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The measurement of polarization of the fluorescence of solutions under high pressure is rendered uncertain by the photoelastic birefringency of the windows, and needs a correction dependent upon the fraction of the light emerging from a window with polarization normal to that of the incident light (scrambling coefficient). This correction, which increases rapidly with applied pressure, differs according to whether the method of measurement employs rotation of the excitation polarizer (T format) or the emission polarizer (L format), and the latter is shown to involve the more reliable and the smaller correction. A four window pressure bomb housing a 1 ml volume cuvette that permits absorption and fluorescence measurements up to 4 kbar is described. The polarization correction up to 2.5 kbar was determined. Its reliability was demonstrated by measurements of the fluorescence polarization spectra of fluorophores in solvents of high viscosity at all pressures and by comparison of isopiestic and isothermal measurements of a fluorophore in isobutanol, a fluid solvent with viscosity that changes rapidly with pressure or temperature.

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INTRODUCTION

Measurement of linear polarization of fluorescence is a recognized method to study Brownian rotational motion of molecules in solution and a number of instruments have been described for this purpose.[1-12] All but one of them were designed to study samples at atmospheric pressure in which case one must rely on changes in the temperature or viscosity of the sample to characterize the rotational properties of the systems under study. Isothermal increase of hydrostatic pressure provides, on the other hand, a direct method to increase the viscosity of the system without changing the composition or the total energy of the medium. Recently a high-pressure apparatus (Chryssomallis *et al.*[13]) was described to allow polarization measurements under pressures of $10^{-3}-10$ kbar. To introduce the necessary corrections it requires an independent determination, at the beginning and at the end of the pressure run, of the absolute value of the polarization at atmospheric pressure.

The physical requirements of a high-pressure bomb for fluorescence polarization determinations are in our view: simplicity of construction, an internal volume as small as possible, pressure transmitting fluid transparent to the wavelengths of excitation and emission, total isolation of the sample from the hydrostatic fluid; polarization scrambling due to the birefringence of the windows at high pressure kept at a minimum or accounted for by a correction factor. Additionally the polarization values obtained from the use of this type of bomb should be absolute, that is, independent of determinations by another instrument. We describe here a high pressure bomb meeting these conditions and set out the corrections required by the depolarization owing to birefrigency of the windows. The high pressure bomb was part of a photon counting fluorescence polarization instrument.

I. EXPERIMENTAL APPARATUS AND PROCEDURE

A. High pressure bomb

The high pressure bomb was made of Vasconax 300 (Teledyne Vasco Company, Chicago, IL). As it can be seen in Figs. 1 and 2 the bomb body is cylindrical, with four optical ports and one pressure inlet (Fig. 3). One of the windows is used to transmit the excitation light, two more perpendicular to the first are used to detect the fluorescence emission. The fourth permits exit of the

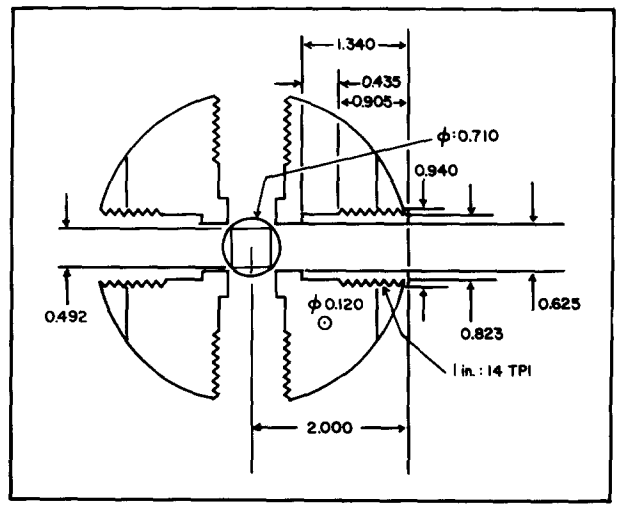

FIG. 1. Top view diagram of the high pressure bomb. All dimensions are in inches. Tolerances are omitted. In the critical places they were usually +0.005, and −0.000 in.

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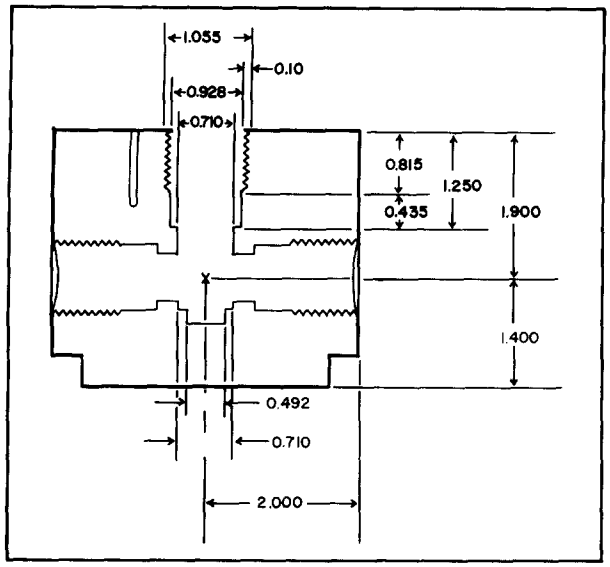

FIG. 2. Side view diagram of the high pressure bomb. Tolerances omitted. In the critical places they were usually +0.005, and −0.000.

excitation light minimizing its entrance into the detection system by multiple reflections.

All the high pressure seals were made using Bridgman's principle of unsupported area.[14] The windows were made of fused quartz of the following characteristics: T19 Suprasil 1, Birefrigency 5 nm/cm, practically fluorescence free and having optically flat surfaces, with the following dimensions: diameter 0.50 ± 0.05 in., thickness 0.30 ± 0.05 in. The seals between the windows and the plug ports are made by mirror-polishing the metal port surface upon which the window will rest. The pressure will make the seal by pressing the surfaces against each other. The window cap gives the window a mechanical support when the cell is handled at atmospheric pressures.

It can be seen (Fig. 4) that in the interior of the bomb there is enough space to accommodate a fused quartz cylindrical cell of about 1.5 ml of volume and 10 mm in diameter. This cell is attached to a brass base which prevents its movement with respect to the four window plugs. The stopper of this cell is made of a polyethylene tube with one of its ends closed by heating. The purpose of this collapsible tube is to allow pressure equalization between the hydrostatic medium (ethanol) and the sample inside the cell, preventing at the same time the mixing of both liquids. Experiments were performed to demonstrate the absence of all liquid flow into or out of the inner cell. In one such experiment, dyes highly soluble in ethanol were placed in the sample compartment, the bomb was pressurized and depressurized several times, and the presence of the dye in the ethanol was investigated by absorption spectrophotometry. We also used a dilute solution of bacterial luciferase (~0.25 mg/ml) in 0.10 *M* Tris buffer, pH 7.0. This enzyme is known to be sensitive to ethanol (Holzman, 1979). The activity which was expected to be low if an ethanol leak were to occur, was measured before and after a pressure increase with identical results. By these tests, a previous inner cell design was tested and rejected. This inner cell was made using a regular square cuvette with a standard conical opening at the top. A teflon stopper was perforated and a collapsible plastic tube was mounted on it. This design presented two sealing surfaces, one between the stopper and the cuvette walls and the other between the stopper and the plastic tube, and this arrangement facilitated the circulation of material into and out of the inner cell. Although fluorescence polarization observations are made usually using cuvettes with parallel walls, a cylindrical cuvette was not found to be a drawback. Being surrounded completely by alcohol its curved surface almost disappears owing to the closeness

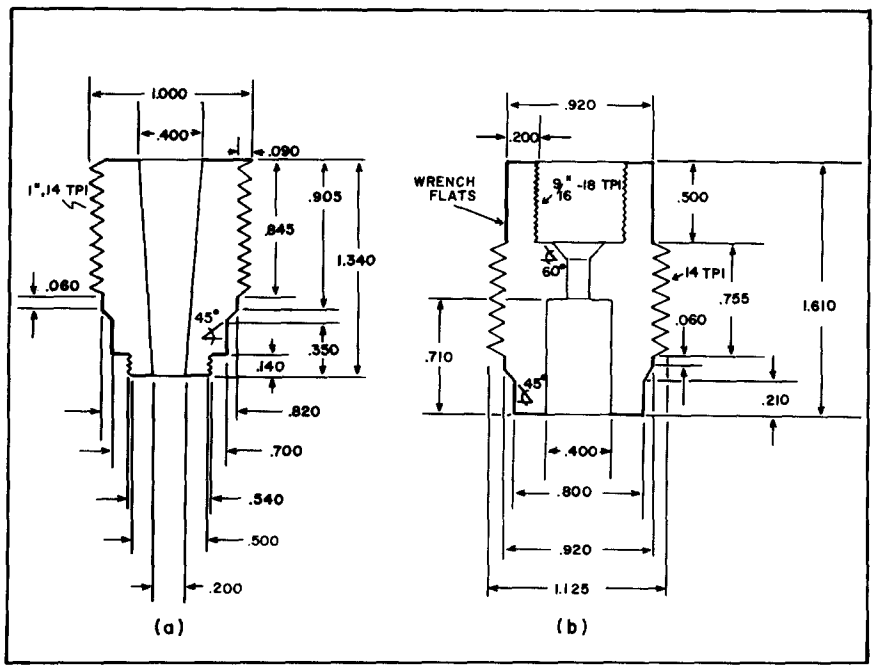

FIG. 3. Side view diagrams of the window port (a) and of the pressure inlet port (b). Dimensions are in inches.

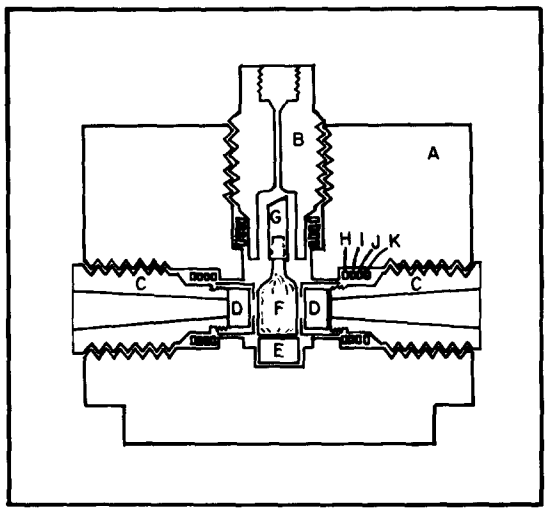

FIG. 4. Side view diagram of high pressure bomb (A) with the inner cell (F), and its holder (E), the window ports (C) and the pressure inlet (B). Sealing rings I, J, K, and extractor H are shown making no contact with the walls and ports to facilitate visualization; (D) quartz windows, (G) polyethylene flexible stopper.

of the indeces of refraction of quartz and ethanol. Figure 4 shows the sample cell within the pressure bomb. It can be appreciated that the aperture of the bomb is maximized by the conical shape of the plug openings. It can also be seen that the top plug is shaped so as to allow enough space for the inner cell top without unduly increasing the bomb inner volume (about 10 ml). The pump used (High Pressure Equipment, Erie, PA) consists of a manually operated piston screw pump. It is specially designed for use in applications where a liquid is to be compressed within a small volume in order to develop pressure. The pressure gauge, from Olson Engineering and Sales Corporation, Burlington, Iowa, had a 40 000 psi (2.72 kbar) maximum rating with a minimum graduation of 50 psi (3.4 bar). The high pressure line arrangement was such that the pump was easily taken out of the bomb pressure line for alcohol refilling or changes required during experiments.

A thermostatic bath model LT-50 from Neslab was used to vary and hold the temperature of the bomb between +30°C and −40°C within less than one-half degree of temperature variation. The thermostatic fluid, methanol, was circulated through copper tubing soldered to four steel plates attached to the bomb body. The efficiency of the setup in introducing or removing heat, was not high, taking almost one hour to achieve a 10°C change. A faster and more efficient system of heat transfer would have required an inconveniently large bomb size to accommodate the tubing within the bomb body. The bomb base was machined square to permit its clamping with a vise for easy removal of the window plugs and a Teflon square plate was fixed to this base to minimize thermal contact between the cell and the polarization instrument. A precision thermistor model YSI 44008 (batch AC48) with an absolute error of ±0.2°C was used to monitor the cell temperature. A homemade thermocouple of CrAl was also built and used when more precise temperature determinations were required.

In order to monitor the cell temperature a conduit of 2 mm in diameter and 23 mm depth was drilled in the bomb body. A thermocouple inserted in it will therefore follow the temperature variations of the bomb and not of the sample inside it. To ensure that this is also the sample temperature it is necessary to wait until the whole system reaches equilibrium. A temperature-sensitive transducer in close contact with the cell would be more appropriate, but it was decided against because of the technical problems arising from an extra plug just for temperature monitoring. Several experiments involving temperature changes proved that the measured bomb temperature does not differ significantly from the sample temperature provided 5 to 10 extra minutes of stabilization are allowed after each temperature change.

B. Handling, loading, and servicing the high pressure bomb

When filling the bomb with alcohol, care was taken to prevent getting air bubbles trapped. These bubbles, if allowed to stay inside the bomb, reduce the pumping efficiency and introduce systematic errors upon decompression by shaking the inner cell. In order to avoid them, the bomb was placed in the normal horizontal position, filled with alcohol, and then tilted and shaken several times until no more air bubbles were seen. The inner cell was then introduced and the top plug secured. The filling of the inner cell has to be done with the same precautions described for the bomb. This was a critical conditon: any residual air within the cell will dissolve with increasing pressure but will form bubbles on decompression, thereby giving rise to a large scattering signal. To prevent this, a technique was developed which requires a certain amount of dexterity and/or practice: First, the quartz sample cell was filled using a syringe until a convex meniscus formed, then the tube that acts as the stopper was completely filled while partially compressed with two fingers, then cell and stopper were brought together simultaneously while releasing the fingers' pressure. In this way, it is possible to have the sample completely confined to the cell and the stopper without any air bubbles.

This stage achieved, the cell is washed with ethanol and introduced into the bomb. The pressure inlet plug is torqued down and the bomb is ready for use. There were almost no maintenance problems with this type of bomb. We usually cleaned the windows using a Q-tip moist with ethanol. Under normal working conditions the samples do not get in contact with the bomb; therefore, alcohol rinsing was the only standard care procedure for the bomb.

C. Photon counting polarization photometer

A full description of the instrument used has been published by Jameson *et al.*[16] The only major modifications of the instrument described by the already men-

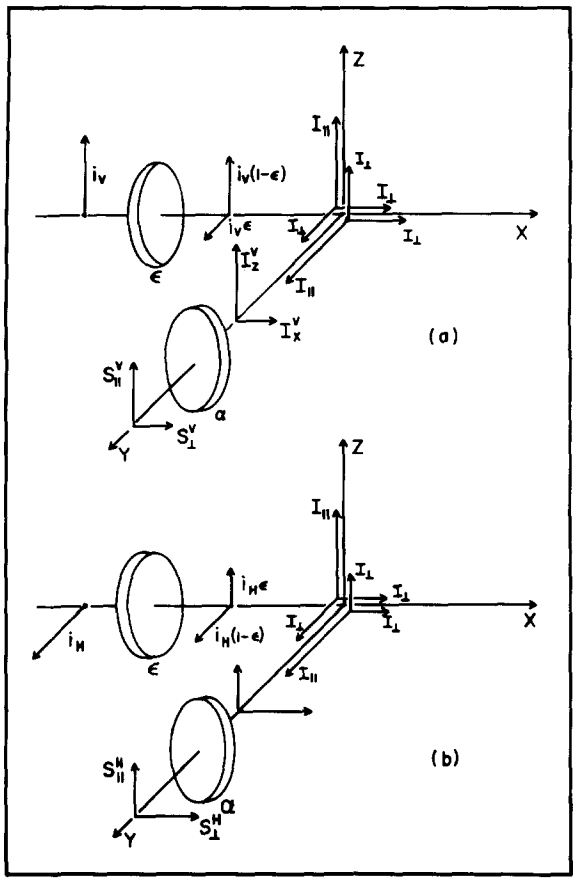

FIG. 5. Diagrams of the effect of window birefrigency upon the intensity of a completely polarized beam of light. For simplicity only two windows were drawn. Vectors were not drawn to scale. ϵ and α are the scrambling coefficients of the excitation and emission windows, respectively: (a) case in which excitation is polarized vertically (b) case in which excitation is polarized horizontally.

tioned authors was the use of commercially available photon counting electronics. Our design used a nonlinear dynode chain with higher voltages in the latter stages of the PMT base (EMI 6256S). Capacitive coupling to a fast amplifier discriminator: Ortec Model 9302 was used. This model was selected due to its wide band, high gain amplifier and integral discriminator capable of counting rates up to 100 MHz. Typically this unit has a rise time of 3 ns and with the gain used (20×) it has a linear response for input voltages of up to 50 mV. The discriminator output was connected to an Ortec Photon Counter model 9315. This model features two counters, thus allowing the detection of the parallel and the perpendicular signals simultaneously. Each counter has a range of 10^7 counts. It is also possible to use one of them as a timer, counting input impulses from a 100 kHz internal crystal-controlled clock with a time base switch-selectable for increments of 1 ms or 0.1 min. This counter can also be reset and will stop a counting interval in both sections when it reaches the desired time or number of input pulses.

E. Window birefrigency correction

The recent availability of high-pressure apparatus and the techniques developed for making spectroscopic measurements, while the sample is subjected to pressure, have been largely responsible for the increased interest in this important field. Some of the more important advances are described by Fishman and Drickamer,[17] Drickamer,[18] and Drickamer and Balcham.[19] In this type of instrumentation the optical properties of the windows used in the high pressure bomb are among the most important considerations. The windows show a pressure-dependent scrambling of polarization as a result of strain-induced anisotropies in the window material (photoelastic effect), see Campbell.[20] Sapphire windows are the most common choice for high-pressure experiments due to their high tensile strength and transparency in the u.v. region. However, these two advantages are counterbalanced by a very high percentage of polarization scrambling under conditions identical, as regards window size and pressure, in comparison with fused quartz. Our final choice was F19 Suprasil fused quartz. According to Cantor *et al.*[21] fused quartz (Ultrasil T-16, now discontinued, supplied by Karl Lambrecht Corp., Chicago, IL) shows low polarization scrambling in the range of 0–2 kbar.

Experiments were performed to check the window scrambling effects upon the measured polarization employing samples that are not expected to change the state of polarization of the emitted light between 0 and 2 kbar. For this purpose we chose a solution of fluorescein in glycerol which, already at atmospheric pressure, has a value close to the maximum (p_0) and should be insensitive to further increase in viscosity of the solvent caused by the applied pressure. The results obtained for fluorescein in glycerol with the system kept at −25 °C showed that at 2 kbar the window scrambling resulted in a 30% decrease of the polarization value observed at 1 bar (0.45).

The following treatment shows the theoretical approach used in the calculation of a window scrambling factor called α, its experimental determination, and the influence that it has in the choice between alternative formats through which the fluorescence polarization of a sample can be measured. The so-called "T" format involves the use of two photomultipliers that measure the intensity of the parallel and perpendicular polarized emitted fluorescence light respectively, see Jameson.[16] The "L" format also involves the use of two photomultipliers but in this case one of them measures alternatively the intensities emitted parallel and perpendicular while the other monitors the total fluorescence intensity emitted by the sample.

1. "T" format calculations

When the polarization photometer is used in the "T" format, the measurements are made by determining the ratio between parallel and perpendicular polarized components of the emission when excited by vertically polarized light ($S_{\parallel}^{V}/S_{\perp}^{V}$) or horizontally polarized light ($S_{\parallel}^{H}/S_{\perp}^{H}$). The state of polarization of the excitation will be defined as: Vertical when the electric vector of the wave is in the z–x plane and horizontal when it is

in the $x-y$ plane. Polarization measurements are obtained from the intensities emitted along the y axis with polarization directions defined as parallel ($\|$) when the electric vector oscillates in the z direction and perpendicular ($\perp$) when it oscillates in the y direction. Let

$$\gamma \equiv \frac{S_{\|}^{V}/S_{\perp}^{V}}{S_{\|}^{H}/S_{\perp}^{H}} . \tag{1}$$

The polarization experimentally measured (through the scrambling windows), p', will be given by:

$$p_T' = \frac{\gamma - 1}{\gamma + 1} . \tag{2}$$

The S intensity values refer to the components of the fluorescence after passing through a depolarizing observation window characterized by a scrambling coefficient α. Before passing through this emission window the components of the fluorescence excited by light which is originally vertically polarized, but only incompletely polarized after passing through the excitation window, will be denoted I_z^V, I_x^V, I_z^H, I_x^H in accordance with the scheme of Fig. 5.

The values of S and I are related through the following equations:

$$\begin{aligned} S_{\|}^{V} &= I_z^V(1-\alpha) + I_x^V\alpha, \\ S_{\perp}^{V} &= I_z^V\alpha + I_x^V(1-\alpha), \\ S_{\|}^{H} &= I_z^H(1-\alpha) + I_x^H\alpha, \\ S_{\perp}^{H} &= I_z^H\alpha + I_x^H(1-\alpha). \end{aligned} \tag{3}$$

In turn $I_z^V \ldots I_x^H$ have been generated by excitation with partially polarized light originating by passage of perfectly polarized light through a window with scrambling coefficient ϵ. If we designate as $I_{\|}$ and $I_{\perp}$ the components of the fluorescent light with electric vector, respectively, parallel and perpendicular to the incident exciting radiation, and if this radiation is polarized vertically, that is in the $0Z$ direction, the following relations can be written:

$$I_z^V = I_{\|}; \quad I_x^V = I_y^V = I_{\perp}. \tag{4}$$

Similarly, if the excitation is polarized horizontally, that is, in the $0Y$ direction:

$$I_z^H = I_x^H = I_{\perp}; \quad I_y^H = I_{\|}. \tag{5}$$

These relations are shown in Figs. 6(a) and (b).

On vertical excitation, through a window with scrambling coefficient ϵ, as shown in Fig. 5, the values of I_z^V and I_x^V are intermediate between those shown in Figs. 6(a) and (b), namely:

$$I_z^V = (1-\epsilon)I_{\|} + \epsilon I_{\perp}, \tag{6}$$

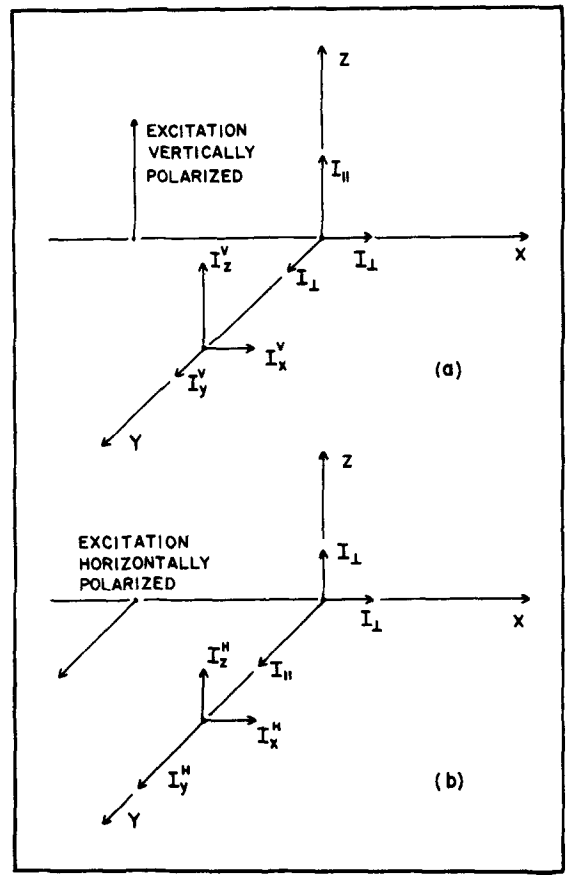

FIG. 6. Limiting cases for excitation polarized vertically (a) and horizontally (b).

$$I_x^V = \epsilon I_{\perp} + (1-\epsilon)I_{\perp} = I_{\perp}. \tag{7}$$

Similarly, on horizontally polarized excitation passing through the excitation window:

$$I_z^H = \epsilon I_{\|} + (1-\epsilon)I_{\perp}, \tag{8}$$

$$I_x^H = I_{\perp}. \tag{9}$$

If the values of I_z^V, I_x^V, I_z^H, and I_x^H given in Eqs. (6)–(9) are introduced into Eq. (3), we can obtain by means of Eq. (1) an expression for the observed polarization of the fluorescence as a function of the components $I_{\|}$ and $I_{\perp}$ that would be excited by perfectly polarized light and the distorting effects of the excitation and emission windows, described by ϵ and α, respectively. Proceeding in this way we find:

$$\begin{aligned} S_{\|}^{V} &= I_{\|}(1-\epsilon-\alpha+\epsilon\alpha) + I_{\perp}(\epsilon+\alpha-\epsilon\alpha), \\ S_{\perp}^{V} &= I_{\|}(\alpha-\epsilon\alpha) + I_{\perp}(1-\alpha+\epsilon\alpha), \\ S_{\|}^{H} &= I_{\|}(\epsilon-\epsilon\alpha) + I_{\perp}(1-\epsilon+\epsilon\alpha), \\ S_{\perp}^{H} &= I_{\|}\epsilon\alpha + I_{\perp}(1-\epsilon\alpha), \end{aligned} \tag{10}$$

and therefore:

$$\gamma = \frac{I_{\|}^2(\epsilon\alpha - x) + I_{\perp}^2(\alpha+\epsilon-\epsilon\alpha-x) + I_{\|}I_{\perp}(1-\epsilon-\alpha+2x)}{I_{\|}^2(\epsilon\alpha-x) + I_{\perp}^2(1-\epsilon-\alpha+3\epsilon\alpha+x) + I_{\|}I_{\perp}(\epsilon+\alpha-4\epsilon\alpha+2x)} , \tag{11}$$

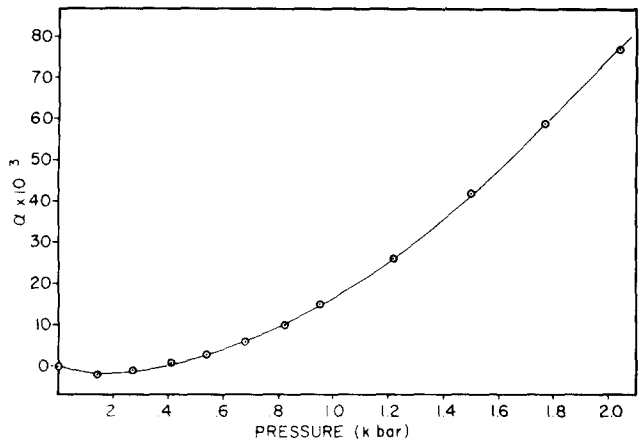

FIG. 7. Scrambling coefficient (a) as a function of hydrostatic pressure for the "L" format configuration only.

where

$$x \equiv \epsilon\alpha^2 + \epsilon^2\alpha - \alpha^2\epsilon^2.$$

The polarization (p) that would be measured under ideal conditions, that is, without scrambling windows or at atmospheric pressure, is given by:

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}). \tag{12}$$

Using this equation and Eq. (2), we can rewrite Eq. (11) as:

$$p_T' = P\,\frac{(1 - 2\epsilon)(1 - 2\alpha)}{1 + [4P^2/(1 - P)](\alpha\epsilon - x)}. \tag{13}$$

Finally assuming that α and ϵ are equal and neglecting all the terms involving the square of the scrambling coefficients we can simplify the latter equation to

$$p_T' \simeq P(1 - 4\alpha). \tag{14}$$

This equation permits calculation of the true polarization values of samples measured under scrambling conditions induced by pressure, within the approximations already stated and when the "T" format is used.

2. "L" format calculations

When polarization photometer is used in the "L" format, the measurements are made by determining the ratio between parallel and perpendicular components excited by vertically polarized light ($S_{\parallel}^V/S_{\perp}^V$) and calculating the apparent polarization value (p_L') by use of the following equation:

$$p_L' = \frac{(S_{\parallel}^V/S_{\perp}^V) - 1}{(S_{\parallel}^V/S_{\perp}^V) + 1}. \tag{15}$$

The S values can be replaced in terms of $I_{\parallel}$ and $I_{\perp}$ using the first two equations in Eq. (10), obtaining:

$$p_L' = P(1 - \epsilon)(1 - 2\alpha)/(1 - P\epsilon). \tag{16}$$

And assuming $\alpha = \epsilon$ and small we can simplify Eq. (18) to:

$$p_L' = P(1 - 3\alpha)/(1 - P\alpha). \tag{17}$$

This equation permits calculation of the true polarization values (P) of samples measured under scrambling conditions induced by pressure, within the approximations already stated, and when the "L" format is used. The intensities $S_{\parallel}^V$ and $S_{\perp}^V$ are measured through the same emission window in the "L" format. In the "T" format each component is measured through a different emission window and therefore any difference in the scrambling coefficient between these windows will show up in the corrected polarization value as a systematic error for which no correction is available. Secondly, the depolarizing effects of the windows in the "T" format have a weight of $4/(3 - P)$ greater than in the "L" format. Clearly the "L" format is to be preferred.

If the polarization of a pressure-independent sample like fluorescein dissolved in glycerol is known at atmospheric pressure ($\alpha \simeq 0$) and at any given pressure, then the correction factors for the windows can be calculated using Eq. (13) in the "T" format or Eq. (16) in the "L" format.

F. Azimuthal dependence of the birefrigency effect

The theoretical approach sketched in the last section assumed that when subjected to pressure the windows are uniformly affected on their surface and consequently that there is no azimuthal dependency on this effect. In other words, one assumed the absence of preferential polarization directions along which the depolarizing effects would be reinforced or minimized.

To check for the effects of pressure upon the homogeneity of the windows, the polarization instrument was modified, so that a beam of polarized light could pass through the emission windows and be analyzed by means of a rotating polarizer. The azimuthal dependency was studied by analyzing the polarized intensity distribution transmitted for several polarization planes of the first

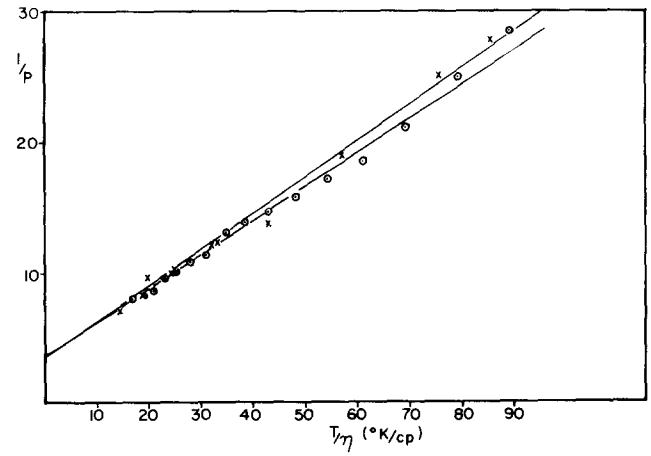

FIG. 8. Perrin plots for PRODAN in isobutanol (Lot DPK, Mallinkrodt spectral grade). Isopiestic measurements (⊙). Isothermal measurements (×). Excitation bandwidth: 0.9 nm. Emission filters Corning 3-74. Pressure data obtained with instrument in "L" format; temperature data obtained in a different photon counting instrument using "T" format configuration.

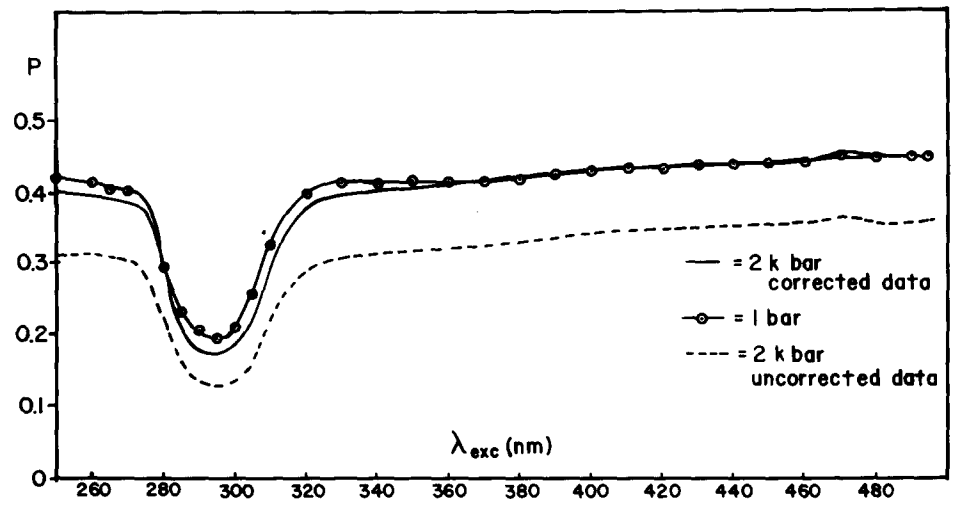

FIG. 9. Excitation polarization spectrum of PRODAN in glycerol. Temperature +23°C. Measured with "L" format. (⊙) data obtained at 1 bar; (- - -) data obtained at 2 kbar (uncorrected); (——) corrected data for 2 kbar. Excitation resolution 5 nm (250–280 nm); 3 nm (280–340 nm); 1.5 nm (340–495 nm).

polarizer. As expected, the $\cos^2\theta$ law (θ being the angle determined between the planes of transmission of the polarizers) was observed, within the experimental error, at atmospheric pressure. Deviations from it were found to be present symmetrically distributed about θ when 2 kbar of pressure were applied to the windows. The intensity distribution was studied for various angles of the plane of polarization of the excitation polarizer, and lack of azimuthal symmetry was found to be less than 10% when 2 kbar of pressure was applied to the windows. However, when a sample is studied under real conditions it is placed *between* the emission windows and therefore their error contribution to the experimental polarization obtained using the "T" format will be much smaller than 10% (probably less than 5%). The use of the "L" format will free us from having to take this error into consideration.

G. Viscosity considerations

Changes in the viscosity of the solvent induced by pressure will result in polarization changes, see Perrin.[22] In order to calculate the scrambling coefficients it is necessary to estimate the polarization changes produced by the increase in viscosity with pressure.

Using Perrin's equation[22,23]

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{RT}{\eta V}\tau\right), \qquad (18)$$

where p_0 is the limiting polarization, that is, the polarization in the absence of all extrinsic causes of depolarization, R the gas constant, T the temperature in absolute degrees, τ the lifetime of the excited state, η the viscosity of the solution, and V the molar volume of the molecule supposed spherical. This equation permits calculation of the polarization that would be observed at any given pressure if the viscosity of the solvent at this pressure is known, under the additional assumption that p_0, τ and V are pressure independent. The experimental fluorescence p' will differ from the calculated value, P on account of the photoelastic birefringency of the windows and under the assumption $\alpha = \epsilon$, this coefficient can be calculated by Eqs. (13) or (16). For the reasons sketched above we chose the "L" over the "T" format and from experiments of the type described the value of α was determined for the interval of pressures up to 2 kbar.

Figure 7 shows the plot of α vs pressure. To facilitate further comparison a least-squares fitting of the birefrigency coefficient was done with a polynomial of third degree ($\alpha = b_0 + b_1X + b_2X^2 + b_3X^3$). Best fitting was obtained for the following parameters: $b_0 = -5.32 \times 10^{-4}, b_1 = -1.25 \times 10^{-2}, b_2 = 3.48 \times 10^{-2}, b_3 = -5.26 \times 10^{-3}$, X = pressure in kbar.

H. Experimental determinations of polarization

The fluorescence polarization of 6-propionyl-2-dimethylamino naphthalene (PRODAN), see Weber and Farris,[24] in isobutanol was measured as a function of temperature and pressure in two independent experiments. Perrin plots, shown in Fig. 8, are in close agreement despite the fact that two different ways of varying the viscosity of the solvent were used.

Figures 9 and 10 show the excitation polarization spectra for two different samples, one of PRODAN in glycerol and the other, fluorescein in the same solvent. The corrected and uncorrected data are shown as well as the data obtained at 1 bar. Disagreement is restricted to those wavelengths where there is overlap of strong and weak absorption transitions. Pressure could conceivably have the effect of changing their relative contributions, then resulting in intrinsic changes in p_0 with pressure, which are unrelated to depolarization by the windows.

Finally, the excitation polarization spectrum of indole in propylene glycol at room temperature was measured at atmospheric pressure and at 2 kbar. No substantial changes were observed under pressure and excellent agreement with the polarization spectrum of indole measured by Valeur[25] was obtained as it can be seen in Fig. 11. The differences in resolution observed are due

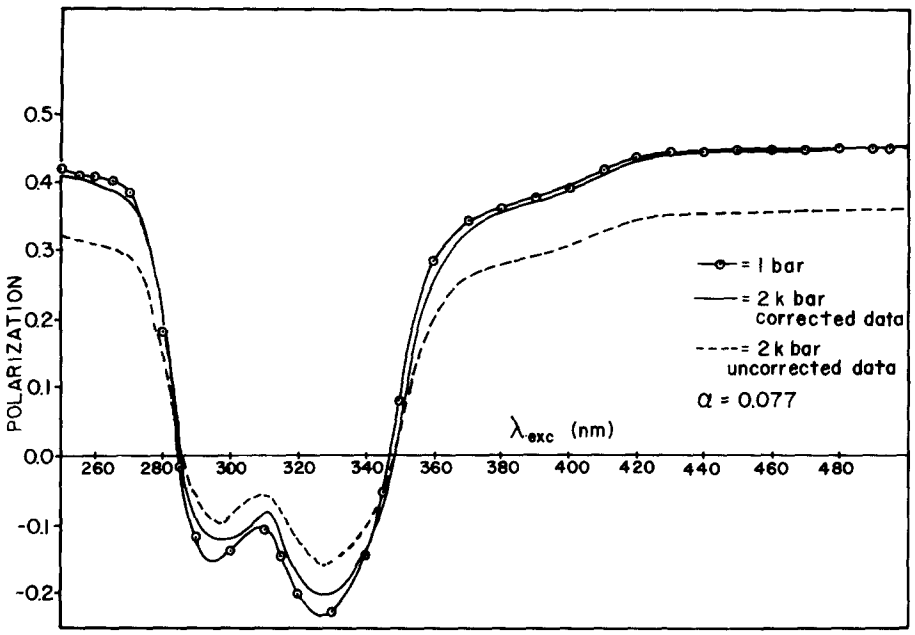

FIG. 10. Fluorescence polarization spectrum of fluorescein in glycerol. Temperature 24 °C. (⊙) measured at 1 bar; (– – –) measured at 2 kbar, uncorrected data; (——) corrected data at 2 kbar. Resolution used was 4 nm from 250 to 310 nm and 1.7 nm from 310 to 490 nm.

to the different slit-width for excitation and the higher temperature (−20 °C as compared with −58 °C in Valeur's experiments) used in our experiments. In all cases in which we could carry out valid comparisons between corrected and uncorrected values the α factors independently determined lead to similar agreement as in the cases shown here.

The same correction factors were assumed to apply independently of the wavelengths of excitation and emission, an assumption justified by the results obtained with fluorescein (excitation at 480 nm, emission at 520 nm) and indole (excitation at 280 nm, emission at 320–380 nm). We do not expect further studies of the wavelength dependence to introduce significant changes in the results already obtained.

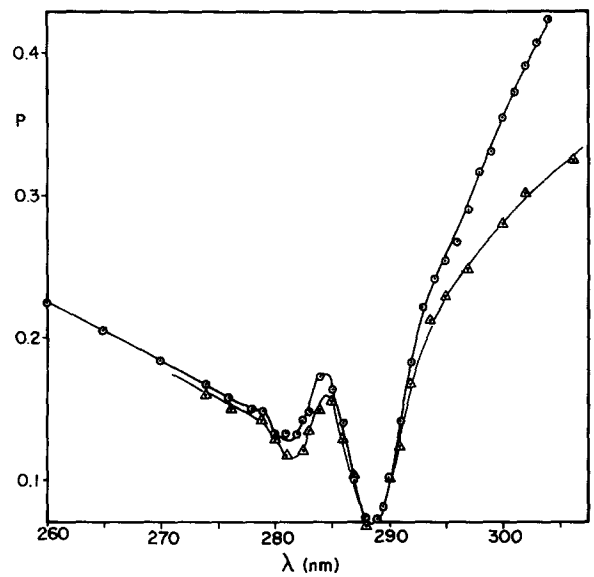

FIG. 11. Excitation polarization spectra of indole in propylene glycol (spectral grade from Eastman, distilled once) at a concentration of 5×10^{-5} *M*. (⊙) measured at 1 bar; (△) measured at 2 kbar. Data was obtained using "L" format, a resolution of 2 nm, emission filters Corning, 7-54 at −20 °C.

III. DISCUSSION

The high-pressure bomb as well as the inner sample cell have proved to be both practical and reliable for measurement of spectroscopic changes in biological systems. It has been used in measurements of the dissociation of enolase,[26] binding of ethididium bromide to t-RNA,[27] and in studies of the effects of pressure on lipid bilayers and natural membranes.[28]

The high-pressure bomb is practical because its dimensions, both internal and external, permit its use in several instruments without major changes. The instruments included: a spectrophotometer, Beckman model ACTA MVI, an analog spectrofluorometer, a photon counting polarization instrument, and a cross-correlation fluorometer. It was also reliable since more than 100 different experiments were performed in less than a year without a single failure due to leaks. This reliability is based on the high degree of precision of the machining of the window plugs and in the fact that loading the samples does not require removal of more than one plug, therefore reducing sealing ring wear. Freedom of contamination from the pressure transmitting fluid and low volume samples are also convenient features of the high-pressure bomb design.

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