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Chemistry

Fluorescence-detected magnetic circular dichroism of fluorescent and nonfluorescent molecules

(tryptophan/cytochrome *c*)

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ABSTRACT **We have modified a spectrometer for the measurement of fluorescence-detected magnetic (and natural) circular dichroism (FDMCD). The instrument can be operated either in a direct mode in which the average and polarization-induced differential fluorescent intensities are recorded separately or in a mode which records their ratio. We have measured the FDMCD of tryptophan, which is itself fluorescent. With the aid of an added fluorescent molecule we also measured the FDMCD of Fe(III) cytochrome *c*, which is nonfluorescent. In both cases, the FDMCD agreed with the conventional transmission-detected magnetic circular dichroism within experimental uncertainty. The FDMCD of both fluorescent and nonfluorescent molecules is potentially a useful technique for investigating optically dense materials and biological molecules in their native environments and systems in which there is inter- or intramolecular energy transfer.**

Magnetic circular dichroism, MCD, is a powerful spectroscopic technique which is finding increasing use in biochemistry. In MCD we measure the difference in absorbance of left and right circularly polarized light induced by an external magnetic field. In the past MCD has always been measured by observing the difference in intensity of the right and left circularly polarized components of a light beam which has passed through the sample. This is transmitted detected MCD.

We have constructed an apparatus which measures MCD by monitoring the fluorescence produced by light absorbed by the sample. This technique is called fluorescence-detected magnetic circular dichroism or FDMCD. Theory predicts and our experiments confirm that FDMCD can be used to measure the MCD of both fluorescent and nonfluorescent molecules. The ability to measure the MCD of nonfluorescent molecules enhances the utility of the technique significantly.

Recently Turner *et al.* (1) reported measurement of fluorescence-detected *natural* circular dichroism, FDCD. While our apparatus is similar to that for FDCD (and indeed can be used for the measurement of FDCD) the information gained from the two techniques is completely different and will frequently be complementary [for reviews of MCD see Djerassi *et al.* (2) and Stephens (3)]. Whereas, CD is sensitive to the conformation and environment of the absorbing molecule, MCD is usually characteristic of the nature of the molecule and relatively insensitive to its environment. Thus MCD, whether detected by emission or transmission, is useful in identifying and quantifying chromophores. FDMCD may also prove useful in energy transfer experiments in which the donor has a large and distinctive MCD.

Abbreviations: CD, circular dichroism; MCD, magnetic circular dichroism; FDMCD, fluorescence-detected magnetic circular dichroism; FDCD, fluorescence-detected (natural) circular dichroism.

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EXPERIMENTAL SECTION

CD and Fluorescence Apparatus. The critical features of the experimental apparatus are shown schematically in Fig. 1. A spectrometer, which will be described in detail elsewhere (J. C. Sutherland, G. D. Cimino, and J. T. Lowe, *Rev. Sci. Instrum.*, in press) was modified to include a photomultiplier (EMI, 9635) which detects light emitted at 90° from the direction of excitation from a sample located between the poles of an electromagnet. This photomultiplier is labeled pm-5 in Fig. 1, a notation consistent with the description of the complete spectrometer. The sample is irradiated with monochromatic light rendered alternately left and right circularly polarized by a photoelastic modulator (PEM). A glass filter prevents light scattered from the incident beam from reaching pm-5 and a 50 mm diameter quartz lens directs emitted light onto the photocathode. The emission photomultiplier is housed in a double magnetic shield consisting of a 1/64 inch (0.4 mm) thick layer of mu metal surrounded by a 1/4 inch (6.4 mm) thick soft iron cylinder. The anode current is converted to a voltage by a pream-

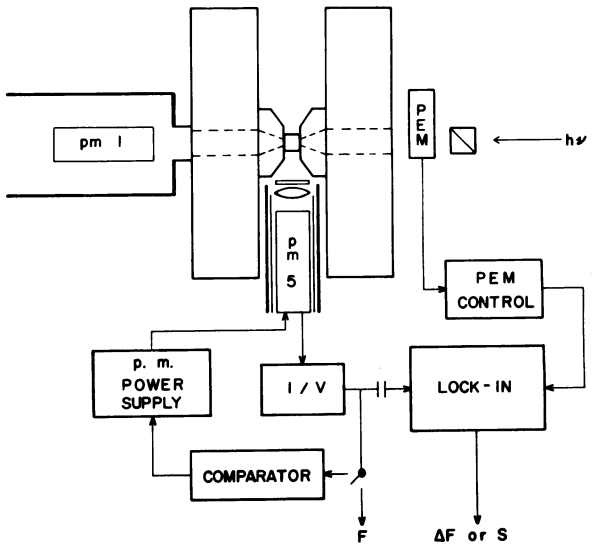

FIG. 1. Schematic diagram for the measurement of FDMCD. In direct or "open loop" mode a constant voltage is applied to the dynodes of pm-5 and signals proportional to F and ΔF are obtained from the outputs of the current to voltage converting preamplifier and the lock-in amplifier, respectively. In ratio or "closed loop" mode the dc output of the preamplifier is connected to a comparator which controls the dynode voltage so that the dc output is constant. In this mode the output of the lock-in is $S = -\Delta F/F$ ln 10. For transmission-detected MCD the power supply and preamplifier are connected to pm-1 and the instrument is operated in ratio mode.

plifier. In the direct recording or "open loop" mode of operation the signal proportional to the average fluorescence intensity, F, is taken directly from the output of the preamplifier while the signal proportional to the (peak-to-peak) modulated component, ΔF, is obtained by multiplying the output of the lock-in amplifier by an experimentally determined calibration factor. In the ratio or "closed loop" mode of operation a servo loop programs the dynode voltage of the photomultiplier so that the dc anode current is constant. In this mode the instrument is calibrated so that the output of the lock-in amplifier, S, is equal to $-\Delta F/(F \ln 10)$. Circular dichroism is measured by switching to pm-1 with the electronics in ratio mode. Except for pm-5 and a two pen chart recorder (Soltec Corp., N. Hollywood, Calif., model B182) which permits simultaneous recording of F and ΔF, all of the components are those listed in the complete description of the instrument.

The direct or "open loop" mode of operation in which F and ΔF are measured separately has the advantage that measurements can be extended to wavelengths outside of the band which excites fluorescence. This is useful in determining the proper baseline for ΔF. To determine that portion of ΔF induced by the external magnetic field, the spectrum is measured first with the field on and then with it off, the field-induced component being equal to the difference. This procedure is correct only if the sensitivity of the photomultiplier is the same when the magnet is on and off. Even though shielded, the sensitivity of pm-5 changes somewhat when the field is turned on. We eliminated this difficulty by adjusting the dynode voltage applied to pm-5 so that the value of F at all wavelengths was the same when the magnet was on as when it was off.

The ratio or "closed loop" mode of operation restricts measurements to those wavelengths which excite fluorescence, since the ratio circuit cannot function when F equals zero. However, in this mode, the instrument automatically compensates for changes in photomultiplier sensitivity caused by the magnet and for changes in the intensity of the exciting beam as a function of time. The latter feature is useful if F and ΔF cannot be recorded simultaneously. This is also the only mode of operation achievable with a commercial spectrometer without extensive electronic modification [see Turner *et al.* (1)].

The signal from the lock-in amplifier in the closed loop mode differs in sign from the signal in open loop mode. Experimentally, the change in sign is achieved by shifting the phase of the lock-in by 180°.

Spectrophotometer. Absorption spectra were recorded with a Cary 118c dual beam spectrophotometer.

Materials. Horse heart cytochrome *c* (Type VI), obtained from Sigma Chemical Corp. (Saint Louis, Mo.), L-tryptophan from K and K Laboratories (Hollywood, Calif.), and fluorescein from Matheson, Coleman & Bell Co. (Norwood, Ohio) were used without further purification. All samples were dissolved in 0.05 M potassium phosphate buffer at pH 7.

THEORY

Assume that the emission photomultiplier (pm-5) responds with equal efficiency to radiation from all regions of the sample, that emitted light from only one molecular species, which shall be denoted by the superscript f, reaches the detector, and that there is no intermolecular energy transfer. The equations developed here are extended to include the effects of intermolecular energy transfer in the appendix. If F_L is the observed fluorescence intensity when the exciting light is left circularly polarized, F_R the corresponding quantity for right circularly polarized excitation, A_L and A_R the absorbances for left and right circularly polarized light, ϕ the probability that an absorbed photon gives rise to fluorescence (assumed independent of the polarization of the exciting beam and the wavelength of excitation) and I_0 is the intensity of the exciting light (also independent of polarization), then

$$F_L = k\phi I_0 \frac{A_L^f}{A_L}(1-10^{-A_L}) \quad [1a]$$

$$F_R = k\phi I_0 \frac{A_R^f}{A_R}(1-10^{-A_R}) \quad [1b]$$

where k is an instrumental parameter. It is useful to introduce the average and differential values of fluorescence and absorbance defined by

$$A = \frac{A_L + A_R}{2} \quad [2a]$$

$$\Delta A = A_L - A_R \quad [2b]$$

$$F = \frac{F_L + F_R}{2} \quad [2c]$$

$$\Delta F = F_L - F_R \quad [2d]$$

Combining Eqs. 1 and 2 and retaining no terms higher than first order in ΔA gives

$$\Delta F = \frac{(kI_0\phi A^f)}{A}\left\{\Delta A\, 10^{-A} \ln 10 + \left[\frac{\Delta A^f}{A^f} - \frac{\Delta A}{A}\right](1-10^{-A})\right\} \quad [3a]$$

$$F = \frac{kI_0\phi A^f}{A}(1-10^{-A}) \quad [3b]$$

Note that A and ΔA represent the net absorbance and circular dichroism of the sample (i.e., the sum of the absorbances and CD of all species) while A^f and ΔA^f are the absorbance and net CD of the fluorophore only. Turner et al. (4) have recently given equations equivalent to **3a** and **3b**.

By combining equations **3a** and **3b** it is easy to solve for ΔA^f as a function of ΔF, F, ΔA, A, and A^f, each of which can be determined experimentally. However, ΔA^f is independent of k, I_0, and ϕ.

Eq. **3a** gives the net differential fluorescence which is due to the sum of the natural and magnetic components. Both ΔA and ΔA^f can be written as the sum of natural and magnetic components (e.g., $\Delta A = \Delta A_n + H\Delta A_m$. For a discussion of the separation of natural and magnetic circular dichroism and the system of units used in the present experiments see Sutherland *et al.* (5). Since **3a** is linear in ΔA and ΔA^f, ΔF can also be written as

$$\Delta F = \Delta F_n + H\Delta F_m \quad [4]$$

In FDMCD, ΔF_m, the field-induced differential fluorescence, normalized by the strength of the magnetic field, is the parameter of interest. Eq. **4** presumes that ΔA is the only parameter on the right-hand side of Eqs. 3 affected by the magnetic field.

Eq. **3a** reveals that ΔF, the modulated fluorescence intensity, is due to two competing mechanisms. First, the fluoro-

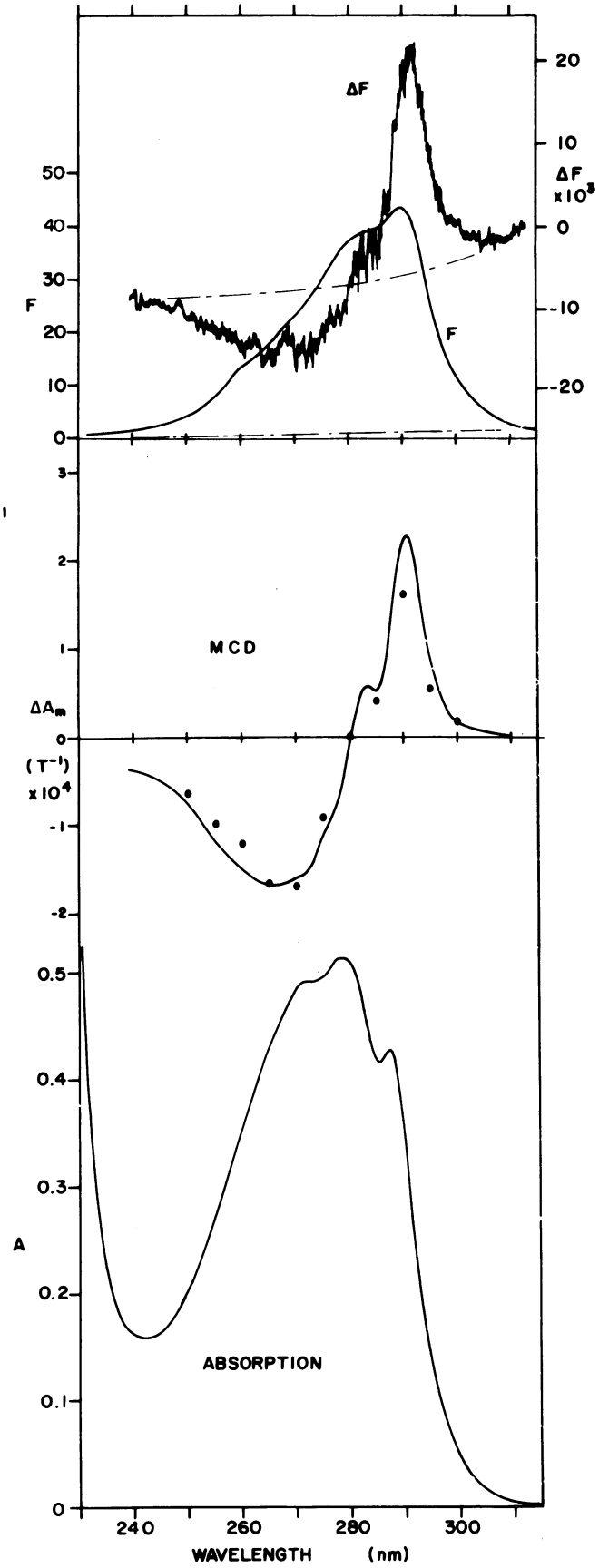

FIG. 2. The absorption, A, magnetic circular dichroism, ΔA_m, fluorescence, F, and differential fluorescence, ΔF, of a 90 μM solution of L-tryptophan. The FDMCD calculated from F, ΔF, A, and the strength of the magnetic field according to Eqs. 4 and 5 is shown as closed circles. The transmission-detected MCD is shown as a solid line. The ΔF spectrum measured with the field off (— - —) has been smoothed, but the field-on spectrum is shown as recorded to indicate the signal-to-noise ratio. The ΔF spectrum was recorded with a time-constant of 30 sec and a scan rate of 0.02 nm/sec. The spectral band width was 2.5 nm. The values of F and ΔF are plotted in units of mV.

phore can exhibit circular dichroism. The modulation of its fluorescence intensity follows directly from this differential absorption. We call this the "absorption modulated" contribution to ΔF. Second, the intensity of the exciting light bathing a fluorophore will be amplitude modulated due to the differential absorption of circularly polarized light by that portion of the sample between the fluorophore and the front of the cuvette. We describe this as "excitation modulation" of the fluorophore. The distinction between absorption and excitation modulation is illustrated by considering the limiting cases in which ΔF is due mainly or entirely to a single mechanism.

Case I, Absorption Modulation. The fluorescent species is the only absorber, i.e., $A^f = A$ and $\Delta A^f = \Delta A$.

Then

$$\Delta F = kI_0\phi\Delta A\, 10^{-A} \ln 10 \qquad [5a]$$

and

$$F = kI_0\phi(1 - 10^{-A}) \qquad [5b]$$

Solving for ΔA and using the definition of S given above yields

$$\Delta A = \frac{-S(1 - 10^{-A})}{10^{-A}} \qquad [6]$$

which is the expression given by Turner *et al.* (1) for this case.

The maximum value of ΔF, calculated by equating the derivative of ΔF with respect to the concentration of the fluorophore to zero, is obtained when A equals $(\ln 10)^{-1} = 0.434$. Absorption modulation dominates when A is low. However, excitation modulation reduces the magnitude of ΔF as A increases. When A becomes large, ΔF approaches zero.

Case II, Excitation Modulation. If the fluorophore does not exhibit observable circular dichroism, i.e., $\Delta A^f = 0$, Eqs. 3 become

$$\Delta F = \frac{kI_0\phi A^f \Delta A}{A}\left\{10^{-A} \ln 10 - \frac{(1 - 10^{-A})}{A}\right\} \qquad [7a]$$

and

$$F = \frac{kI_0\phi A^f}{A}(1 - 10^{-A}) \qquad [7b]$$

Solving for ΔA gives

$$\Delta A = \frac{S}{\left\{(A \ln 10)^{-1} - \left(\frac{10^{-A}}{(1-10^{-A})}\right)\right\}} \qquad [8]$$

The expression in braces in Eq. 7a is always negative for $A > 0$ and the corresponding expression in Eq. 8 is always positive. White *et al.* (6) have recently published equations equivalent to Eqs. 7 and 8 and other limiting cases.

Eq. 7a shows that the intensity of the fluorescent emission

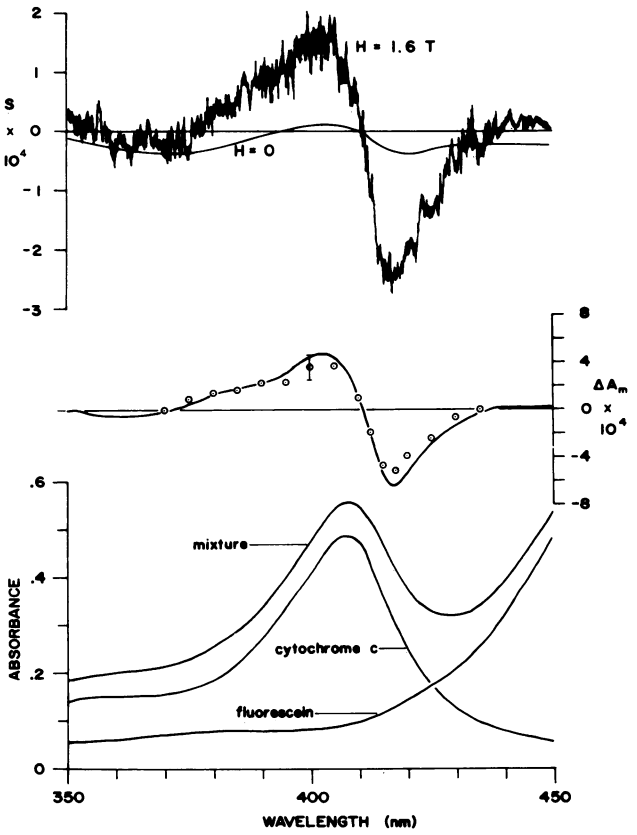

FIG. 3. The absorption, A, magnetic circular dichroism, ΔA_m, and fluorescence ratio spectrum, $S = -\Delta F/F \ln 10$, of a mixture containing fluorescein and 4.8×10^{-6} M Fe(III) cytochrome c. The S spectrum measured with the magnet off ($H = 0$) has been smoothed but the field-on spectrum is shown as recorded to indicate the signal-to-noise ratio. The bottom panel shows that the absorption spectrum of the mixture is the sum of the absorptions of the two components. The MCD of the mixture is characteristic of cytochrome c only [see Sutherland and Klein (8)], the MCD of fluorescein being negligible in comparison. All fluorescence was from the fluorescein, but the FDMCD (shown as the open circles) calculated from Eq. 8 agrees within experimental uncertainty with the transmission-detected MCD and is characteristic of cytochrome c.

of the fluorophore is modulated by the differential absorption of other molecules in the sample. Note that a positive value of ΔA yields a negative value of ΔF. Thus Case II can be described as "excitation modulation," since modulation of the fluorescence is due to the modulation of the exciting light reaching the fluorophore and not to differential absorption by the fluorophore. This is the phenomenon which permits the use of fluorescence to detect the circular dichroism of nonfluorescent molecules. The fluorescent and modulating molecules are assumed to be randomly distributed throughout the sample and not to interact directly.

RESULTS AND DISCUSSION

Tryptophan

The fluorescent amino-acid tryptophan has a strong MCD [Barth *et al.* (7)]. Using the direct or "open loop" method, we measured the fluorescence, F, and differential fluorescence of a solution containing 90 μM tryptophan. These spectra and the transmission-detected MCD and absorption spectrum of this sample are shown in Fig. 2. The field-induced differential fluorescence, $H\Delta F_m$, is the difference between the signal measured with the field on and off. Measurements of ΔF were extended to wavelengths which excited little or no fluorescence. This procedure, which is possible in the direct but not in the ratio mode of operation, is useful in determining the proper baseline.

Since tryptophan was the only absorbing species at the wavelengths of excitation, Case I applies. The FDMCD calculated from the experimental values of F and ΔF by means of Eqs. **5a** and **b** is plotted as the closed circles in Fig. 2. The figure shows good agreement between the FDMCD and the transmission-detected MCD.

Cytochrome *c*

Heme proteins such as hemoglobin, myoglobin, cytochromes, and peroxidases have large and distinctive MCD spectra [see e.g., Djerassi *et al.* (2)] which can be used as a means of detection and quantification. Fluorescence is often superior to absorption experiments in studying biological materials *in situ*. Unfortunately, the heme group is nonfluorescent. However, Case II discussed in the Theory section suggests a procedure for measuring the FDMCD of hemeproteins and other nonfluorescent molecules. A highly fluorescent molecule with little or no CD (natural or magnetic) of its own is mixed with the nonfluorescent molecule whose CD is to be studied. The emission intensity of the fluorophore is "excitation modulated" by the CD of the nonfluorescent molecule.

Results of a typical experiment are shown in Fig. 3. Oxidized cytochrome c was mixed with the fluorescent dye fluorescein. The net CD of the dye was negligible compared to that of the cytochrome c and the absorption spectrum of the mixture was the sum of the individual components indicating no interaction between the cytochrome c and the dye. In addition to the absorption spectrum of the mixture, Fig. 3 contains the transmission-detected MCD, the "S" spectrum (i.e., the differential fluorescence measured in ratio mode) and the values of FDMCD calculated by means of Eq. **8**. The MCD of the mixture is similar to the MCD of the isolated cytochrome c [see e.g., Sutherland and Klein (8)]. The S spectrum qualitatively resembles the MCD and the FDMCD agrees within experimental uncertainty with the transmission detected MCD. These data confirm the theoretical analysis leading to Eqs. **7** and **8**.

APPENDIX

Suppose that the ith molecular species transfers excitation energy to the fluorophore with probability ψ_i (which may be zero for some values of i). Then $A = A^f + \Sigma_i A_i$ and $\Delta A = \Delta A^f + \Sigma_i \Delta A_i$.

Proceeding as before gives:

$$\Delta F = \frac{kI_0\phi}{A}\left\{A^f\left[\Delta A\, 10^{-A}\ln 10 + \left(\frac{\Delta A^f}{A^f} - \frac{\Delta A}{A}\right)(1-10^{-A})\right] + \sum_i \psi_i A_i\left[\Delta A\, 10^{-A}\ln 10 + \left(\frac{\Delta A_i}{A_i} - \frac{\Delta A}{A}\right)(1-10^{-A})\right]\right\}$$

and

$$F = \frac{kI_0\phi}{A}(A^f + \sum_i \psi_i A_i)(1-10^{-A}).$$

Note that the sign of the energy transfer term in ΔF can depend on the anisotropy ratio, $\Delta A_i/A_i$, of the donor. Turner *et al.* (4) also generalized their expressions to include energy transfer.

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