Fluorescence Polarization Measured with a New Simple Instrument

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Polarization of fluorescence was developed into a useful tool in biochemistry by G. Weber some twenty years ago.[1,2] Several instruments of varying complexity have been constructed, and polarization of fluorescence has been successfully applied in a wide range of fields. Still, however, there is only a very limited number of laboratories throughout the world, which have the proper equipment. There are two reasons for this: those instruments which have been described in the literature are usually too complicated to be copied by biochemists, and commercially available fluorometers equipped with polarization adaptors do not yield accurate results and they usually suffer from lack of thermostat facilities.

As discussed here, the polarization, p, is defined as

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are, respectively, the vertically and horizontally vibrating components of the light emitted at right angle to the incident beam of vertically polarized, exciting light.

(For an explanation of the physico-mathematical principles of polarization the reader is referred to Pesce *et al.*[3]) $I_{\parallel}$ and $I_{\perp}$ may be measured separately and consecutively and p calculated according to eqn. (1), but it is realized that errors involved in such measurements will be magnified by the application of this equation. Experience shows that the only principle which gives a sufficient degree of accuracy is that one introduced by Weber,[4] which employs separate polarizers and photomultipliers for the isolation of $I_{\parallel}$ and $I_{\perp}$. These two light components may be compared by means of an optical compensation method,[4] or by the application of electronic operations of varying degrees of sophistication all the way to the complete calculation of p.[5-8] The essential step in reducing the instrument error is the introduction of a device which gives the ratio of $I_{\perp}$ and $I_{\parallel}$. Recent developments in electronic components make it possible to build at a very modest price an instrument which performs this operation. The inexpensive analog divider employed in the instrument described below makes it possible to display the ratio of $I_{\perp}$ and $I_{\parallel}$ on a simple voltmeter. Polarization is then obtained as

$$p = \frac{1 - I_{\perp}/I_{\parallel}}{1 + I_{\perp}/I_{\parallel}} \qquad (2)$$

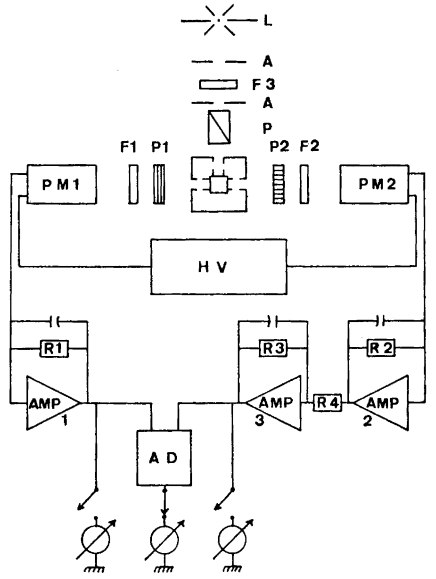

Fig. 1. Schematic plan of polarization fluorometer. L, 100 W mercury lamp (Gates MLA-100); A, apertures; F1, F2, F3, broadband filters; P, Glan polarizer (air type); P1, horizontally oriented polarizing filter; P2, vertically oriented polarizing filter; PM1 and PM2, photomultiplier tubes (EMI 9524S); HV, 1000 V supply; R1, R2, R3, variable feedback resistors; R4, fix input resistor; AMP, operational amplifiers (1 and 2=Philbrick/Nexus 1009, 3=Philbrick/Nexus Sq−10a); AD, analog divider (Intronics D401).

Design of the instrument. The principal features of the instrument are presented in Fig. 1. The Glan prism may be turned through exactly 90° to permit either vertically or horizontally

polarized light to enter the sample cuvette, which is contained in a water-jacketed housing, at the bottom of which is a magnetic stirrer (not shown in Fig. 1). Tunnels through the cuvette housing permit light to exit in the horizontal plane at $\pm 90°$ to the exciting beam. The emitted light passes in one direction through a horizontally oriented filter polarizer and in the other direction through a vertically oriented filter. Selection of wavelengths for both exciting and emitted light is effected by suitable filters. For most dyes employed in biochemical studies isolation of the mercury 365 nm line by means of a broadband filter provides for adequate exciting light. Both photomultiplier tubes are fed by the same power supply containing ten 100 V batteries. This arrangement together with other features in the construction makes the instrument reliable and accurate even in laboratories with less stable line voltage.

The electronics of the instrument is all composed of compact, inexpensive solid state components. Operational amplifiers convert the weak (10^{-9}—10^{-6} A) high-impedance signals into signals fitting the analog divider, which operates in the ± 10 V range. These amplifiers were selected for their extraordinary input impedance ($>10^{12}$ Ω). The feedback resistors (R1 and R2) are stepwise variable between 1 and 100 MΩ. This way the analog divider may always be fed with optimal signals of a few volts. The second amplifier in one arm simply serves as a voltage inverter, since the divider gives the value of $-10X/Y$, where Y must be negative. The feedback resistor of this amplifier (R3) is continuously variable (10—50 kΩ) to allow for accurate matching of the photomultiplier responses during calibration. The three volt meters (10 V) on the instrument panel display values proportional to $I_{\perp}$, $I_{\parallel}$, and $I_{\perp}/I_{\parallel}$, respectively.

The analog divider has built-in calibration circuitry, and switches are provided on the instrument to make possible the calibration with X/X or X/Y signals. After proper calibration the full-scale linearity remains better than 1 % for months. Prior to polarization measurements the photomultiplier responses are matched in the following way. With a fluorescent solution in the cuvette the Glan prism is turned so as to transmit horizontally polarized light, the signals are attenuated to ~2 volts, and the ratio volt meter, which now registers $I_{\perp}/I_{\parallel}$, is then adjusted to read exactly 10 V.

Test of performance. The accuracy of absolute determinations of p was checked by measurements of identical samples in the new instrument and in the Weber [4] instrument. A 10^{-5} M solution of 1-anilino-8-naphthalenesulfonate in propylene glycol was placed in the cuvette, and polarization of the fluorescence was measured while viscosity was varied by a variation of the temperature. As predicted by theory, a straight line, which coincided very well with the one previously determined with the Weber instrument,[9] was obtained when $1/p$ was plotted *versus* T/η (Fig. 2).

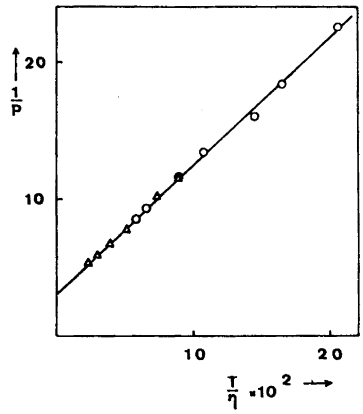

Fig. 2. Reciprocal of polarization, $1/p$, as a function of temperature and viscosity, T/η, for 1-anilino-8-naphthalenesulfonate in propylene glycol. △, values obtained with new instrument; ○, values obtained with the Weber fluorometer.

Applications in protein chemistry. Our instrument was constructed with the main purpose of facilitating the study of structural alterations in irradiated proteins. Such alterations may be of several fundamentally different kinds, and there is no one technique available for the study of all such changes. Fluorescence polarization comes in here as a useful complement to analytical ultracentrifugation and gel filtration, optical rotatory dispersion and light-scattering.

Bovine serum albumin undergoes a partial dissociation into subunits in the pH interval 4—2, and at pH values below about 3 the molecule is assumed to be composed of a small number of roughly spherical subunits, which are linked by flexible peptide chains (*cf.* Weber and Young [10]). If fluorescent groups with sufficient lifetime of the excited state are

attached to albumin, this partial dissociation may be observed as a decrease of the fluorescence polarization due to the shorter rotational relaxation times of subunits (Fig. 3, *cf.* Refs. 2 and 10). Radiation-induced structural changes will be detectable as deviations from the normal titration curve provided the reproducibility of values obtained with the instrument is satisfactory. Measurements such as those summarized in Fig. 3 (*cf.* Weber,[2] Weber and Young [11]) show that our instrument well satisfies such requirements.

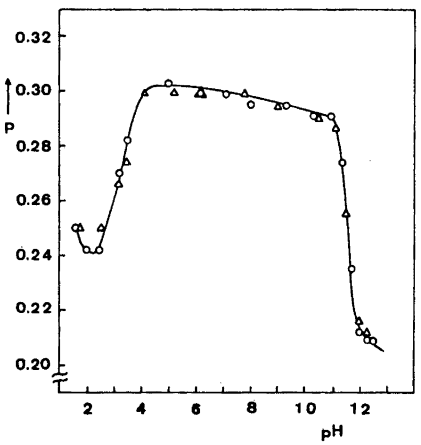

Fig. 3. Polarization of fluorescence as a function of pH for 1,5-dimethylaminonaphthalenesulfonate conjugated with bovine serum albumin. Different symbols signify measurements on different occasions of identical samples.

We have made a series of experiments, in which solid bovine serum albumin has been γ-irradiated and then fractionated on Sephadex G-100. One diffficulty in the evaluation of the yields of such changes as cross-linking and fragmentation by means of gel filtration or ultracentrifugation techniques is that such separations are disturbed by increases of the cross-sections of the molecules due not only to intermolecular cross-links but also to unfolding of the peptide chains. Fluorescence polarization measurements will among other things distinguish between such phenomena. Experiments we have made show that a clearer picture of radiation effects in proteins is obtainable if a separation method like gel filtration is complemented with polarization measurements. The results of those investigations will be published elsewhere.

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3-Exoamino-2-endohydroxybornane

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Preparations of three out of four 3-amino-2-hydroxybornane isomers have recently been described in two independent publications.[1,2] However, attempts to prepare the title compound (*2*) were reported to be unsuccessful.