

unique direction determined by the transition moment of the excited molecule (see Fig. 1). The transition moment of a molecule, which is determined by its electronic distribution, is the direction in which the molecule most easily absorbs light. Therefore, molecules absorb and emit light along preferred linear directions within their molecular framework; i.e., light is preferentially absorbed when the electric vector of the exciting light is parallel to the transition moment of the molecule (Fig. 1A). Fluorescence emission is exactly analogous to absorption; i.e., the electric vector of the emitted light will be parallel to the transition moment of the excited molecule (18, 32). Note that the transition moment may or may not be the same for absorption and emission, however, certain assumptions remedy this dilemma. Assuming that (a) the absorption and emission directions are coincident (true for long wavelength excitation), and (b) absorption and emission only occur when the electric vector of the light is parallel to the absorption and emission direction (oversimplified, but corrected for in a more rigorous treatment) the following model (Fig. 1C) and discussion demonstrate the essential features of fluorescence polarization.

When a molecule is excited with polarized light, the electric vector of the light emitted will be parallel to the electric vector of the exciting beam if two conditions are fulfilled. First, the molecule should be excited only to its lowest excited state (and hence, the excitation wavelength is chosen to be the longest absorption band of the fluorophore). Second, the molecule must remain stationary during the lifetime of the excited state. The second condition above is the basis of the fluorescence polarization technique, i.e., rotation of a molecule during its excited state will depolarize emitted light with respect to the exciting beam. The fluorescent lifetime of the molecule determines the degree of depolarization observed. For example, if two molecules with equal rotation rates are labeled with probes with short and long fluorescence lifetimes, the molecule with the long fluorescence lifetime will display a greater depolarization of emitted light since it can rotate more during its extended life-

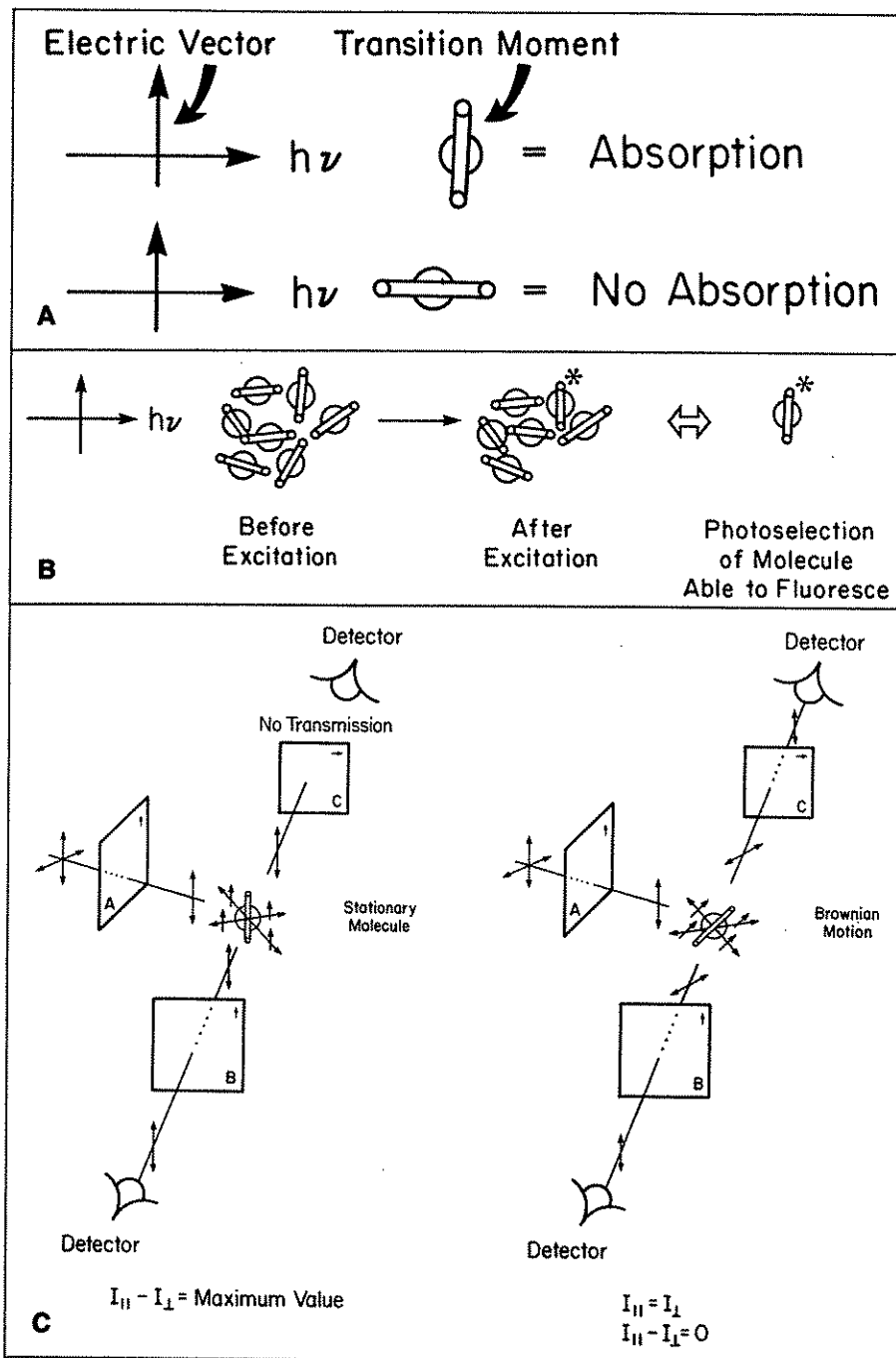


Figure 1. Molecular basis of fluorescence polarization. **A.** Light absorption by a molecule occurs optimally when the electric vector of exciting light is parallel to the transition moment of a molecule; in contrast, no light is absorbed when the electric vector and transition moment are perpendicular. [Note that molecules whose transition moments are not perfectly parallel to the electric vector of light can absorb light (with a probability which decreases as the transition moment departs from a parallel orientation).] It is important to note that the electric vector of emitted light will be parallel to the transition moment of the excited molecule (under conditions described in the text). **B.** Polarized light photoselects a subpopulation of molecules whose transition moments are parallel to the electric vector of incident light. Most randomly oriented molecules fail to absorb light and are unable to fluoresce. **C.** When a molecule is excited to its lowest excited state the absorption and emission transition moments will be parallel. If the molecule remains stationary during the excited state, light will be transmitted by polarizer B (which is oriented in parallel with the excitation polarizer) but not transmitted by the perpendicularly oriented polarizer C. If rapid rotational Brownian motion occurs during the excited state, the electric vector of emitted light will be oriented randomly which will result in nearly equal amounts of light traversing polarizers B and C (hence the term " $I_{\parallel} - I_{\perp}$ " will approach zero).

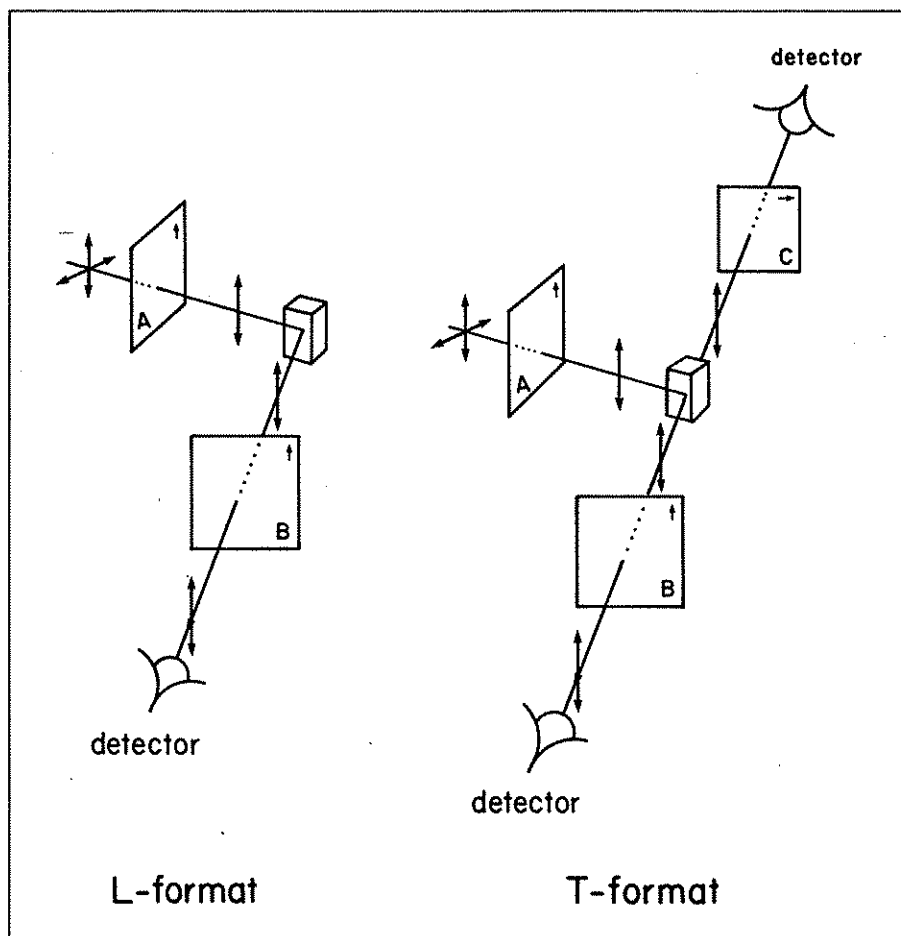


Figure 2. Diagram of L-format (panel A) and T-format (panel B) spectrofluorometers. In the T-format, the four intensity readings required (vv, vh, hh, hv) can be obtained by one rotation of polarizer A.

time in the excited state.

As indicated above, the molecular basis of fluorescence polarization depends on the orientation of the transition moment of a molecule with respect to the electric vector of light. Thus, when light excites a population of molecules, a group of molecules are photoselected whose transition moments are non-random; i.e., the photo-selected molecules have a transition moment that is parallel or near-parallel to the electric vector of light. Molecules whose transition moments are oriented in other directions are not excited (Fig. 1B).

In studies carried out between 1929 and 1936, Perrin (50) derived an equation, to be presented later, which utilizes (a) the concepts presented in the 1906 Einstein equation (equation 1), and (b) the two phenomena, presented above,

involved in the fluorescent emission of light. The basic principles involved in the experimental measurement of fluorescence polarization are presented diagrammatically in Fig. 1C. As indicated in Fig. 1C, the fact that emitted light is polarized in the same direction as the exciting beam will result in more light being transmitted by polarizer B if the molecule is stationary. In contrast, if the excited molecule is free to move randomly under the rules governing rotational Brownian motion (equation 1), light received by polarizer B will be depolarized and, hence, less light will be received by the detector (Fig. 1C). Thus, as the molecular motion in a system increases, emitted light will be increasingly depolarized and, hence, one can estimate the rotational relaxation time of a molecule by measuring the depolarization of its fluorescent signal.

MEASUREMENT OF FLUORESCENCE POLARIZATION VALUES

Fluorescence polarization values (P) are measured by (a) exciting a molecule with polarized light, and (b) observing the intensity (I) of emitted light passing through a polarizer held parallel (||) or perpendicular (⊥) to the direction of the electric vector to the polarized excitation (Fig. 1C). Polarization values (P) are computed as indicated in equation 2:

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}} \quad (\text{eq. 2})$$

where $I_{||}$ is the intensity of light when polarizers A and B in Fig. 1C are in a parallel orientation, and $I_{\perp}$ is the intensity when polarizers A and B are in a perpendicular orientation (39, 74). As indicated in Fig. 1C, the term " $I_{||} - I_{\perp}$ " in eq. 2 will approach zero if no molecular motion occurs. Hence, polarization values (P) reflect the degree to which the photoselected population of excited molecules becomes randomized during the excited state.

In practice, polarization measurements are made by taking fluorescence intensity readings with the excitation and emission polarizers in combinations of vertical (v) and horizontal (h) positions. These combinations are vv, vh, hh, and hv, where the order of the letters represents the positions of the excitation and emission polarizers, respectively. In equation 2, $I_{||}$ and $I_{\perp}$ correspond to intensity readings taken in the vv and vh laboratory settings, respectively. The hh and hv readings are used to correct for optical artifacts introduced by the instrument. Thus, in actual practice, polarization values are calculated from equation 3:

$$P = \frac{I_{vv} - I_{vh} (I_{hv}/I_{hh})}{I_{vv} + I_{vh} (I_{hv}/I_{hh})} \quad (\text{eq. 3})$$

Depending on the requirements of the system studied, fluorescence polarization can be performed with virtually any commercially available spectrofluorometer by simply adding rotatable polarization filters to the excitation and emission ports. Fluorescence polarization adaptors are available from the manufacturers of spectrofluorometers. While many types of

polarizers are available, one should be aware that, while inexpensive, film polarizers are unacceptable for fluorescence polarization measurements at wavelengths shorter than 350 nm. Spectrofluorometers used for fluorescence polarization measurements can be designed in either an L- or T-format. While the L-format uses only one detector, the T-format employs two detectors (Fig. 2). In the case of the L-format spectrofluorometer, one must manipulate the polarizers to produce all four intensity readings in equation 3, while with the T-format instrument only two manipulations of the polarizers are required; i.e., v and h settings at the excitation port. In our laboratory we have simplified data acquisition by interfacing a personal computer to the signal processing unit of the spectrofluorometer via a data acquisition unit.

Polarization data can be expressed as polarization (P) values (eq. 2) or as anisotropy (r) values (eq. 4):

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (\text{eq. 4})$$

(Note the similarity between equations 2 and 4; anisotropy values can be converted into polarization values via the relationship $r = 2P/3 - P$). Analysis of data based on polarization (P) values can be unwieldy because polarization values are not simply additive; in contrast, expression of data as anisotropy values expedites data analysis since anisotropy values are additive. For example, if a solution contained 50% free ligand ($r = 0$) and 50% bound ligand ($r = 0.4$) and assuming that the bound and free ligand were equally fluorescent, the observed net anisotropy would be 0.2. Due to the additive nature of anisotropy values there has been a trend in the recent literature to use anisotropy rather than polarization values. Nevertheless, the technique itself is commonly referred to as fluorescence polarization even though anisotropy values are derived from its application.

Since the basic phenomenon involved in fluorescence polarization involves the depolarization of the fluorescence emission signal as a consequence of molecular motion, it should not be surprising that experimental conditions which affect molecular motion also affect the

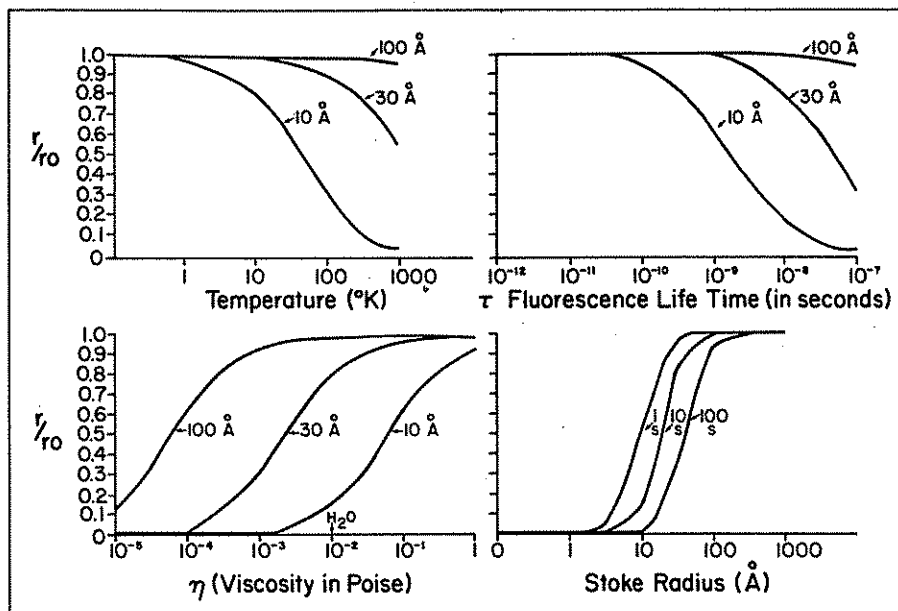


Figure 3. Theoretical anisotropy values derived from the Perrin-Weber equation. Values of r/r_0 were calculated from the Perrin-Weber (eq. 6) equation by varying each of the experimentally manipulatable terms individually. The Perrin-Weber equation was reformulated as $r/r_0 = 1/(1 + RT\tau/\eta V)$. The values for experimental parameters were set at values usually encountered in practice; i.e., $T = 293^\circ\text{K}$ (20°C); $\eta = 0.01$ poise (the viscosity of water); $\tau = 10$ nanoseconds; $R = 8.3 \times 10^7$ ergs mole $^{-1}$ $^\circ\text{K}^{-1}$. For the experimental parameters T , η and τ , the Stokes radius was set at either 10, 30, or 100 Å; in the case of calculations involving varying volume, lifetimes of 1, 10, and 100 nanoseconds were chosen.

derived values of P and r . The influence of experimental parameters on anisotropy is summarized by the Perrin-Weber equation (eq. 5) (38, 50, 73, 74):

$$1/r = 1/r_0 (1 + RT\tau/\eta V) \quad (\text{eq. 5})$$

In the literature, the Perrin-Weber equation is expressed in several alternative forms. In the case of polarization values, the Perrin-Weber equation becomes $(1/P - 1/3) = (1/P_0 - 1/3) (1 + RT\tau/\eta V)$ (50, 73, 74). In the Perrin-Weber equation (eq. 5 above), r_0 is the anisotropy in the absence of molecular motion, R is the gas constant which is equal to 8.3×10^7 ergs mole $^{-1}$ $^\circ\text{K}^{-1}$, T is temperature in $^\circ\text{K}$, τ is the fluorescence lifetime in sec, η is the viscosity of the solution in poise, and V is the molecular volume of the molecule (in Å ^{3}). Note the similarity between the Perrin-Weber equation (eq. 5) and the Einstein equation (eq. 1). In order to illustrate the effect of experimental variables on anisotropy, values for r/r_0 have been computed by varying each of the parameters of

the Perrin-Weber equation while holding all other experimental values constant at values likely to be encountered in actual practice; i.e., $T = 293^\circ\text{K}$ (20°C), $\eta = 0.01$ poise (the viscosity of water), and $\tau = 10$ nanoseconds (see Fig. 3). The theoretical expectations of the Perrin-Weber equation (Fig. 3) indicate that anisotropy values increase as viscosity and molecular volume increase while a decrease in anisotropy occurs when temperature and fluorescence lifetime increase. The Perrin-Weber equation predicts that anisotropy will be more strongly affected by changes in viscosity and molecular volume than by temperature or fluorescent lifetime (Fig. 3).

The theoretical maximum value for $r_0 = 0.4$ ($P_0 = 0.5$). The theoretical maximum value of anisotropy would be observed for randomly oriented "frozen" probes with parallel excitation and emission transition moments (a condition usually approached when a molecule is excited with a wavelength as close as possible to the emission wavelength). Excitation of

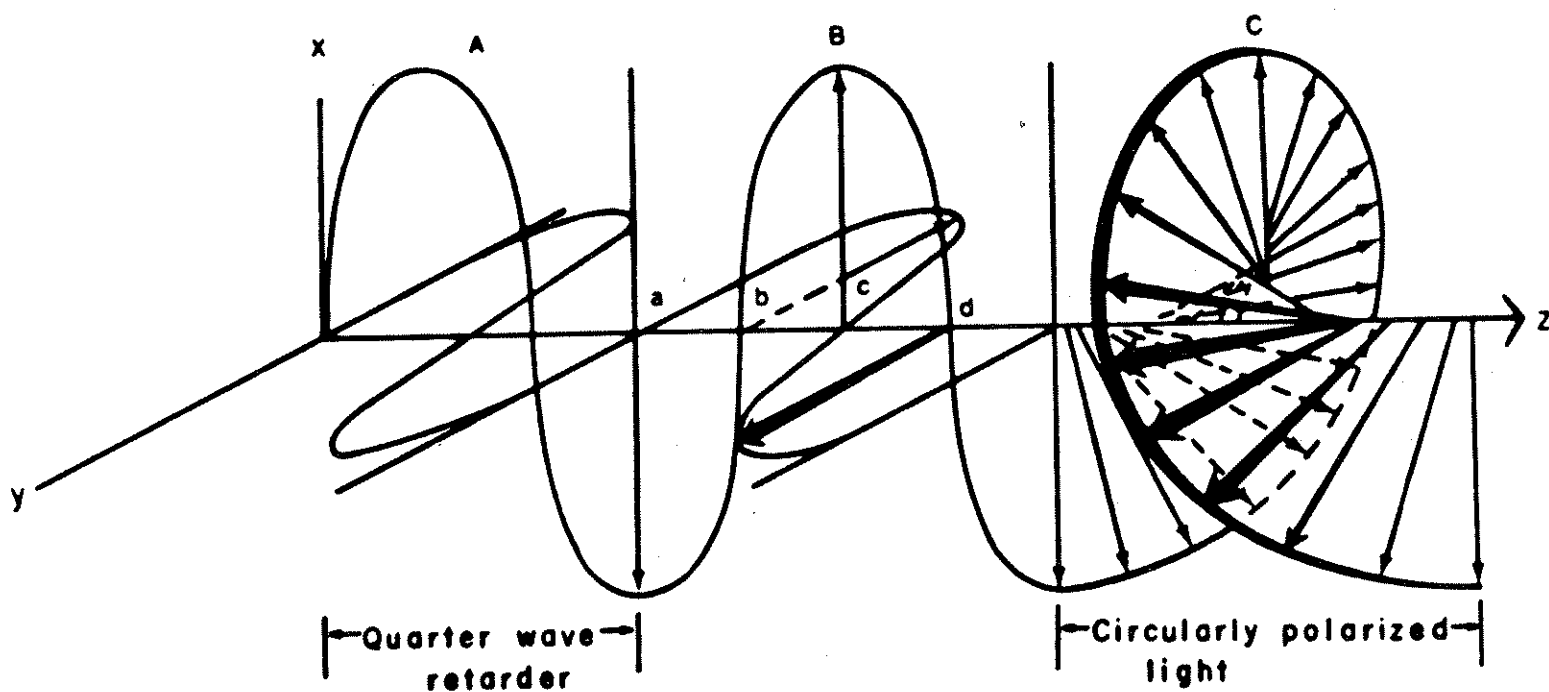


Fig. 3 a-c. Generation of circularly polarized light by means of a quarter wave retarder, (A). Two beams with electric vectors polarized at right angles begin in phase on entering the retarder but on emerging they are 90° out of phase as shown in (B). Addition of the two beams 90° out of phase in (C) gives circularly polarized light (Urry, 1974)

... beam

9. Optical Rotatory

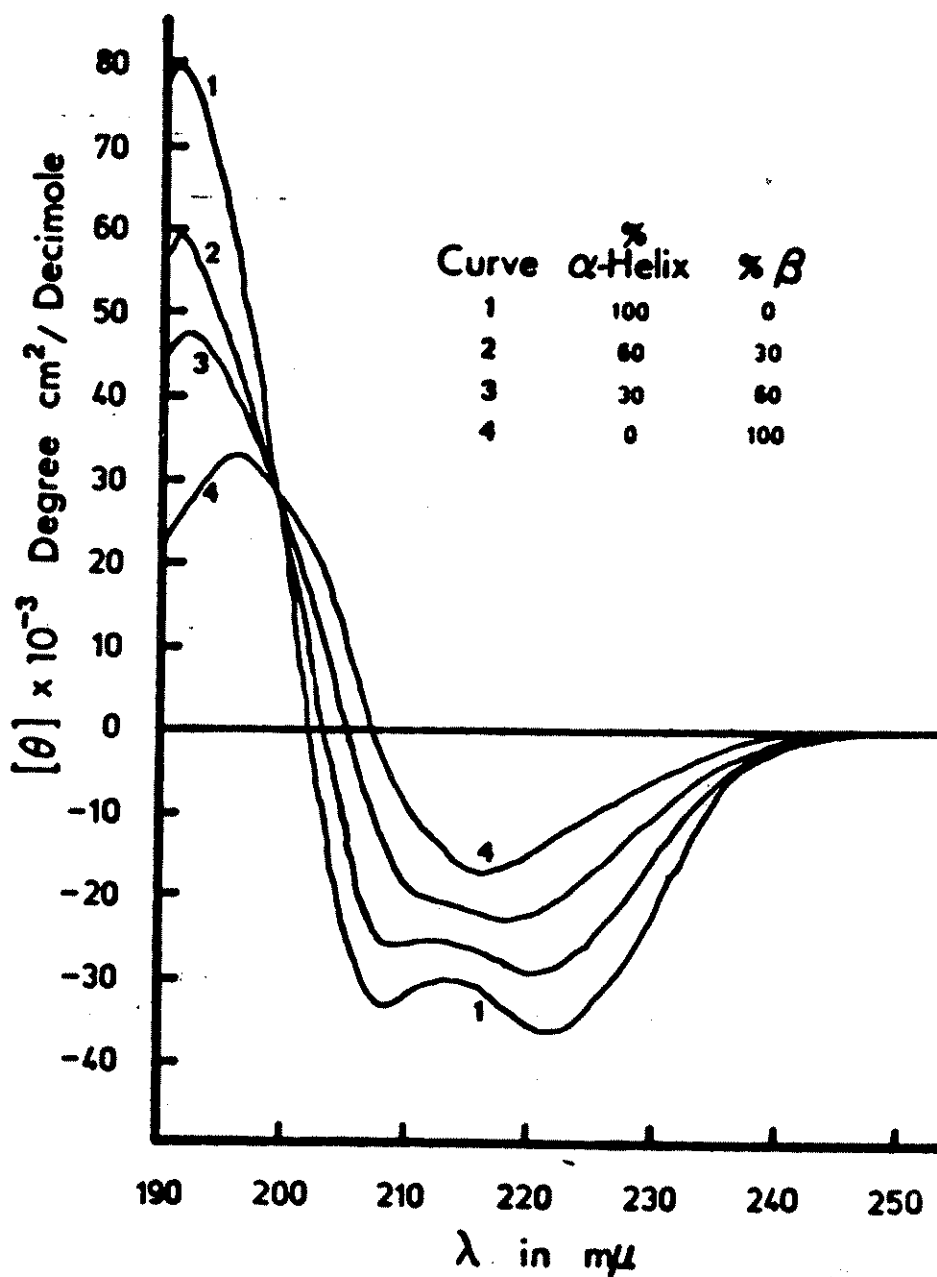


Figure 9.9 Calculated CD of poly-L-lysine containing 0% coil and varying percentag of α -helix and β -sheet structure as indicated.

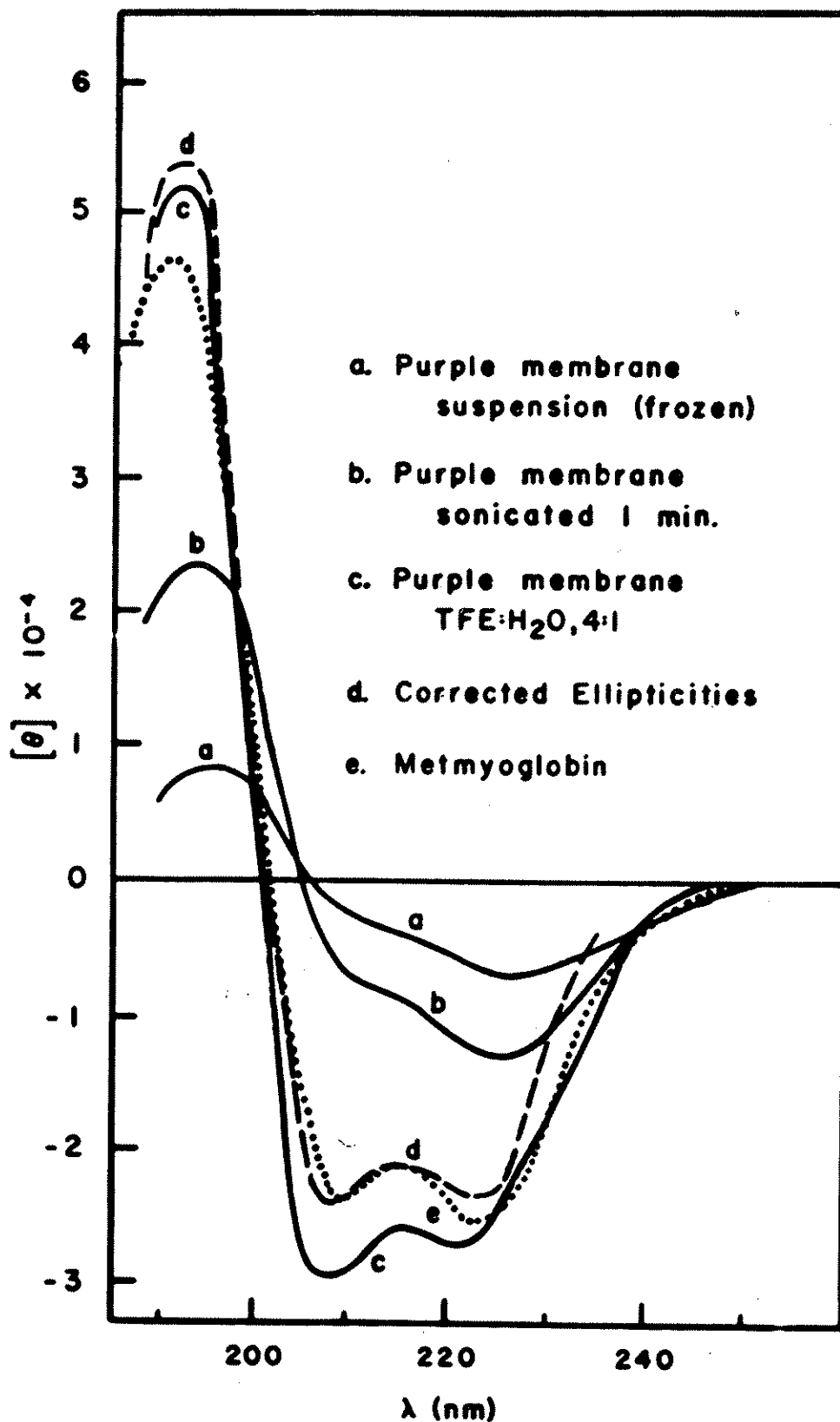


Fig. 14. Ultraviolet CD spectra of the purple membrane after freezing (a), subsequent sonication (b) after subsequent dissolution with trifluoroethanol (TFE) (c). The TFE spectrum is similar to the corrected spectrum (d), which is similar in shape and magnitude to (e), the spectrum of metmyoglobin which is 70% α -helix as shown by x-ray analysis (Long et al., 1968). Curve (e) is reprinted with permission from Quadrifoglio and Urry (1968a). Copyright 1968 by the American Chemical Society.

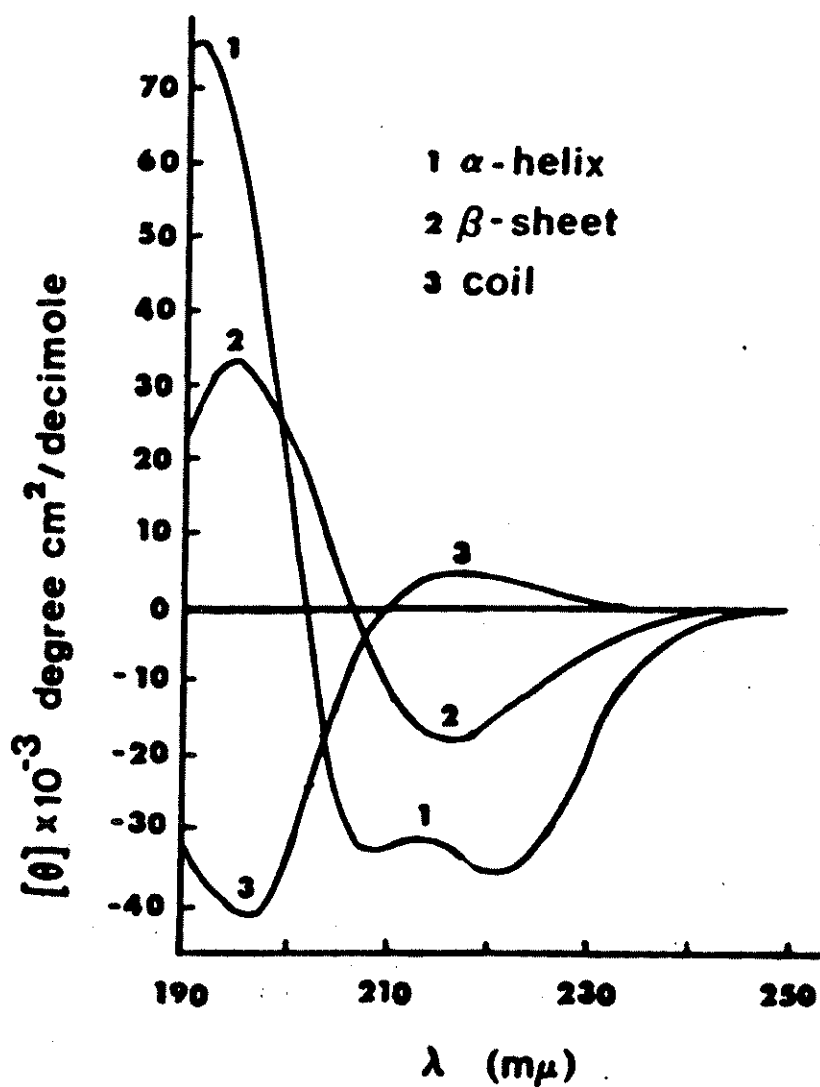


Figure 9.8 CD spectra of lysine in the α -helical (curve 1), β -sheet (curve 2), and coil configurations (curve 3).