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Thermal Coefficient of the Frictional Resistance to Rotation in Simple Fluorophores Determined by Fluorescence Polarization†

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ABSTRACT: Experimental data are presented by employing organic fluorophores, tryptophan and tyrosine among them, that substantiate a logarithmic expansion of the viscosity as a function of temperature for the determination of the thermal coefficient of the viscosity by measurements of the polarization of the emitted fluorescence. The values obtained agree within experimental uncertainties with those determined by flow viscometry, and the agreement extends to a range of glycerol–water mixtures for which the thermal coefficient of the viscosity is an irregular function of the glycerol content. Prodan, a fluorophore that forms strong bonds with the hydroxylic solvents employed, yields values similar to those obtained with perylene, which interacts with solvent by weak dispersion forces alone.

A number of studies have shown that the polarization of the fluorescence of tyrosine and tryptophan in proteins is determined by the overall rotation of the protein molecules as well as by rotations of local character in which the individual residues undergo motions independent of those of the whole particle. Such partial motions are revealed by Perrin plots (Perrin, 1926) with limiting polarization values smaller than those that correspond to a truly immobile fluorophore (Weber, 1952) or by an initial slope that corresponds to a kinetic rotational volume much smaller than that of the whole protein (Wahl & Weber, 1967; Lakowicz & Weber, 1980). Both characteristics are equally unequivocal in those cases in which the emission is due to a single excitable fluorescent residue, but the decrease in apparent limiting anisotropy may contain

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0006-2960/84/0423-6785$01.50/0

a contribution from energy transfer if several amino acid residues are responsible for the fluorescence emission. When Perrin plots cover a sufficiently large range of external viscosity, they reveal a complexity that may be understood in terms of rotational units that vary both in their effective volume and in the amplitude that the surrounding elements in the protein permit to the rotations. We have presented an analysis of the internal motions of bovine pancreatic trypsin inhibitor (BPTI)[1] in these terms (Kasprszak & Weber, 1982). While there is nothing incorrect in this approach, the conclusions of such analysis, both in in this case and in the more recent study of Lakowicz et al. (1983), were equally disappointing: No general view emerged, apart from the complexity of the individual cases and their specific differences. We have therefore undertaken a different approach, introducing a method of analysis that attempts to derive parameters characteristic of the environment rather than those of the rotating unit itself: As is shown in the two following papers (Rholam et al., 1984; Scarlata et al., 1984) we are able to derive the thermal coefficient of the frictional resistance to the rotations of tyrosine or tryptophan and the characteristic amplitude at which the surrounding amino acids become the determinant of that frictional resistance. In this paper we substantiate the validity of our analysis by application to some simple organic fluorphores, including tyrosine and tryptophan, dissolved in pure solvents and solvent mixtures. In successive papers we extend this approach to a study of the local rotations of peptides and proteins. Glycerol–water mixtures, examined here in some detail, are appropriate solvents for the study of the local rotations in peptides and proteins on account of both the absolute value of their viscosities and their use as stabilizers of protein structure (Gekko & Timasheff, 1981a,b).

Materials and Methods

L-Tryptophan and L-tyrosine were purchased from Sigma. Spectral-grade butanol and glycerol were from Aldrich. Purities were checked spectroscopically. Glycerol was stored under vacuum with a desiccant. Prodan was prepared as described by Weber & Farris (1979). Perylene, zone refined, of 99.99% purity was from Princeton Chemicals.

Fluorescence polarization was measured with the photon counting instrument of Jameson et al. (1976). Polarization values were corrected for background fluorescence when this exceeded 0.5% of the total intensity. The standard deviation of independent polarization observations was ±0.0012. Fluorescence lifetimes were measured by the cross-correlation method of Spencer & Weber (1969) with updated electronics (SLM Instruments, Urbana, IL). The exciting wavelength, additional excitation filter, and emission filter (all Corning Glasses) employed with the different fluorophores are given in the following table:

fluorophore	excitation (nm)	excit. filter	emission filter
tyrosine	280	7-54	0-53
tryptophan	297	7-54	0-54
Prodan	360	7-37	3-75
perylene	433	7-59	3-71

Optical modules were purged with dry nitrogen to prevent frosting at the lower temperatures. Temperatures were regulated by a methanol circulating bath (Neslab LT-50). Viscometric data for glycerol were from Miner & Dalton (1953). Those of butanol were from the *CRC Handbook of Chemistry and Physics* (1980).

Analysis of the Data. We attempt to extract from the fluorescence polarization data the characteristic thermal coefficient of the frictional resistance to the rotations of the fluorophore. The decrease in fluorescence polarization with temperature commonly observed is due both to the increase in the average kinetic energy of the fluorophore and to the decrease in the viscosity of the solvent. These quantities are related to the polarization by the Perrin law

$$1/p - \tfrac{1}{3} = [1/p(0) - \tfrac{1}{3}][1 + RT\tau/(\eta V)] \qquad (1)$$

where $p(0)$ is the polarization of the motionless fluorophore, R is the gas constant, T is the kelvin temperature, τ is the lifetime of the excited state, V is the effective volume of the fluorophore, and η is the viscosity of the solvent. Setting

$$A = 1/(1/p - \tfrac{1}{3}) \qquad A(0) = 1/[1/p(0) - \tfrac{1}{3}]$$

eq 1 may be written as

$$[A(0)/A] - 1 = RT\tau/(\eta V) \qquad (2)$$

Over a limited range of temperatures about a temperature $T(0)$ the dependence of the viscosity of liquids upon the temperature is given to a close approximation by the relation

$$\eta = \eta(0)\exp[b[T(0) - T]] \qquad (3)$$

where $\eta(0)$ is the viscosity at $T(0)$ and b is the thermal coefficient of the viscosity, that is, the fractional change in viscosity per degree. Introducing the value of η in eq 2 and taking logarithms

$$\ln [[A(0)/A] - 1] - \ln (RT\tau/V) = -\ln [\eta(0)] + b[T - T(0)] \qquad (4)$$

The quantities entered in the left-hand side of this equation $[A(0), A, T, \tau]$ are all directly measurable. R, $\eta(0)$, and V are constants. Accordingly a plot of

$$Y = \ln [[A(0)/A] - 1] - \ln (RT\tau/V) \qquad (5)$$

vs. $t = T - T(0)$ should give a straight line with slope b equal to the thermal coefficient of the viscosity of the solvent. In a fluorophore immersed in a heterogeneous medium a linear relation between Y and t over an interval of temperature Δt characterizes the resistance that the environment offers to the rotation of the fluorophore as having a thermal coefficient b over the interval Δt.

Results and Discussion

Observations of the polarization of the fluorescence were made in mixtures of 80% glycerol and 20% 0.05 M, pH 7.0, phosphate buffer (w/w) and in butanol. Figure 1 shows plots of the natural logarithm of the viscosity coefficient determined by flow as a function of temperature. We can see that eq 3 holds well in the temperature range −40 to +20 °C. The absolute values of the slopes are approximately N 0.08/°C for 80% glycerol buffer and 0.04/°C for butanol. The thermal coefficient of the frictional resistance to the rotation of the fluorophores tyrosine, tryptophan (Figure 2), and Prodan in the same solvent are presented in Table I. All the fluorophores display linear plots with slopes that are remarkably close to the thermal coefficient of the solvent viscosity. When the absolute polarizations of the solutions are small, the error in Y becomes prohibitively large. Because of this circumstance we collected data only in the temperature range in which errors in Y are small enough to permit reliable calculation of the slope. The list of values of Table I indicates that with butanol as solvent the slopes are virtually the same for perylene, which interacts with the solvent through dispersion forces and Prodan

[1] Abbreviations: Prodan, 2-(dimethylamino)-6-propionylnaphthalene; BPTI, bovine pancreatic trypsin inhibitor.

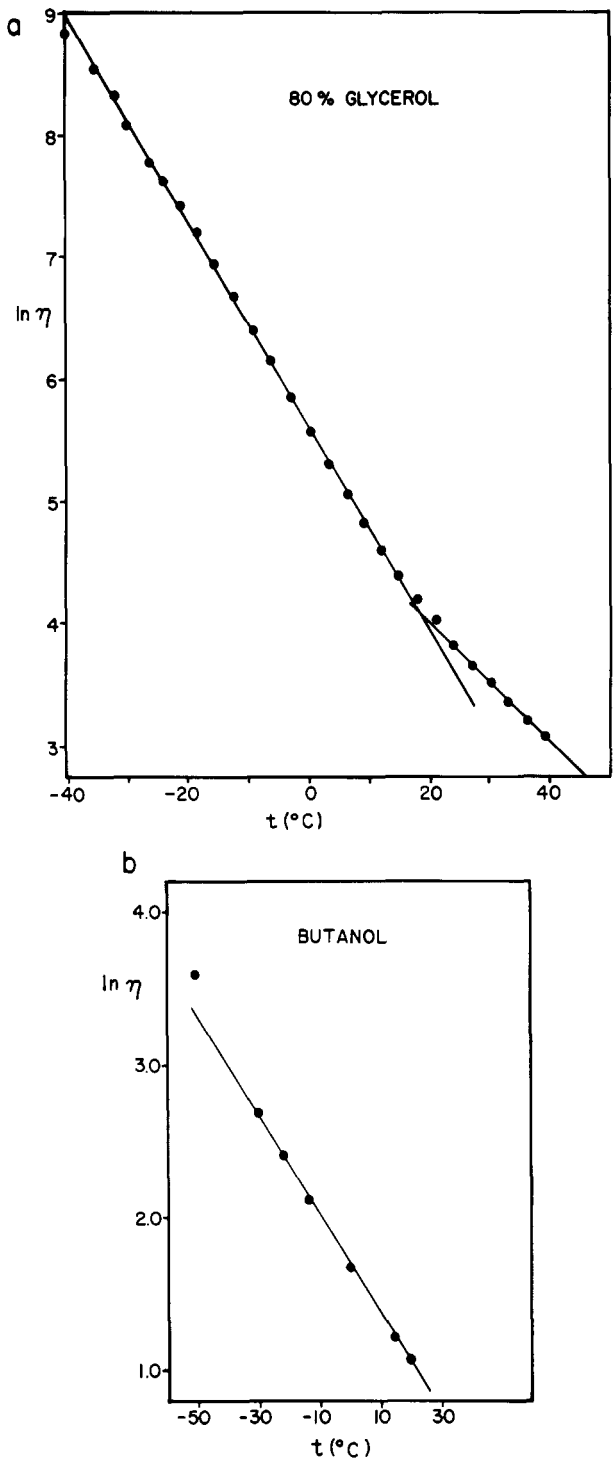

FIGURE 1: (a) Plot of natural logarithm of the viscosity, in centipoises, measured by flow vs. the Celsius temperature. (a) 80% glycerol–water; data of Miner & Dalton (1953). (b) Same for butanol; data of *CRC Handbook of Chemistry and Physics* (1980).

Table I: Thermal Coefficients of the Viscosity Measured by Use of eq 4[a]

probes	b
80% Glycerol/Water (8%)	
tyrosine	6.7
tryptophan	7.0
prodan	7.2
Butanol (4.1%)	
prodan	3.7
perylene	4.3

[a] All values are given in percent change per degree. The figures in parentheses are the thermal coefficients measured by flow viscometry.

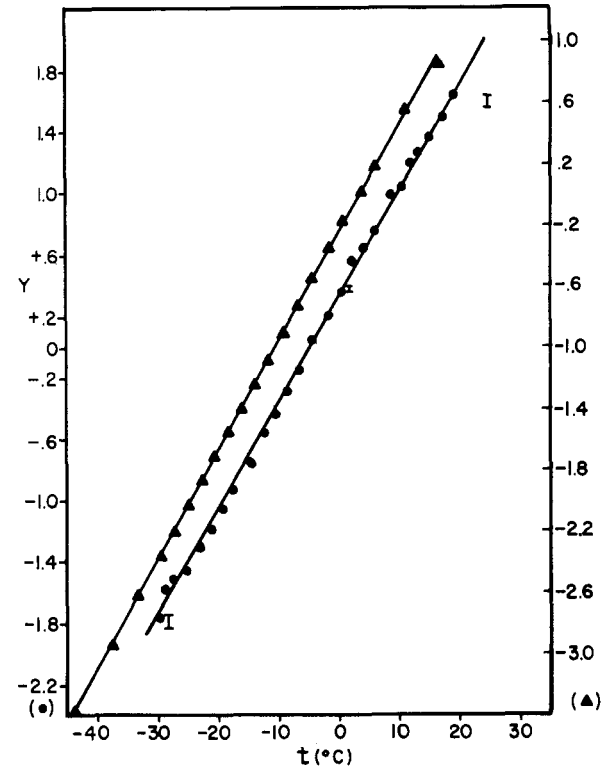

FIGURE 2: Plots of Y (eq 5) vs. the Celsius temperature for tryptophan (●) and tyrosine (▲) dissolved in 80% glycerol–20% 0.05 M phosphate buffer, pH 7.0.

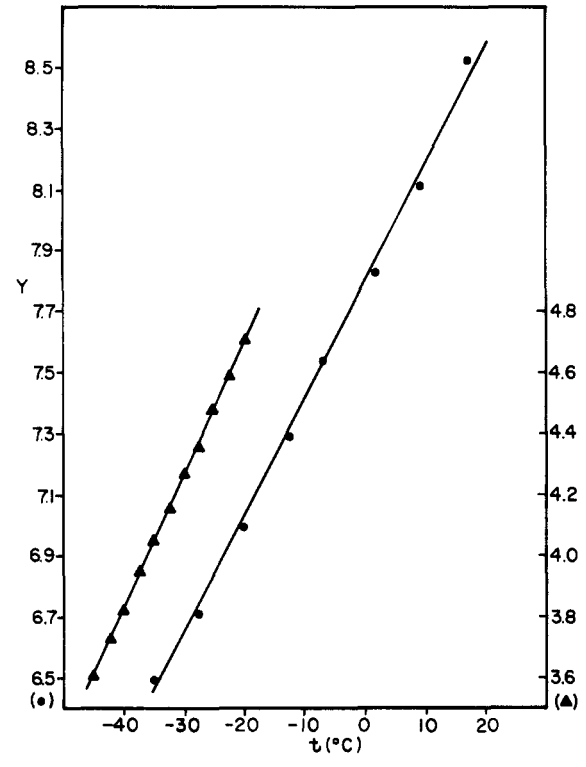

FIGURE 3: Plots like those of Figure 2 for Prodan (●) and perylene (▲) in butanol solution.

which interacts through the much stronger dipole–dipole energies (Macgregor & Weber, 1981). Their Y plots are shown in Figure 3. Table II shows the thermal coefficient of the viscosity measured by flow for a series of glycerol–water mixtures of 67–90% glycerol content (w/w), according to data taken from Miner & Dalton (1953). It is apparent that the thermal coefficient of the viscosity does not change mono-

Table II: Thermal Coefficients of Frictional Resistance in Various Glycerol–Water Mixtures

glycerol (%)	thermal coefficients of viscosity	
	by flow	by fluorescence depolarization[a]
67	8.4	8.3
70	9.5	9.9
75	8.65	8.4
80	8.0	7.0
90	9.0	9.9

[a] Measured with tryptophan as the probe. Conditions were those shown in Figure 2.

tonically with increasing glycerol proportion but rises, falls, and rises again in these circumstances. The detailed molecular interactions responsible for these changes are not known but they may be reasonably thought to arise in changes in the number and nature of various glycerol–water aggregates present in these mixtures. This irregular behavior permits us to further estimate the applicability of our eq 4, which assumes the identity of the thermal coefficient of the macroscopic solvent viscosity with that of the frictional resistance that the medium opposes to the fluorophore. As shown by Table II there is good agreement between the macroscopically and microscopically derived thermal coefficients. The small differences observed between them in these and in the other cases shown in Table I, are significant: The flow viscosity is determined by the interactions among solvent molecules; the fluorescence depolarization depends additionally upon solvent–fluorophore interactions. Although these latter interactions are all important in determining the symmetry and amplitude of the rotations (Mantulin & Weber, 1977), they seem to play only a minor role on the changes in rotational amplitude with temperature. Very similar values are found for Prodan and perylene solutions in butanol, though Prodan interacts with butanol through very strong dipole–dipole interactions (Macgregor & Weber, 1981) and perylene interacts with the solvent exclusively through the much weaker dispersion forces.

Registry No. Prodan, 70504-01-7; tyrosine, 60-18-4; tryptophan, 73-22-3; perylene, 198-55-0.

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