Design and Performance of a Differential Spectrofluorometer*

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SUMMARY
A differential spectrofluorometer has been designed and built. This instrument is capable of measuring and recording the difference fluorescence spectrum between two samples which share a common light source, monochromator, and photomultiplier. The two fluorescent signals are synchronously demodulated, filtered, and subtracted in a differential voltmeter.

The model compounds, indole and N-acetyl-l-tryptophanamide, as well as lysozyme and an inhibitor have been studied. Difference fluorescence spectroscopy offers a sensitive method to detect protein conformational changes involving the aromatic residues and to reveal small emission differences in aromatic molecules. Used concurrently with solvent perturbation it can be used to probe the topology of the surface to evaluate the accessibility of aromatic residues.

The determination of conformation and conformational changes in biological systems has become of central interest in relation to biological function. Many physicochemical techniques have been applied to such determinations (1), e.g. optical rotatory dispersion, circular dichroism, nuclear magnetic resonance, etc. The presence of the aromatic side chain of the amino acids tyrosine, tryptophan, and phenylalanine has made the ultraviolet spectral region particularly advantageous for such determinations.

As the ultraviolet absorption spectra of tryptophan and tyrosine are related to the environment about them, a change in the environment about either amino acid will cause a shift in the absorption spectrum (2, 3). Consequently, a conformational change of a protein containing these residues will result in a spectral shift if the environment about either residue is altered, or solvent perturbation of exposed residues will likewise cause spectral shifts. Thus intrinsic tryptophan and tyrosine moieties can be used as probes for detecting conformational changes in proteins. A particularly useful way to follow these changes is by difference spectroscopy (2, 3). In this method the difference between a perturbed and unperturbed protein molecule is measured. This difference technique has allowed investigators to determine, among other things, the fraction of exposed tyrosine and tryptophan residues in a native protein, the effect of substrate binding, and to study the kinetics and thermodynamics of denaturation. In particular, it has allowed the observation of very small differences.

Another technique for studying changes in protein conformation, which is related to absorption, is fluorescence. This technique allows the use of much smaller samples because of the greater sensitivity of the method. Changes in the environment (e.g. solvent perturbation, substrate binding, conformational changes) about a tyrosine or tryptophan residue, either incorporated in a protein molecule or free in solution, have been shown to lead to changes in the fluorescence emission spectrum of these amino acids (4-6). In addition to these causes of fluorescent alteration, specific quenching effects induced by environmental factors can cause large fluorescent changes. The correlation of the fluorescence spectrum with the absolute immediate medium surrounding these moieties is difficult. However, this method is excellent for observing small changes occurring about these residues. In analogy with difference absorption spectroscopy, difference fluorescence spectroscopy offers a highly sensitive method to measure conformational changes in proteins, or to probe the surface to locate aromatic residues. Any system which possesses a fluorescent moiety is amenable to study. The usefulness of difference fluorometry was shown by the work of Lehrer and Fasman (7) in their study of the effect of pH and inhibitors on the fluorescence of lysozyme. These workers were able to separate the fluorescence of 3 different tryptophan residues, and to show the effect of binding on their fluorescence.

This technique, although powerful in principle, has not found widespread use. One obvious reason for this is the lack of availability of a difference spectrofluorometer. This paper describes in detail a difference spectrofluorometer built in this laboratory. In addition, the use of this instrument to measure fluorescence differences of N-acetyl-l-tryptophanamide in various solvent systems, indole in two different solvents, and a comparison of some of the difference spectra from the paper of Lehrer and Fasman (7) on lysozyme is reported.

OPTICS
In Fig. 1 is seen an optical schematic of the instrument. The light source is a 150-watt direct current xenon lamp (Osram XBO 150 W/1) powered by a regulated power supply (G. W. Gates and Company, Long Island, New York, No. P150D).
Additional heavy filtering was installed between the power supply and the lamp to reduce ripple to less than 1%. The two sample times share the light source, by the "can"-type rotating chopper. This is a cylinder with a 180° section milled out and fitted over the xenon lamp. Some difficulties were initially encountered in igniting the lamp. The high voltage, high frequency pulse delivered by the power supply arced to the chopper and prevented lamp ignition. Rounding of all sharp edges, increasing the diameter of the chopper, and reducing stray capacitances solved this problem.

The excitation wave length is selected by a pair of matched interference filters (Thin Film Products Division of I-R Industries, Waltham, Massachusetts) which have a transmission of 20% at 280 nm and a half-band width of 100 nm. The filters were matched to within 2% for band width and peak transmission.

The exciting light is focused at the center of the cuvettes by fused quartz condensing and focusing lenses (Bausch and Lomb, Rochester, New York). The same set of lenses is used throughout the instrument. The sample holders are thermostatted and accommodate cuvettes of 1-cm internal light paths or smaller. An adjustable shutter between the light source and the sample allows initial adjustment of the exciting light.

Fluorescence emission is observed at 90° to the direction of the exciting light. This fluorescence is focused on a plain front surface mirror which in turn reflects the light to a second mirror and is subsequently collimated at the entrance slit of the monochromator. The monochromator is a Bausch and Lomb 250-mm focal length grating type with a constant resolution of 16 A per mm of slit width. It was coupled to the instrument on its "side."

That is, the entrance slit was in the direction of the two fluorescent beams so that each beam illuminated a section of the grating along the rulings. Placing the monochromator in its normal way would have produced spectral shifts caused by the displacement of the two beams on the grating along a line perpendicular to the rulings. A field lens was used at the exit slit which focuses and superimposes the two slit images on the same spot of the photomultiplier photocathode.

**DETECTION AND RECORDING**

The chopper was rotated by an 1800-rpm synchronous motor (Bodine type NSY-12) and therefore the output of the photomultiplier (EMI 6255S) is a 30 Hz square wave (8), which is preamplified by an operational amplifier (Philbrick SP24A). In Fig. 2, 2 seen the oscilloscope photograph of the various wave forms. A disc with the same mechanical cut out as the chopper is mounted on the shaft of the chopper with a small tungsten lamp (6 volts) and a phototransistor (G.E. L14A502) on either side of the disc. The collector leads of the phototransistors are high speed mercury-wetted contact relays (C.P. Clare HGS 5000) which have operate and drop-out times of 1 msec with no contact bounce. The contacts of the relays switch the alternate half-cycles of the photomultiplier output into three-stage R-C boxcar integrators. Time constants of these integrators are variable from 100 msec to 3 sec. For most of the spectra 100-msec time constants were sufficient at the scanning speed of 0.5 mm per second. During the dark half-cycles the contacts of the demodulating relays are grounded.

Several lock-in techniques could be used including commercial tuned amplifiers. However, it was found that the relatively inexpensive phototransistors and mercury relays formed a good demodulation circuit. The filtered direct current voltages are subtracted by a Keithley model 153 differential voltmeter and its output is recorded on a Hewlett Packard 7550B (RS) X-Y recorder. The X input voltage of this recorder is derived from a 10-turn potentiometer and a voltage source. The potentiometer is coupled to the wave length drum of the monochromator by a precision gear. The spectrum is scanned by a stepping motor, each step corresponding to a 0.5-mm increment. The speed of scanning is controlled by a low frequency oscillator.

In typical operation the photomultiplier is operated at 1200 volts and the outputs of the filters at the peak of the emission band are around 8 volts. To amplify small differences the sensitivity of the differential voltmeter is switched to scales which are 10 or 30 times more sensitive.
For the present work the excitation wave length was always 280 mp while the emission was scanned from 300 to 430 mp. Light output of the lamp decreases appreciably at this excitation wave length and even though the monochromator grating was blazed for 3000 A, the over-all efficiency of the system is quite low. With these conditions a 10% difference could be measured with a 1% accuracy.

**EXPERIMENTAL PROCEDURE**

*N*-Acetyl-L-tryptophanamide was purchased from Mann (m.p. 192-194°) and used as such. Lysozyme was obtained from the Worthington (lot LYSF 7CB) and used without further purification. Indole (Matheson Coleman and Bell, lot 384224) was recrystallized from methanol in the dark.

The solutions of *N*-acetyl-L-tryptophanamide were made by first preparing a stock solution in twice distilled water and by diluting with either twice distilled water or reagent grade methanol to equal volumes. The O.D.279 was 0.152 for *N*-acetyl-L-tryptophanamide in methanol (1% H₂O) and 0.143 in water. The solutions of indole were made by preparing a stock solution in ethanol (USP-NF grade) and then diluting with twice distilled water or reagent grade methanol. The O.D.270 was 0.157 for indole in methanol (1% ethanol) and 0.151 in water (1% ethanol). The solutions for lysozyme were prepared by making stock solutions of twice the desired concentration in 0.2 M NaCl, and diluting in half with either 0.2 M NaCl or tri-*N*-acetyl-D-glucosamine (the gift of Dr. J. Rupley) dissolved in 0.2 M NaCl at the appropriate pH. The final lysozyme solutions had an O.D.270 of about 0.2 (when using the split cells) or 0.1 when using standard fluorescence cells, and the final concentration of inhibitor was slightly greater than 0.2%.

For all but one of the set of spectra reported standard fluorescence cells (40 x 10 x 10 mm) were used. For the set of spectra of lysozyme plus tri-*N*-acetyl-D-glucosamine versus lysozyme, split cuvettes were used (40 x 10 x 10 mm was divided with a 1-mm quartz plate giving each compartment a 4.5-mm light path). One split cell contained protein solution in one compartment and solvent plus inhibitor in the other; the second split cell contained protein plus inhibitor in one compartment and solvent in the other. The blank solutions were placed facing the excitation source. In this way the absorbing or fluorescing effect of a perturbant is cancelled out. Both sets of fluorescence cells were purchased from the Optical Cell Company, Beltsville, Maryland.

It is necessary to adjust for small differences in the total optical paths of the two components. Mechanical shutters, attached directly to the two cell compartments, enable the operator to equalize the fluorescence intensity of a control solution placed in each compartment at a single wave length. The difference spectrum between two such control solutions constitutes the base line of the instrument. This baseline is subtracted from the difference between the control solution and a sample solution to give the true difference.

The spectra obtained from the difference fluorometer are not corrected for variation in sensitivity of the photomultiplier tube, the xenon lamp, or the efficiency of the monochromator. It is possible to obtain a correction curve over a limited spectral range by measurement of the corrected fluorescence intensity of, say, indole dissolved in diethyl ether, in methanol, and in water, on a Zeiss ZFM 4C spectrofluorometer (9). This corrected value is divided by the fluorescence intensity of the same solution measured on the difference fluorometer, when the appropriate solvent is used as the reference solution. This ratio (f) which varies with wave length constitutes our correction curve. To obtain the corrected fluorescence value of a sample at a particular wave length one uses the relationship

\[
f_r = f(f_d)
\]

where \( f \) is the correction factor, \( f_d \) is the fluorescence intensity of the sample measured on the difference fluorometer, and \( f_c \) is the

**TABLE I**

| Corrected peak wave length fluorescence values | \( \lambda_{max} \) | \( \lambda \) of difference spectra |
|-----------------------------------------------|-----------------|---------------------------------|
| Lysozyme, pH 7.0                              | 346             | 328±                           |
| Lysozyme, pH 11.4                             | 345             |                                 |
| Lysozyme + tri-*N*-acetyl-D-glucosamine, pH 5.6| 335             | 310±                           |
| Lysozyme, pH 5.6                              | 338             | 365±                           |
| *N*-Acetyl-L-tryptophanamide in water          | 350             | 331±                           |
| *N*-Acetyl-L-tryptophanamide in methanol       | 341             | 417±                           |
| Indole in water                               | 340             | 300±                           |
| Indole in methanol                            | 318             | 370±                           |

* Maximum.

* Minimum.
**FIG. 4.** Fluorescence spectra of N-acetyl-L-tryptophanamide. 

\(a\), in water, \(O.D.\lambda = 0.143\); \(b\), in methanol, \(O.D.\lambda = 0.143\); 
\(b - a\), the difference spectrum. Instrumental conditions: the sensitivity of the difference spectrum is 1.7 times that of the absolute spectrum; difference spectrum, photomultiplier tube voltage, 1120 volts; full scale of the Keithley voltmeter, 3 volts; 
Y range of the recorder, 100 mv per inch; absolute spectrum; photomultiplier tube voltage, 1120 volts; full scale of the Keithley voltmeter, 10 volts; Y range of the recorder, 50 mv per inch.

**FIG. 5.** Fluorescence spectra of N-acetyl-L-tryptophanamide in water at two different concentrations. 

\(a\), \(O.D.\lambda = 0.143\); \(b\), \(O.D.\lambda = 0.140\); \(b - a\), the difference spectrum at the sensitivities indicated relative to the absolute spectra. Instrumental conditions for the \(\Delta \lambda\) difference spectrum are: photomultiplier tube voltage, 1150 volts; full scale of the Keithley voltmeter, 3 volts; 
Y range of the recorder, 50 mv per inch. Instrumental conditions for the \(\lambda\) difference spectrum are: photomultiplier tube voltage, 1150 volts; full scale of the Keithley voltmeter, 10 volts; Y range of the recorder 10 mv per inch. The conditions for the absolute spectrum are: photomultiplier tube voltage, 1150 volts; full scale of the Keithley voltmeter, 10 volts; Y range of the recorder, 50 mv per inch.

**FIG. 6.** Original tracing of the fluorescence spectra of lysozyme and inhibitor in water at pH 5.1. 

\(a\), pure enzyme; \(b\), with tri-N-acetyl-D-glucosamine; \(b - a\), the difference spectrum. Instrumental conditions for the difference and absolute spectra are: photomultiplier tube voltage, 1300 volts; full scale of the Keithley voltmeter, 3 volts; Y range of the recorder, 100 mv per inch.

**FIG. 7.** Fluorescence spectra of lysozyme in water. 

\(a\), at pH 7.0; \(b\), at pH 11.4; the difference spectrum \(b - a\). Instrumental conditions for the difference and absolute spectra are: photomultiplier tube voltage, 1300 volts; full scale of the Keithley voltmeter, 3 volts; Y range of the recorder, 100 mv per inch.
corrected fluorescence intensity of the sample. The correctness of this method was shown by use of a solution such as L-tryptophan in water. The peak value of the corrected fluorescence curve ($f_c$) was $351 \text{ m}\mu$ (10, 11).

For all the spectra measured the following variables were constant: cell compartment temperature, 24°; monochromator entrance and exit slit widths, 4 mm; slit height, open. Other instrumental variables are given in the figure legends.

APPLICATION AND DISCUSSION

It is known from previous work that a change in the solvent medium can cause a corresponding change in the fluorescence intensity as well as a shift in the spectrum of a fluorescent solute (12). In general a decrease in the polarity of the solvent causes an increase in fluorescence and a corresponding blue shift in the fluorescence spectrum. Utilizing this fact, the usefulness of the difference technique in fluorescence can be easily shown. In Fig. 3 is shown the fluorescence of indole in methanol and in water as well as the difference fluorescence spectrum. Lowering the polarity, e.g. water to methanol, of the media causes a blue shift and increase in fluorescence. The difference curve yields a long wave length negative band and a short wave length positive band. The corrected peak values for this and following figures are found in Table I. The fluorescence spectra of N-acetyl-L-tryptophanamide in methanol, in water, and their difference fluorescence spectra is shown in Fig. 4. These spectra are similar to those of the indole curves; the peak shift is not as large, while the increase in fluorescence in methanol is relatively larger. The difference spectrum is composed of a small negative long wave length band and a large positive short wave length band. The spectra in Fig. 5 are of N-acetyl-L-tryptophanamide in water at two concentrations. Here the negative difference curve is the equivalent of that expected for quenching without a shift in emission. The utility of the instrument may be viewed from the small difference between the absolute spectra, which may be expanded in the difference mode by switching to a higher sensitivity. In this particular case there is no spectral shift, but a small difference may be accompanied by a shift, which could be easily evaluated by enlarging the difference spectrum.

It has been shown from the work of Lehrer and Fasman (7) that 3 of the 6 tryptophan residues in lysozyme are affected by changes which occur in or near the active site of the enzyme. In Fig. 6, which is a photograph of the original tracing, the effect of inhibitor on the enzyme at pH 5.1 is seen. In this pH region a carboxyl group is protonated, and causes a fluorescence decrease in 1 of the tryptophan residues when inhibitor is added. This can be observed from the negative portion of the difference spectrum. In addition, the binding of inhibitor causes the environment about 1 or 2 tryptophan residues to become less polar, and is seen as the positive band of the difference spectrum which is shifted toward the blue relative to the absolute spectra. In the free enzyme a change in pH from 7.0 to 11.4 causes a quenching of 1 of the tryptophan residues without a corresponding change in the conformation of the enzyme (7). The fluorescence curve obtained for lysozyme at pH 7.0 is seen in Fig. 7, Curve a, and at pH 11.4 as Curve b. The negative difference Curve (b - a) is that expected for quenching of a tryptophan with emission maximum at $330 \text{ m}\mu$, namely a tryptophan moiety in a nonpolar environment. This quenching is most likely the result of energy transfer to an ionized tyrosine residue (13, 14). It should be stressed here that only through use of the difference technique could the fluorescence contributions of these 3 tryptophan residues be separated in lysozyme.

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