Reports

Fluorescence Polarization: Measurement with Ultraviolet-Polarizing Filters in a Spectrophotofluorometer

Abstract. *Ultraviolet-polarizing filters with large apertures facilitate the use of spectrophotofluorometers for measuring fluorescence polarization. Such filters (i) allow the use of polarized exciting light even in the ultraviolet region, (ii) make it possible to correct for grating anomalies which influence the observed polarization, and (iii) because of good transmission properties allow the entrance of sufficient light so that small slits and dilute solutions can be used. Under optimum conditions, polarization can be measured with a precision of ±0.002. Results obtained are in excellent agreement with values cited elsewhere and indicate that standard spectrophotofluorometers can easily be adapted for accurate measurement of polarization.*

Fluorescence polarization is a useful analytical technique for studying the Brownian motion of molecules in solution (*1*) and for measuring the binding of coenzymes to proteins (*2*). In addition, measurement of fluorescence polarization of highly viscous solutions as a function of activating wavelength to obtain "polarization spectra" is valuable in determining the relative orientation of the electrical dipole oscillators responsible for absorption and emission (for example, *3*). A need for instrumentation to measure polarized emission therefore exists. Most workers in this field have either devised their own polarization fluorometers or have modified light-scattering photometers for this purpose (*4, 5*). Since the development of the spectrophotofluorometer in this laboratory some ten years ago (*6*) commercial instruments of this type have become popular, and it is surprising that only two studies (*2, 7*) of luminescence polarization have appeared in which commercially produced spectrophotofluorometers were employed.

The main problems in the use of such instruments for measuring polarization appear to be the depolarizing effects of the gratings (*8, 9*) and the lack of sensitivity due to the low efficiency of the birefringent crystals often used to polarize light. Recently, polarizing filters with large apertures for use in both the visible and ultraviolet regions have become available (*10*); we have found that these filters greatly facilitate the measurement of polarization with a commercially produced spectrophotofluorometer.

An Aminco-Bowman spectrophotofluorometer (*11*) was fitted with an Osram XBO-150 xenon arc lamp and an RCA 1P28 multiplier phototube. The photocurrent was amplified with an Aminco microphotometer and recorded on an X-Y recorder (*12*). The monochromators were calibrated with a mercury arc. Samples were held in a microcuvette (*11*) having an internal cross-sectional area 2.9 by 2.9 mm and a capacity of 0.2 ml (*13*). Copper tubing was soldered to the brass cell holder to allow circulation of water from a thermostat-controlled bath. In the activating beam, quartz Polacoat (*14*) polarizing filters, formula 105 UV (ultraviolet), were employed; these polarizers have been described by McDermott and Novick (*10*). Transmission of our filters was 29 percent at 370 mμ and 13 percent at 225 mμ, and dichroism was good from 225 mμ to over 600 mμ, with transmission of two crossed filters reaching a maximum of about 0.5 percent at 350 mμ. Bleaching was not a problem in filters used over a period of 8 months. The emitted light was analyzed with type HNP'B Polaroid film (*15*) which was less expensive than the Polacoat filters, but could not be used in the exciting beam because of bleaching (*10*). The Polaroid film has useful transmission down to about 275 mμ (*16*). Figure 1 shows viewing system and the positions of the polarizers and analyzers.

With the incident beam vertically polarized, polarization (P) should ideally be given by

$$P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the analyzers vertically and horizontally oriented. However, the emission must pass through a monochromator whose grating imposes a degree of polarization. Thus, a grating correction factor, G, must be introduced. We obtained G by the method of Azumi and McGlynn (*7*): with the sample excited with *horizontally* polarized light, emission was analyzed as usual to yield I_{HV} and I_{HH}. The ratio of I_{HV} to I_{HH} should be 1 since fluorescence is observed along the direction of the electric vector of the activating light. In practice, however, the ratio is found to vary from about 0.75 to 1 depending on the wavelength setting of the emission monochromator. It should be noted that since I_{HV}/I_{HH} is usually less than 1, and since the

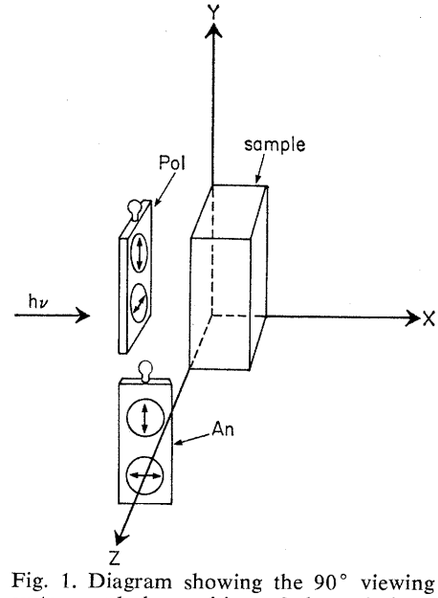
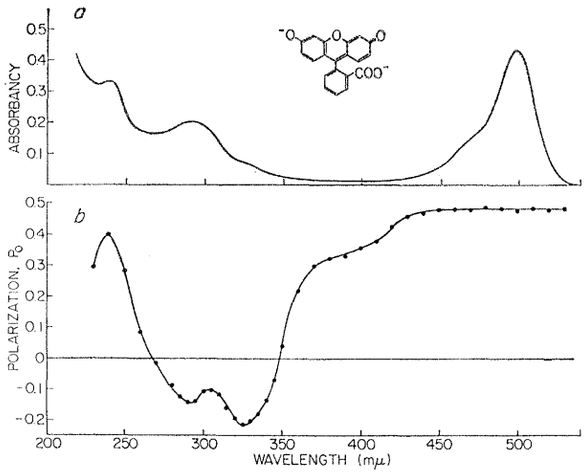


Fig. 1. Diagram showing the 90° viewing system and the position of the polarizers (*Pol*) and analyzers (*An*). Light (*hν*) travels along the *X*-axis through one of two Polacoat discs oriented horizontally and vertically and mounted in a sliding filter-holder. Emission is observed at 90° along the *Z*-axis and is analyzed with Polaroid discs similarly mounted in another holder. The direction in which light is vertically polarized is shown by *Y*.



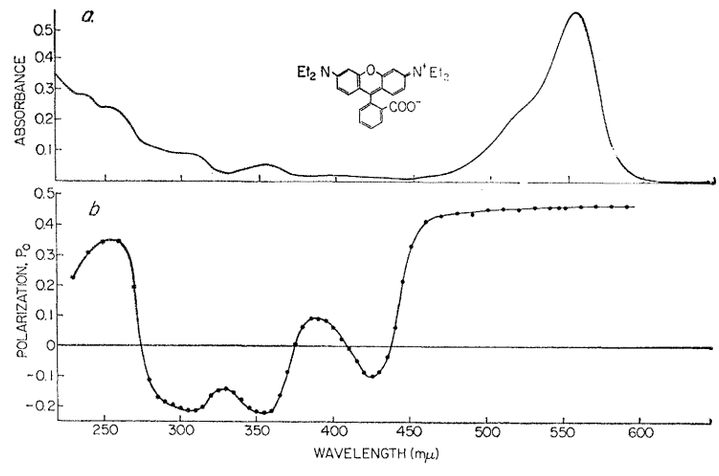


Fig. 2 (left). Fluorescein, $5 \times 10^{-6}M$ in glycerol, 95 percent and 0.10N NaOH, 5 percent (by volume), at 10°C. *a*, Absorption spectrum. *b*, Polarization spectrum. Emission observed at 516 mμ; grating factor, $G = 0.940$. Fig. 3 (right). Rhodamine B, $5 \times 10^{-6}M$ in glycerol, 95 percent, and water, 5 percent (by volume), at 10°C. *a*, Absorption spectrum. *b*, Polarization spectrum. Emission observed at 573 mμ; grating factor, $G = 0.852$.

gratings are ruled vertically, the grating effects may be classified as "parallel diffraction anomalies" (*9*). With $G = I_{HV}/I_{HH}$, the polarization was then obtained from the formula

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

The correction factor G needs to be determined only once for each series of measurements at a given setting for the emission wavelength, but G also depends slightly on the width of the slit used for emission (since the bandwidth of the emission is dependent on the size of the slit). This method of obtaining G accounts for any anisotropy introduced by the glass of the phototube or the walls of the cuvette and is performed with the same slits as used for measuring I_{VV} and I_{VH}. It should be noted that neither the two Polacoat discs nor the two Polaroid discs need have identical transmission properties.

The fluorescence polarization spectra and absorption spectra of 95 percent (by volume) glycerol solutions of fluorescein and rhodamine B are shown in Figs. 2 and 3. The polarizations are expressed as P_0—that is, P where rotational depolarization is negligible. The polarizations were calculated from the photometer readings corresponding to I_{VV} and I_{VH} after manually setting the activating monochromator. Emission was observed through a Corning 3-73 filter to eliminate scatter below 400 mμ, and wavelengths were sorted with a Corning 7-54 filter when activating light of short wavelength was employed. Small but significant corrections were made for readings from a solvent blank. The polarization spectrum of fluorescein shown in Fig. 2*b* shows minima at 290 mμ and 325 mμ, a maximum in the negative region at 305 mμ, and a broad shoulder around 370 mμ. In the long wavelength region, the polarization reaches a value of 0.477. Recently, Szalay *et al.* (*17*) reviewed published data on the polarization of fluorescein emission and calculated that P_0 for the long wavelength region should be 0.4922 ± 0.0050 in basic glycerol solutions if corrections are included for depolarization due to secondary fluorescence (this, in turn, being due to reabsorption of emitted light) and residual Brownian motion. The agreement of our value with the most reliable estimates must be considered good, since a slight amount of depolarization due to secondary fluorescence might still occur under our conditions.

The intricate polarization spectrum of rhodamine B (Fig. 3*b*) agrees in form with that published by Feofilov (*18*). The polarization reaches a maximum of 0.462 at long wavelengths, in agreement with Singleterry and Weinberger (*4*), who employed a dye purified by chromatography on alumina columns. Our samples of rhodamine B and fluorescein (*19*) were recrystallized from ether-ethanol mixtures and reprecipitated from ethanol by addition of water. The effect of any remaining impurities on the polarization of fluorescence was minimized in our instrumentation by the presence of the emission monochromator which was set at the peak of the dye fluorescence. In fact, since it is known that the polarization of a pure substance should be independent of the emission frequency (*20*), examination of the polarization of the emission band may be used as one criterion of purity. We found with our instrumentation that the polarization of the emission bands for glycerol solutions of rhodamine B and fluorescein were essentially constant.

The precision of the polarization measurements depends on the signal-to-noise ratio. For substances with high quantum efficiency, P can be measured

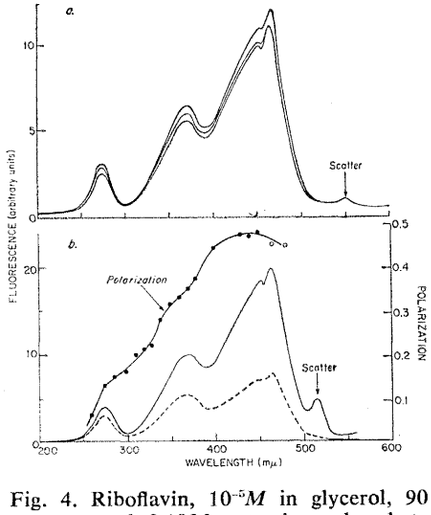


Fig. 4. Riboflavin, $10^{-5}M$ in glycerol, 90 percent, and 0.10M potassium phosphate buffer, 10 percent, pH 7.0, at 8°C. *a*, Repeated tracings of I_{VV}; 20 mμ excitation bandwidth, emission at 550 mμ. *b*, I_{VV} (——) and I_{VH} (– – –) obtained as in *a*, but with attenuating screen in place. All polarization values were obtained from these curves with emission observed at 515 mμ ($G = 0.940$) except for two points (open circles) made from separate readings with emission at 550 mμ ($G = 0.900$). The ordinate scales of *a* and *b* are not comparable.

to about ±0.002, even with 1/64-inch (0.04-cm) slits in the activating beam to yield a nominal bandwidth of 5 mμ. The present system was sufficiently sensitive to record the small polarizations of xanthydrol dyes in aqueous solutions at room temperature (23°C). The emission at 526 mμ of $1 \times 10^{-5}M$ fluorescein in 0.10*M* potassium phosphate buffer, *p*H 7.0, activated by 436 mμ light (5 mμ bandwidth) yielded photometer readings for I_{VV} and I_{VH} of 89.5 ± 0.2 and 94.0 ± 0.2. Since *G* was found to be 0.915, *P* was given by (3.5 ± 0.4)/(175.5 ± 0.4) = 0.020 ± 0.002, in agreement with Weber's data (*21*).

Since the polarizers with large apertures allowed polarized excitation to be used even at 230 mμ, a given absolute error in *P* results in a smaller percentage error than would be obtained if unpolarized excitation were used. The absolute value of *P* is always greater when polarized light is employed ($-\frac{1}{3} \leq P \leq +\frac{1}{2}$) instead of natural light ($-1/7 \leq P \leq +\frac{1}{3}$) (see *1*). Because of the good transmission properties of the Polacoat filters, the spectra in Figs. 2*b* and 3*b* were obtained with a bandwidth of 5 mμ throughout the ultraviolet and visible regions. Polarization spectra of these dyes at low concentrations ($5 \times 10^{-6}M$) have not, to our knowledge, been obtained previously by polarized excitation with this degree of resolution and precision.

The spectra in Figs. 2*b* and 3*b* were obtained by taking readings of I_{VV} and I_{VH} directly from the photometer at each wavelength setting. Similar spectra have been obtained by recording I_{VV} and I_{VH} continuously and subsequently calculating *P* from the tracings; but little time is saved by doing it in this manner, and the precision of the measurements is considerably less. This method of recording I_{VV} and I_{VH} rapidly as a function of wavelength is particularly useful for samples which are light-sensitive and cannot be exposed for long periods of time. For example, riboflavin is rapidly decomposed by exposure to light; Fig. 4*a* shows that repeated tracings of I_{VV} decreased in amplitude even though the intensity of the light was reduced some 70 to 87 percent by the Polacoat filter. By placing a 30-mesh blackened copper screen in front of the second mirror of the activating monochromator, the intensity of the light was reduced 10-fold more, so that repeated tracings of I_{VV} and I_{VH} were reproducible to within 2 percent. The polarization spectrum is shown in Fig. 4*b*. The apparent peak in the activation spectrum of riboflavin at 470 mμ is due to a sharp increase in energy output in the xenon-arc continuum at this wavelength, as was verified by calibrating the excitation system according to the method of Argauer and White (*22*). Calibration for variation in excitation energy with wavelength is not necessary for polarization measurements, however.

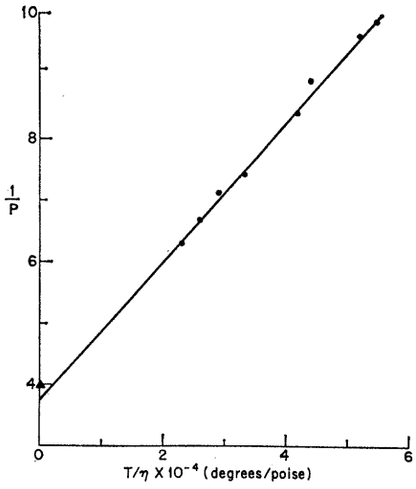


Fig. 5. Perrin plot for fluorescence polarization of β-anthryl conjugate of bovine serum albumin having 4.7 anthryl residues per mole of protein. Protein concentration, 0.12 mg/ml; 0.10*M* potassium phosphate buffer, *p*H 7.0. Activation at 380 mμ (5 mμ bandwidth); emission at 416 mμ (10 mμ bandwidth) where *G* was 0.825. The value of $1/P$ at $T/\eta \approx 0$ was obtained with protein dissolved in 90 percent glycerol at 8°C. The other points were obtained by raising the temperature from 11.8° to 46°C.

Our instrumentation is applicable to studies of rotational motion by means of fluorescence polarization (*1*). When a dye is attached firmly to a macromolecule, the mean harmonic rotational relaxation time, ρ_h, may be calculated if one knows the lifetime of the excited state of the fluorophor, τ, by means of the modified Perrin-Levshin equation (*23*):

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right)$$

Here, P_o is the polarization at infinite viscosity, and *P* is the observed polarization under the same conditions for which ρ_h is measured. P_o is conveniently obtained by plotting $1/P$ against T/η, the ratio of the absolute temperature to the viscosity, and extrapolating to infinite viscosity. We have repeated the experiment of Harrington *et al.* (*24*) who studied the rotational relaxation time of bovine serum albumin by labeling it with β-anthryl isocyanate. β-Anthryl isocyanate was prepared and reacted with crystalline bovine serum albumin (*25*) as described by Laurence (*26*), and the unreacted fluorescent material removed with charcoal (*27*). The results shown in Fig. 5 gave P_o = 0.267 and the rotational relaxation time at 25°C was calculated from the value of $\tau = 4.4 \times 10^{-8}$ second (*24*). The value of $\rho_h = 1.20 \times 10^{-7}$ second at 25°C is in close agreement with that found by Harrington *et al.* (*24*) who employed a polariscopic measurement system and unpolarized exciting light from a mercury arc lamp. Visual methods of measuring fluorescence polarization, however, are inherently less precise than photoelectric means (*21*), and the sensitivity of the measurements by Harrington *et al.* (*24*) must have been relatively low since a high P_o value was not obtained. According to their data, all experimentally measured points for *P* fell between 0.035 and 0.077. In our work, the combination of polarized excitation and selection of excitation at 380 mμ (where no strong mercury line is available) resulted in a high P_o value, and the data in Fig. 5 consist of *P* values ranging from 0.100 to 0.250.

Azumi and McGlynn (*7*) employed an Aminco-Bowman spectrophotofluorometer fitted with Glan prisms for measurement of phenanthrene luminescence polarization at low temperatures. In comparison to the Glan prisms available to us, the polarizing filters were much less expensive, had better transmission, and were less limited in their viewing angle. Glan prisms have also been noted to deviate the beam of light, thus causing an apparent shift of wavelength in the spectrophotofluorometer (*7*), but this effect is absent with polarizing filters. Selected prisms, however, might prove satisfactory.

We have neglected the fact that in our system, the exciting and emitted light beams are not strictly collimated. Light is focused by means of two concave mirrors of 250-mm focal length in each monochromator, so that there is a 1-meter light path for each side. Masking of the second mirror of the activating monochromator should have decreased the already small convergence angle; but when this was done, no significant effect on the fluorescence polarization was observed.

Our optical arrangement could also be used in a spectrophotofluorometer with a phosphorescence attachment to yield information on the polarization of

triplet state transitions. Since spectrophotofluorometers are now in wide use and can so easily be adapted to the measurement of fluorescence polarization, this technique should now be readily available to many laboratories.

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Pressure Dependence of the Alpha-Beta Transition Temperature in Silver Selenide

Abstract. *The pressure dependence of the α-β transition temperature in Ag_2Se was determined by observing the temperature at which the sharp change in resistivity occurs when Ag_2Se is transformed from the low-temperature orthorhombic to the high temperature body-centered-cubic form. The transition temperature increased from 133°C at 1 atmosphere to 298°C at 47 kilobars. The value of ΔH_t, the heat of transformation, of 2.19 kcal/mol measured calorimetrically agreed well with the value calculated from dT/dP of the transition.*

The silver and copper chalcogenides have well-characterized (*1*, *2*) polymorphic transitions to cubic structures at elevated temperatures. A sharp change in resistivity occurs when Ag_2Se is transformed from the low-temperature orthorhombic form (*3*, *4*) (α-Ag_2Se) (*5*) to the high-temperature body-centered-cubic form (β-Ag_2Se). Under atmospheric pressure, this transition occurs at about 133°C. Pressure dependence of the transition temperature up to 47 kb was determined by resistance measurements. The samples were polycrystalline cylinders and rectangular parallelepipeds cut from an ingot prepared by the horizontal Bridgman technique and then cooled slowly through the transition region. The sample had carrier concentrations of about 2×10^{18} cm^{-3} at 77°K. Measurements of resistivity versus temperature were made at atmospheric pressure by a four-lead method. At higher pressures, measurements were made in the tetrahedral-anvil press by a two-lead method previously used to investigate the P-T diagram of InSb (*6*). The high pressure cells were calibrated with the Bi I-II (25.4 kb) and the Tl I-II (37 kb) transitions (*7*). The four-lead method was also used to measure resistance at room temperature as a function of pressure to 40 kb. No appreciable change in resistance was observed. X-ray powder patterns taken at more than 45 kb also showed no phase change at room temperature.

Typical data for relative resistance versus increasing temperature are given in Fig. 1 for atmospheric pressure and 47 kb. At all pressures the resistance increased sharply over a range of 10° to 20°C when the samples were heated through the transition point (Fig. 1). Baer *et al.* (*8*) also observed an increase in resistivity on heating a sample which had been converted to the low-temperature form by cooling from a temperature a little above transition. This treatment is similar to those used in preparation and measurement of our samples. In experiments on samples

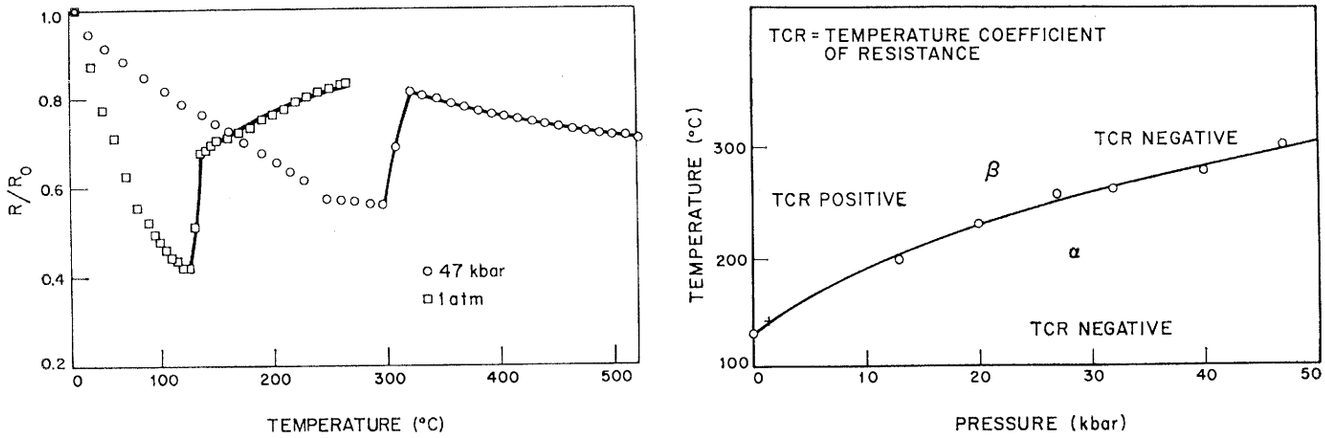

Fig. 1 (left). Relative resistance (R/R_0) versus temperature for Ag_2Se at atmospheric pressure and at 47 kb. At atmospheric pressure, resistivity in ohm-cm is equal to $(1.02 \times 10^{-3}) \times (R/R_0)$. Fig. 2 (right). Temperature of α-β transition in Ag_2Se versus pressure. Cross indicates data of Roy *et al.* at 1115 atm (*9*).