized probe molecule. The sensor is based on a measurement of the fluorescence of the basic form when excited at two different wavelengths. The spectra show that at λ_B the basic form will be selectively excited, while at λ_A the acidic form will be more selectively excited. The parameter measured by the sensor is the ratio of the fluorescence intensity emitted at λ_F when excited at λ_B to the intensity emitted when excited at λ_A. This ratio is measured as a function of pH. Under ideal conditions the logarithm of the measured ratio will be linearly related to pH:

$$\log \left(I_{F(\lambda_B)}/I_{F(\lambda_A)}\right) = pH + \text{constant} \qquad (2)$$

There are several advantages to using this ratio measurement. Not only will the change in this ratio with pH be greater than the change in fluorescence emission of either single form with pH, but, also, a ratio measurement is insensitive to source fluctuations, drift, temperature, quenching, ionic strength, and small loss of reagent or degradation, all of which can affect a single intensity measurement (7). Additionally, because this ratio will only be finite when there is unprotonated form present in the ground state, the sensor only "tracks" the ground state pKa and is independent of the excited state pKa, which may be affected by such factors as buffer concentrations.

The results given previously for 4,5-DHN-2,7-DSA illustrate the desired relationship between the measured ratio and pH (Figure 5). We were originally attracted to dihydroxynaphthalene compounds since, with two ionizable groups having different pKa's, we thought that they would extend the pH region over which an optical sensor would be suitable. Although they were determined to have pKa's in the usable range (Table 4), unfortunately, the dihydroxynaphthalene compounds we evaluated photodecompose very readily, probably forming quinones.

The problem of rapid decompostion also occurred in the evaluation of 1-naphthol-2-sulfonic acid. Compounds that photodecompose would not be a practical choice for use as the probe molecule in the optical pH sensor.

In our evaluations of possible probes we found Alizarin Red S particularly attractive because it had pKa's both above and below the physiological pH range. The absorption and fluorescence data given in Table 5 are for the acid and base forms at the higher pKa. Although this compound is stable on the membrane, the fluorescence signal is too weak. In order to check whether concentration quenching is occurring, we tried three different amounts of the Alizarin Red S on the membrane: 10E-5 moles, 10E-6 moles, and 10E-7 moles. Although the fluorescence intensity does increase slightly as the amount on the membrane decreases, it is still too weak. A weak signal would be more subject to error and interference; therefore, Alizarin Red S would not be a practical choice for the sensor.

Our goal is to extend the usable range of the pH sensor previously developed to the regions above and below the physiological pH range. Two compounds we thought would be usable below this range were DANS and DADS acid (Table 6). Each one was immobilized on the anion-exchange membrane. In both of these the ionizable group is an amino group rather than a hydroxy group. We discovered that at low pH values, below the pKa of these

acids, they come off of the anion-exchange membrane, presumably due to charge-charge repulsion between the protonated amino group and the cations on the anion-exchange membrane. Therefore, neither of these would be a desirable probe molecule for the optical pH sensor.

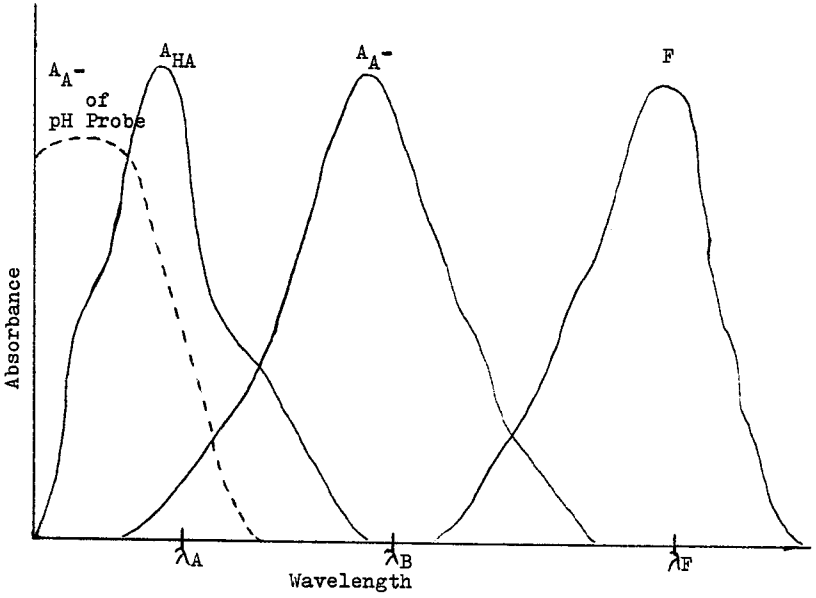
Four of the compounds evaluated, namely, Rhodamine B, Safranine O, 8-Anilino-1-Naphthalene Sulfonic Acid, and Nuclear Fast Red, were determined to have pKa's less than two (Table 7). Since a probe molecule is limited to measuring a pH range of +/-1 pH unit about its pKa, these would not lead to the development of a sensor for a commonly measured pH region. In the case of Rhodamine B, it could be that the acid and the base form do not have significantly different spectral properties. However, if that is so, it would still not be usable as a pH probe in the optical sensor.

We examined the structures of the four compounds we found to be non-fluorescent on the membrane (Table 1). At least in the case of the Alizarin Blue Black B, Sulfonazo III, and Quinaldine Red, the non-rigidity of the molecule very likely promotes very efficient non-radiative decay. Of course, a non-fluorescent molecule could not be used alone to develop an optical pH sensor based on fluorescence.

FUTURE WORK

We are currently pursuing a new approach of immobilizing two molecules on the ion-exchange membrane simultaneously. The absorption properties of one of the molecules must be dependent on pH. This would act as the pH probe. The other molecule, which would act as the reporter probe, is required to have fluorescence properties independent of pH, but dependent on the absorption properties of the pH probe. Therefore, the pH probe would respond to pH in such a way as to effect the fluorescence properties of the reporter probe. The pH region over which such a dual probe system could be used would depend on the pKa of the pH probe.

Figure 7 shows the spectral properties required for the dual probe approach. We are now evaluating a system using 2-naphthol-7-sulfonic acid, which is more stable on the membrane than the previous naphthol compounds evaluated, as the pH probe. For the reporter probe we are using HOPSA, the molecule previously used by Seitz. As the pH approaches the pKa of the naphthol, the absorption of the naphthol attenuates the lower of the two excitation wavelengths for the HOPSA molecule, while not effecting the other excitation wavelength. Thus, the



Parameter Measured: $\frac{I_{F(\lambda_B)}}{I_{F(\lambda_A)}}$

Figure 7 Required Spectral Properties for Dual Probe System.

measured ratio should change in response to the pKa of the naphthol, which upon immobilization on the anion exchange membrane is 8.5. The attractive advantage of this system is that in combination with HOPSA it would extend the usable region of the previously developed sensor, based on HOPSA alone, to the region of pH 6 to 10. Work on this dual probe will continue in the future.

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