EXCITED STATE INTRAMOLECULAR GEOMETRICAL RELAXATION POTENTIAL USE IN MICROFLUIDITY PROBING

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ABSTRACT

EXCITED STATE INTRAMOLECULAR GEOMETRICAL RELAXATION

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BY

JOSEPH KORDAS

Trans-1,1,4,4-tetraphenyl-2-methyl butadiene (TPMB) undergoes an intramolecular twisting relaxation after excitation. The extent of this relaxation depends on the viscosity of the medium. The fluorescence energy maximum exhibits a blue shift and the fluorescence intensity is greatly enhanced as the medium becomes rigid.

We have focused our attention on the quantitative aspects of fluorescence energy and intensity dependences on viscosity and temperature of the medium with the purpose of explaining the effect of each of these parameters on radiationless processes of the excited state of TPMB and similar molecules. Of particular interest is the separation of viscosity and temperature effects. While the fluorescence energy depends only on the viscosity of the medium, the quantum yield depends both on viscosity and temperature. Fluorescence intensity variation as the viscosity is changed by lowering temperature is interpreted in terms of solvent as well as solute activation energies. Solvent activation energies correspond to viscosity activation energies obtained from the macroscopic viscosity dependence on temperature. Solute activation energies correspond to torsional frequencies in the excited state of TPMB.

Joseph Kordas

A selective red-quenching mechanism is proposed to account for anomalous shifts that are dependent on the temperature and polarity of the medium. Selective red quenching appears to be a general phenomenon occurring in situations where excited molecules undergo geometrical relaxation during their lifetime.

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To my wife

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CHAPTER 1

GENERAL INTRODUCTION

Many membrane processes are dependent on the fluidity of the membrane (1). Membrane fluidity determines the passive permeability of both ionic and neutral species. It influences active transport--the movement of carrier species across the membrane or the rotation of carrier enzymes. The ability of a membrane to undergo a conformational change also depends on its fluidity as does cellular development. In fact, any enzymatic process that occurs at the membrane surface may be affected by the membrane's fluidity.

Many studies have been done to demonstrate both the fluidity of membranes and the liquid crystal nature of membranes, selective examples follow:

1. Spin Label Studies.

a.) McConnel et al (2,3,4) found the rate of rotation of a nitroxide radical depends on its attachment site on the fatty acid. The closer it is to the polar end the less it spins.

b.) When the radical is attached near the hydrocarbon end, the medium is found to be more fluid.

2. Rhodospin Studies.

Cone et al have (5) demonstrated that rhodospin is able to rotate in the plane of the rod outer segment disk. Also Blasie (6) found that rhodospin sinks into the hydrocarbon region of the membrane upon bleaching.

3. Cell Fusion Studies.

Frye and Edidis found (7) that after fusing tissue culture cells of mouse and human origin by using Lendai virus the antigens present on the surface of the two cells exchange and result in a mosaic. Their conclusion is that the antigens are free to diffuse along the surface of the cells (lateral diffusion).

4. Model Membranes Studies.

Weber and Shinitzky (8,9) have used fluorescene depolarization (steady state) of perylene and 2-methylanthracene to investigate the fluidity of the interior of micelles. They found that the depolarization of these molecules in micelles is the same as that in hydrocarbons with mascroscopic viscisities of between 17-50cp. They found also that cholesterol increased the rigidity of the model membranes. Gilter (10) using the same technique, found that the depolarization of these molecules in hemoglobin free erythrocytes is equivalent to that in a hydrocarbon with macroscopic viscosity of 100-200cp.

These studies have shown that:

1. Membrane interiors and fluid and that this fluidity depends on the composition of the membrane.
2. Many biological processes are very dependent on the fluidity of membranes.

It is therefore worthwhile to investigate possible methods of determing membrane fluidity. Many people (11,12,13,14,15) have investigated membrane and model membrane structure using ESR and NMR. Both

of these techniques are widely used. However optical methods also have great potential for such use. I will discuss some of the possible fluorescent methods and then in the next chapter characterize a sterically hindered molecule which might be a possible optical probe for environmental fluidity.

There are several excited state processes that maybe useful for investigating medium rigidity. Some of these are listed in figure 1.

Polarization

The most popular fluorescence technique for investigating membrane structure is steady state fluorescence depolarization. Molecules absorb light only when the electric vector of the light is parallel to the transition moment of the molecule. They also emit light with the electric vector parallel to the transition moment of molecule. Therefore if one excites a random rigid sample with polarized light, the fluorescence will also be polarized parallel to the exciting light. However, if the molecules are free to rotate and rotate rapidly compared to the fluorescence lifetime the fluorescence will be depolarized-molecules are able to randomize before emission.

Perrin (16,17) derived the following relationship:

$$(1/p - 1/3) = (1/Po - 1/3)\ (1+6R\tau)$$

where $p=(I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$, Po = limiting polarization, τ = fluorescence lifetime and R = rate of rotation.

Einstein (18) found that if the molecule is spherical and in an isotropic medium $R=1/6(kT/\eta V)$ where V= volume cf the molecule.

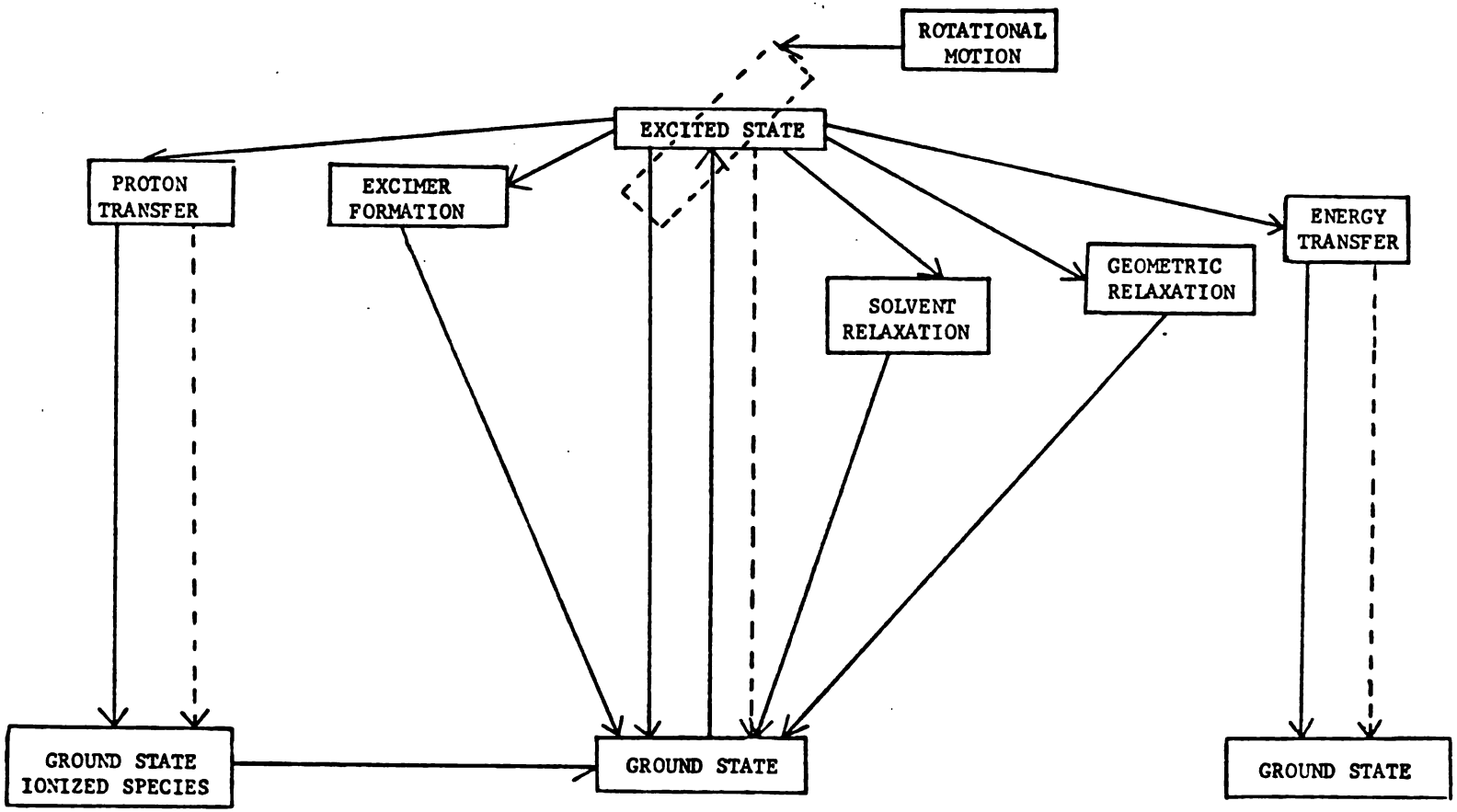

FIGURE 1

SELECTIVE EXCITED STATE PROCESSES

Hence by determining the fluorescence polarization p, one can get an approximation of η . In order to get p_o, p must be studied as a function of T/η and extrapolated to $T/\eta = 0$. This type of measurement cannot be made with biological systems. Typically one determines p in the membrane environment and finds a hydrocarbon in which the polarization is the same. One can then say that the molecule experiences a rotational resistance similar to that in a hydrocarbon of a certain macroscopic viscosity.

Weber and Shinitsky (8) using perylene and 2-methyl anthracene, found that for micelles made from the series of detergents lauryltrimethyl-ammonuum bromide, myristyltrimethylammonium bromide, cetyltrimethylammonium bromide and stearyldimethylbenzylammonium bromide, the polarization at 27°C is equivalent to that in a hydrocarbon with a macroscopic viscosity in the range of 17-50cp. The change in fluidity with temperature was found to follow a single exponential with an activation energy in the range of 6.1-9.6 kcal/mole^{-1}. Also they found that the microfluidity increases rapidly with the addition of cholesterol. Cogan et al (9) has used this same technique to investigate the microenvironment of lecithin dispersions and observes a phase transition for the dipalmitoyllecithin dispersions.

Perrin's relationship only holds for spherical molecules. Most flurescent aromatic molecules are planar rather than spherical and therefore have two axes of rotation, in-plane rotation and out-of-plane rotation. Weber (8) had derived the following relationship for this case:

$$r_o/r = 1+6/\lambda \; (R_p(2\cos^2\alpha - 1) + R_{op}\cos^2\alpha) \; / \; (3\cos^2\alpha - 1)$$

Where r is the anisotropy defind as $r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$. Rp is the rate of rotation about an axis normal to the ring plane (in-plane rate of rotation), R_{op} is the rate of rotation about an axis contained in the ring at right angle to the absorption oscillator, λ is the rate of fluorescence of the molecule and α is the angle between absorption and emission ascillators.

This relation reduces down to an equation in only one rate of rotation for particular values of p_o:

when p_o — 1/7 $\qquad r_o/r = 1+6R_{op}/\lambda$

when p_o — -1/3 $\qquad r_o/r = 1+6R_p/\lambda$

Therefore from studying the polarization at wavelengths of excitation at which p_o=1/7 and p_o=-1/3, one can obtain the in-plane and out-of-plane rates of rotation and learn both about the microfluidity of the environment and the degree of anisotropy of the medium.

Thomas studied (19,20,21) the steady state polarization of pyrene and 2-methyl anthrancene to determine the microviscosity of micellar interiors and Escherichia coli membrane vesicles. He finds that the polarization date agrees with fluorescence decay quenching data.

Time Dependent Polarization

Jablonski (22) in 1961 derived a relationship for the time dependent anisotropy which he defined as $A(t) = (I_{\parallel}(t) - I_{\perp}(t))/(I_{\parallel}(t) + 2I_{\perp}(t))$. He found that for rigid spherical molecules in an isotropic medium

$$A(t)=A_o\exp(-t/\phi)$$

where $\phi=V\eta/kT=1/6D$, is the rotational correlation time, $A_o=.4(3\cos^2\alpha-1)/2$. α is the angle between emission and absorption moment, and D is the rotational diffusion coefficient. For ellipsoids the expression becomes more complicated (23,24,25,26,27).

$$A(t)=A_o\sum_{i=1}^{3}f_i\exp(t/\phi)$$

Here ϕ's are related to the rotational diffusion coefficients of the ellipsoid.

This simplifies to $A_o\exp(t/\phi)$ if the major axis is parallel to the emission moment. For completely asymmetric molecules A(t) is a sum of five exponentials (23,24).

By studying the time dependent anisotropy, one can determine if the molecule which is rotating is spherical and if the medium is isotropic. If this is the case, one gets a single exponential decay for A(t) and can then determine ϕ and from ϕ get η.

Until recently, time dependent anisotropy measurements have only been made on large macromolecular systems (23). For instance, Stryer (24) studies the time dependent anisotropy of dansyl-lysine bound to an antibody. Also Wahl and Timasheff (28) used fluorescence time dependent anisotropy to study the aggregation of β-lactoglobulin A. From the rotational correlation times obtained, they were able to estimate the size of the various aggregates.

Recently Vanderkooi and Cehelnik (29,30) have begun to use time dependent depolarization of small molecules to probe liquid crystal structure (29,30). Cehelnik studies the time dependent anisotropy of

all-trans-1,6-diphenylhexa-1,3,5-triene in several solvent systems. In methylcyclohexan at 25°C, he found rotational diffusion to be complete within 1 nano-second after excitation, while in paraffin oil the rotation correlation time =4.83 X 10^{-9}sec. He also found that for a nematic liquid crystal medium (cholesteryl laurate/ cholesteryl chloride) orienated by an electric field A(t) remains constant for a period of $4\frac{1}{2}$ lifetimes (τ =13.0nsec in methylcyclohexane). Vanderkooi uses 12-(9-anthroyl) stearic acid to investigate the fluidity of phospholipid dispersions and red blood cell membranes. She found that the rotational correlation time, for 12-(9-anthroyl) stearic acid fluorescene anisotropy at 37°C is 7.8 X 10^{-9}sec in normal red blood cells and 8.5 X 10^{-9}sec in blood cells containing twice the normal complement of cholesterol. Time dependent depolarization measurements may be a very fruitful method of investigating membrane environment; however, the ratio of ϕ/τ limites this technique. If the lifetime is long compared to the rotation correlation time, fluorescence will be depolarized at all observable times after excitation. This is the case for the simple situation of an aromatic chromophore in a fluid solvent at 25°C. On the otherhand, if the lifetime is short compared to the correlation time, emission will be completely polarized at all observable times after excitation. The latter is the case for diphenylhexatriene in the liquid crystal medium.

Excimer Formation

Another excited state process which is a potential monitor of environmental fluidity or "microviscosity" is excimer formation.

There are two types of excimers, intermolecular and intramolecular. In the first case the excited complex consists of two separate identical monomers, one initially in an excited state and the other in the ground state. In the second case, the excimer is formed by two non-interacting chromophores which are segments of a single molecule. Intermolecular excimer formation is concentration dependent, while intramolecular excimer formation is concentration independent.

The formation of intermolecular excimers in fluid membranes or any other medium is a diffusion controlled process (30,31,32). The value of the coefficient of lateral diffusion, $D_{diff,}$ is obtained from the second order rate constant of excimer formation which can be obtained from the ratio of the excimer to the monomer fluorescence yields (31). A value for the microfluidity of the medium may be obtained directly from the Einstein-Scholuchowski diffusion theory (32). It relates the rate of diffusion to the viscosity of the medium by the expression:

$$k_a = 8(RT/3000\,\eta\,)\ (pa/b)$$

in which pa/b=1 for pyrene. The relationship between the half intensity concentration, $C'_{h'}$ to the viscosity follows:

$$C'_h = k_f = k_r)\ (3000\,\eta/8RT)\ (I_m^{max}/I_e^{max})$$

where $I_e/I_m = C/C'_h$, C'_h is the concentration at which the monomer fluorescene yield is equal to the excimer yield, k_f= rate of fluorescence of monomer, k_{rl}= rate of radiationless decay of monomer and I_m^{max} and I_e^{max} represent the maxium intrinsic fluourscence. Therefore the

value of C'_h can be used as an index of viscosity. Pownall and Smith (32) plotted I_e/I_m versus concentration of pyrene in propanol and obtained C'_h from the reciprocal of the slope. They then plotted C'_h for several solvents versus the kinematic viscosity of the solvents. They found, as expected, that the relationship between C'_h and η is linear and from this they obtained a value for $(k_f + k_{rl})$ (3000/8RT) $(I_m^{max}/I_e^{max}) = .73 \times 10^{-3}$. Therefore by obtaining C'_h, they determined the viscosity of the medium. Pownall and Smith used this technique to study the fluidity of the hydrocarbon region of micelles. They found that η varies from 150-190cp for micelles formed with various detergents. The pointed out that these values are consistently higher than those found by fluorescence depolarization; however, they offered little insight into this descrepancy.

The advantages of this technique over fluorescence depolarization measurements are: 1. Excimer fluorescence is not affected by the depolarizing effect of micellar rotation. 2. Corrections necessary to account for turbid solutions which scatter and depolarize fluorescence are not necessary. and 3. The concentration of surfactant does not appear to be important in the dynamics of excimer fluorescence as long as the concentration is greater than the critical micellar concentration.

One great problem with using intermolecular excimers to investigate medium fluidity is that the measurement made is the concentration value where $I_e = I_m$. It is very difficult to measure the concentration of an aromatic molecule within such systems as micelles. Many assumptions must be made as to the effective volume of the micelle. Also one must assume that all of the fluorophore goes into the micelle. If one

is studying η as a function of temperature, one must assume that the concentration remains unchanged.

The concentration problem can be eleminated by using intramolecular excimers. This type of excimer formation is concentration dependent. However, it is not strictly diffusion controlled. One might say that it is a quasi-diffusion controlled reaction. Consider a molecule like dinapthylpropane. The two phenyl rings must diffuse together to form the excimer, but the diffusion is restricted by the methylene chain (33). One way to use such a molecule would be to calibrate the I_e/I_m versus η, and then use this calibration curve to determine η. We plan to do this in the future. Unfortunately this molecule's sensitivity to oxygen complicates the measurement. Oxygen quenching affects the I_e/I_m ratio. This is, oxygen quenches the excimer more efficiently than the monomer.

Geometric Relaxation

The last excited state process that I will talk about in this introduction is geometric relaxation. I will only talk very briefly about it here. This is the excited state process which I have chosen to investigate as a possible method of measuring medium fluidity. In the next chapter I will talk about the characterization of the fluorescence properties of a sterically hindered molecular which might be appropriate as a fluidity monitor.

Many molecules which normally would be planar in their ground state are forced into nonplanar geometries due to steric hindrance. These molecules in the excited state tend to become more planar. As an example consider tetraphenylmethylbutadiene (TPMB). It is

believed that in its ground state one phenyl ring is forced out of the plane of the rest of the molecule by the steric hindrance between the phenyl ring and methyl group (see structure in chapter 2). The out of the plane rotation in the ground state is about an essential single bond. In the excited state this bond has more double bond character; therefore, the excited state equilibrium configuration is more planar than that of the ground state. The Franck-Condon principle states that nuclear movement is much slower than electronic movement. Therefore on excitation the molecule is not promoted into its excited state equilibrium configuration but into its Franck-Condon state. The molecule then relaxes into its equilibrium excited state geometric configuration. One expects the rate of this relaxation to depend on the viscosity of the medium. I characterize the fluorescent properties of TPMB in the next chapter with the purpose of exploring the possibility of using this type of excited state relaxation to probe fluidity.

CHAPTER 2

EXCITED STATE INTRAMOLECULAR TORSIONAL RELAXATION: VISCOSITY, TEMPERATURE AND MEDIUM EFFECTS ON THE FLUORESCENCE CHARACTERISTICS OF A STERICALLY CROWDED MOLECULE

Introduction

Flexible molecules that undergo intramolecular torsional vibration around a bond linking two interacting mioeties of the chromophore are weakly or non-fluorescent. The low frequency oscillations lead to efficient radiationless deactivation of the excited molecule in fluid media. Upon "freezing" these modes or limiting their amplitudes, the molecule becomes strongly fluorescent. This may be accomplished by using media of high viscosities or by rendering the molecule rigid via a chemical bond.

In addition to the fluorescent enhancement effect, flexible molecules may undergo a change in their equilibrium geometric configuration upon excitation, such as in sterically crowded molecules where the steric strain can be relieved by internal rotation. In these cases a large Stokes shift of the fluorescence maximum is observed in fluid media. The fluorescence emission may originate from the equilibrium excited state, Franck-Condon state or an intermediate geometric configuration depending on the relative magnitudes of the rate constants of the rotational relaxation process (k_r) and fluorescence (k_f). If k_r is viscosity dependent one may expect the fluorescence maximum to be a sensitive function of the viscosity pro-

vided that $k_f < k_r$. As the medium becomes more viscous the fluorescence maximum shifts progressively to higher energies and a maximum blue shift is observed in rigid media.

In an earlier study (34) we have shown that the fluorescence maximum of a sterically crowded molecule, namely trans-1,1,4,4-tetraphenyl-2-methyl butadiene (TPMB), lies 3700 cm^{-1} at higher energies in rigid glass at 77°K compared to its maximum in fluid medium at room temperature as shown in Figure 2. The results were interpreted in terms of an intramolecular twisting relaxation process that is fast in fluid medium. In rigid glass however, the twisting relaxation is slowed down due to the high viscosity of the medium and the emission originates from an excited molecule which has a geometric configuration similar to that of the ground state, i.e. from the Franck-Condon state which lies at higher energies relative to the equilibrium excited state. Potential-energy curves are drawn qualitatively by considering both the variation of the resonance energy and the steric energy as a function of the angle of twist about an essential single bond (34). These curves for the ground and excited state are shown in Figure 3. The curve in the upper state is a steeper function of the angle of twist reflecting the enhanced double bond character of the bond in question. The curves demonstrate the large Stokes shift in fluid medium, compared to that in rigid medium. Emissions from relaxed, intermediate, and Franck-Condon states (shown in Figure 3) demonstrate the blue shift of fluorescence as the medium becomes more rigid.

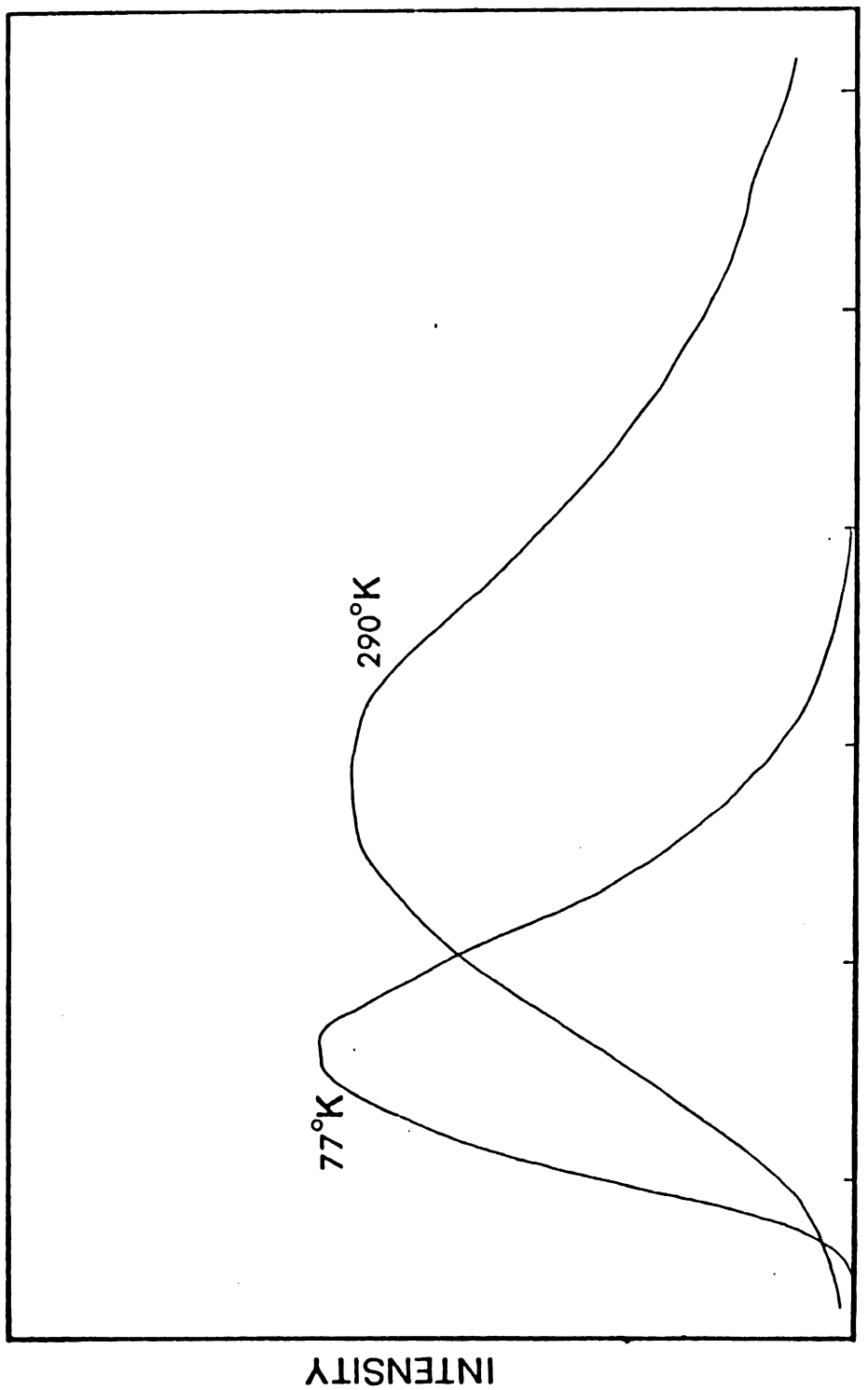

FIGURE 2. Emission spectra of TPNB in 3-methylpentane at room temperature (fluid medium) and at 77°K (rigid glass). Emission at room temperature is much less intense and is recorded at a higher sensitivity.

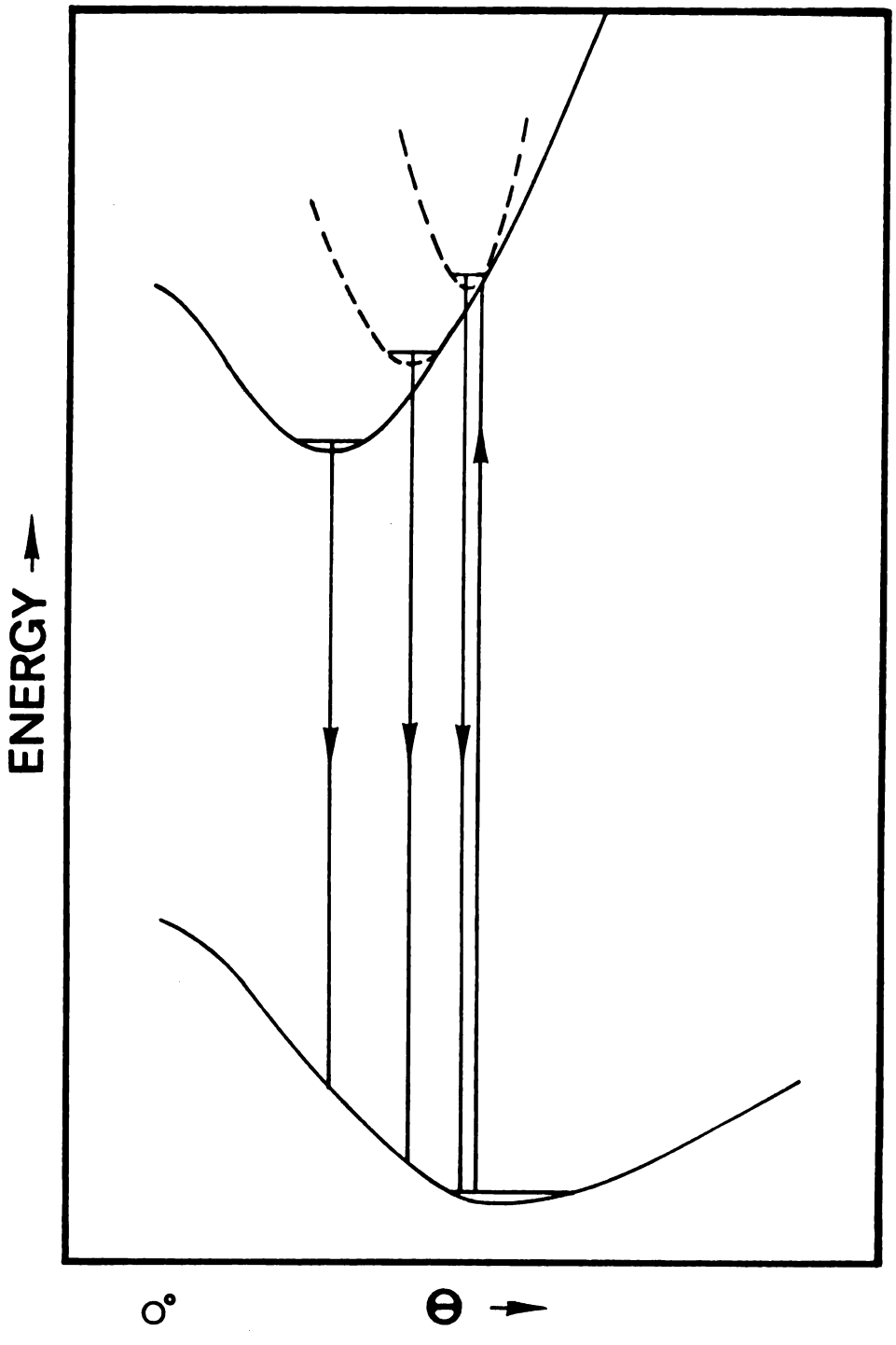

FIGURE 3. Qualitative potential-energy curves for the ground and excited states of TPMB. Emissions from a relaxed state (fluid medium), a Franck-Condon state (rigid medium) and an intermediate state are demonstrated.

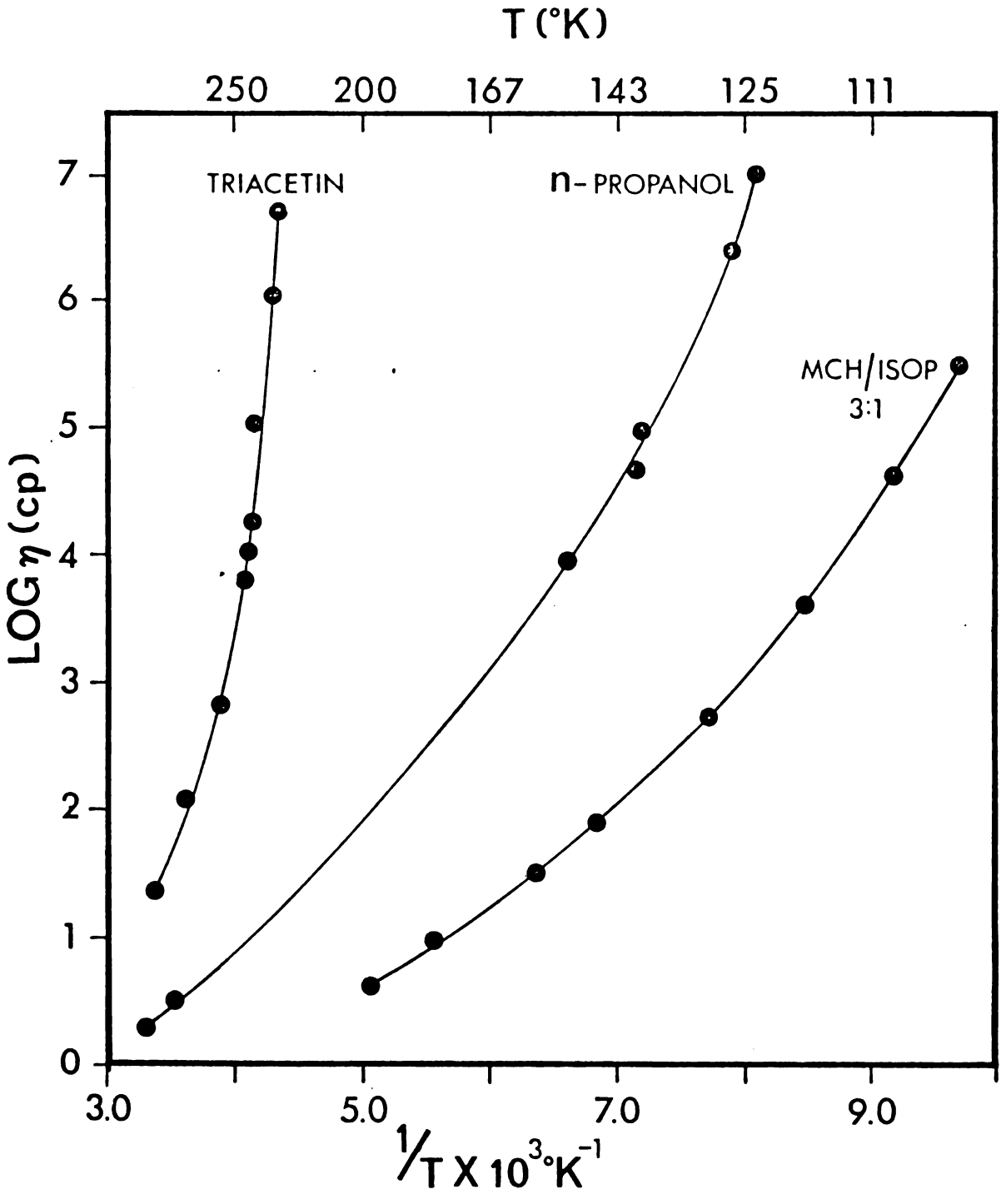

FIGURE 4. Viscosity-temperature data for triacetin, n-propanol and methylcyclohexane/isopentane mixture 3:1. These data are taken from the references cited in the text.

In addition to the blue shift phenomenon, we presently show that TPMB exhibits also a remarkable fluorescence enhancement effect as the viscosity of the medium is increased. We have focused our attention on the quantitative aspects of fluorescence energy and intensity dependences on viscosity and temperature of the medium with the purpose of explaining the role of each of these parameters on the radiationless processes of the excited state of TPMB and similar molecules. Of particular interest is the separation of viscosity and temperature effects. To accomplish this, we used various approaches namely:

1. Use of mixtures of solvents with different viscosities at constant temperature.
2. Use of solvents whose viscosity dependences on temperature are different.
3. Studying temperature effects on luminescence of a sample in a plastic matrix.

Experimental

Materials. Phillips pure grade 3-methylpentane (3MP) was purified by distillation and by passing through an activated silica gel column.

Spectroquality isopentane (IP), methylcyclohexane (MCH) and glycerol (obtained from Matheson Coleman and Bell Company) were used without further purification. Ethanol, n-propanol and butanol were distilled while octanol and triactin were vacuum distilled. Paraffin oil (obtained from Baker) was used without further purification. All solvents were spectrally transparent above 350 nm and exhibited no

fluorescence when excited above 350 nm. In Figure 4 the viscosities of three solvents are ploted as log viscosity versus 1/T. The data for n-propanol are taken from Denny (35) while that for triacetin and methylcyclohexane/isopentane (3:1) are taken from VonSalis (36).

A pure sample of TPMB was further purified by successive recrystallizations from ethanol. The plastic samples were formed by dissolving both TPMB and the plastic (either polystyrene or poly vinyl acetate) in spectroquality $CHCl_3$ and evaporating off the solvent. A thin transparent plastic film was formed with the TPMB embedded in the matrix.

Methods. The Aminco-Keirs Spectrophosphorimeter (1P28 Phototube) was utilized to obtain the emission intensity as a function of temperature. Relative quantum yields were obtained by measuring areas under emission curves and are uncorrected for phototube and monochromator response. In a limited temperature range, where only little shift of emission occurs, these correction factors are identical. A quartz dewar with a flat quartz excitation window and a 1 cm square suprasil cuvette were used. The temperature of the sample was controlled by boiling liquid nitrogen using a power resistor and allowing the N_2 gas to flow into the sample dewar. The temperature of the sample was monitored through a thermocouple (copper, constantan) attached to the outside of the cuvette immediately above the point of excitation. Fifteen minutes were allowed for equilibration at every point after the thermocouple reached the appropriate temperature. Comparing readings of thermocouple on the outside and inside of a cuvette containing

solvent, one finds not more than 1°C difference between thermocouples over wide ranges of temperature (room temperature down to -151°C).

A 750 mm Czerny-Turner Spectrometer (Spex 1700-11) in conjunction with a PAR lock-in amplifier (HR-8) and an EMI 9558 QA phototube was used to obtain the energy of the fluorescence maximum $\bar{\upsilon}_F$. These spectra also are presented uncorrected for phototube and monochromator response.

Absolute quantum yields for TPMB in various solvents at room temperature were obtained by utilizing a double beam quantum yield instrument interfaced with a PDP 11 computer (37). This instrument measures both emission and absorption and corrects for both monochromator and phototube response. It is programmed to correct for innerfilter effects for solutions with optical densities up to 1.0. All solutions used here were 10^{-4} M. The excitation wavelength was again 360 nm. Quinine sulfate (10^{-6} M) in 1 N H_2SO_4 was used as the quantum yield standard with a value of 0.54. Because this apparatus uses phototubes with a weak red response and because the measured quantum yields are very small, these absolute quantum yields are only approximate ($\pm$ 20%).

It was noticed that a slow photochemical process occurs as a result of excitation at short wavelengths. This leads to changes in the intensity of absorption and emission spectra. The excitation wavelength of 360 nm was used throughout our study; at this wavelength, minimal changes occurred upon excitation. Fresh undegassed solutions were used. Degassing the samples produced no change in quantum yields.

Viscosity Dependency of Fluorescence Energy

The fluorescence frequency maximum $\bar{\upsilon}$ F was measured in three different media as a function of temperature. The viscosities of the solvents used, namely n-propanol, triacetin, MCH/IP mixture (3:1), are plotted as a function of temperature in Figure 4. The differences in viscosities of these media at a given temperature enable us to distinguish between temperature and viscosity effects. The fluorescence energy is plotted vs. the logarithm of viscosity in Figure 5. At $\eta \geq 100$ cp the fluorescence maximum exhibits a blue shift, the magnitude of which increases continuously in all three media as the viscosity is increased, reaching a maximum value of about 23,200 cm^{-1} at very high viscosities. Plots of fluorescence energy maximum, $\bar{\upsilon}_F$ vs. temperature in the three media are shown in Figure 6. In general $\bar{\upsilon}_F$ increases rapidly in a narrow temperature region which differs from one medium to the other. In contrast these large changes of $\bar{\upsilon}_F$ occur at nearly the same viscosity. The inflection point of $\bar{\upsilon}_F$ vs. temperature plots occurs at approximately the same viscosity, (the arrows in Figure 6 mark a viscosity of 10^4 cp) in spite of the fact that such viscosity is reach at widely different temperatures varying from 120°K up to 250°K, depending on the medium. This clearly demonstrates that $\bar{\upsilon}_F$ depends primarily on viscosity and not on temperature.

In mixtures of 3MP and paraffin oil, the fluorescence energy maximum of TPMB remains essentially the same up to viscosities of about 30 cp. In pure paraffin oil at 298°K (η =77 cp) the observed

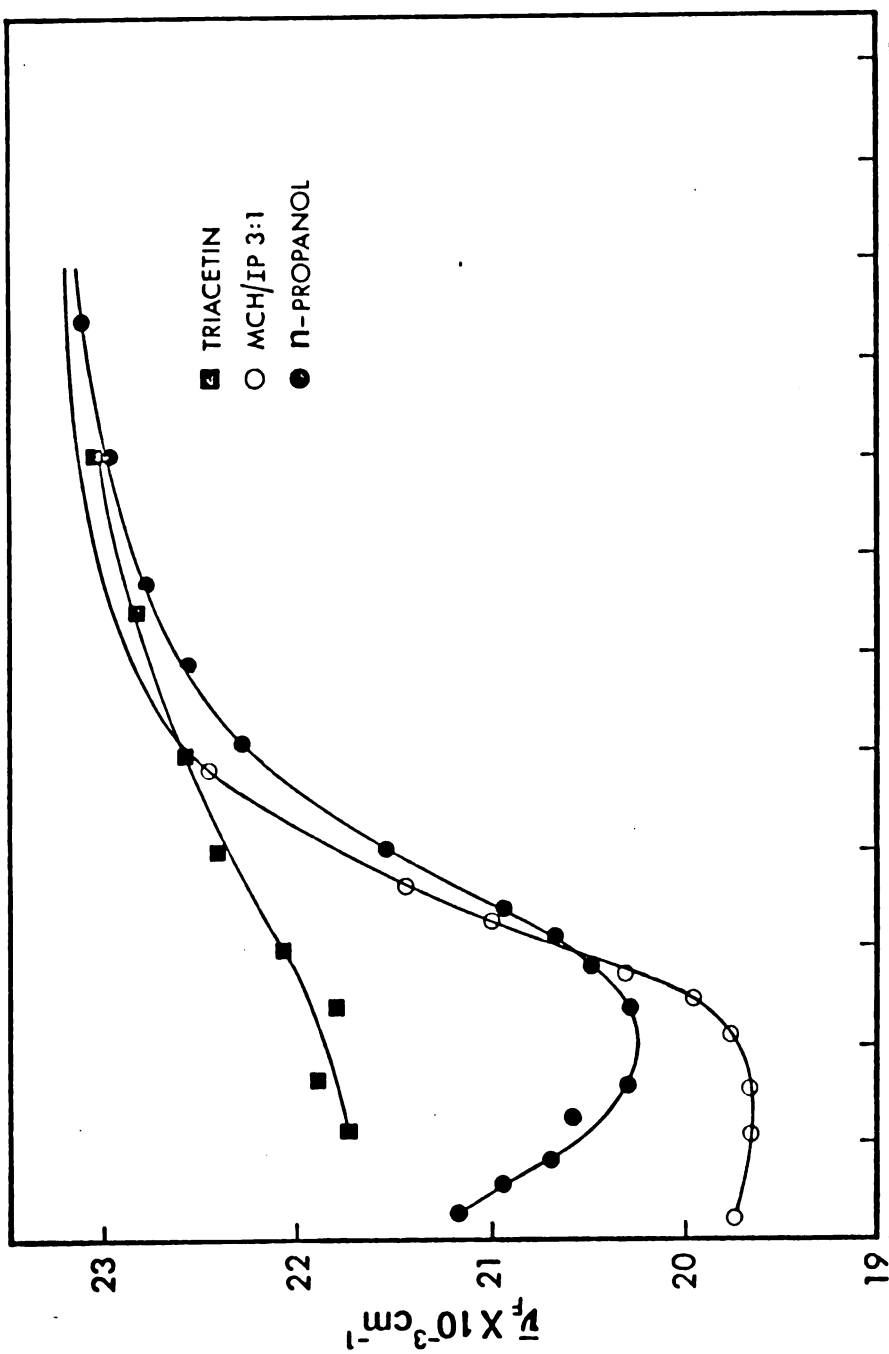

FIGURE 5. The variation of the energy of the fluorescence maximum ($\bar{\nu}_F$) in three different solvents as a function of viscosity. The vicosity was varied by lowering the temperature.

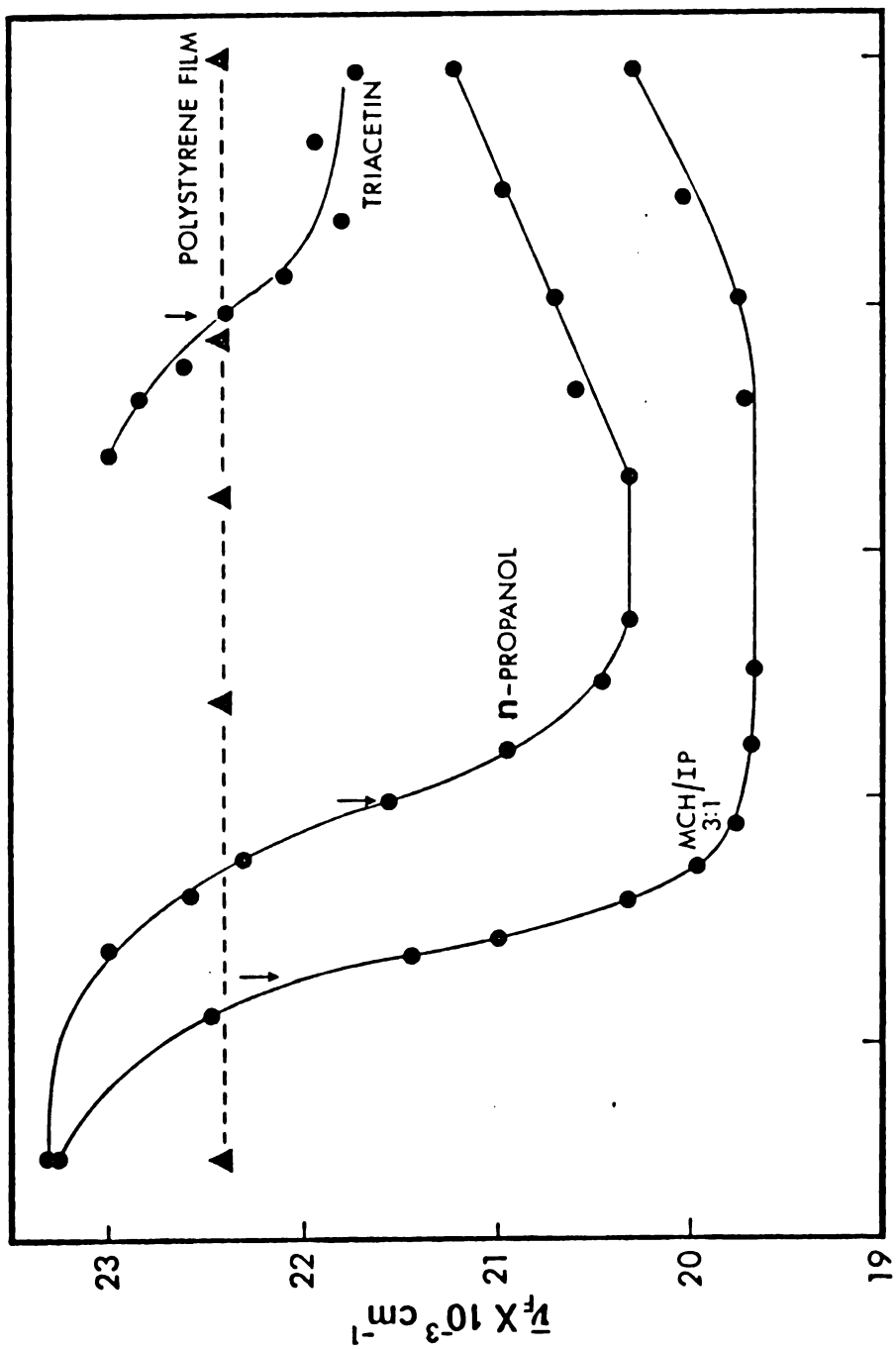

FIGURE 6. The variation of the energy of the fluorescence maximum ($\bar{\upsilon}_F$) as a function of temperature in four different media. Arrows indicate the temperature at which the viscosity of the medium is 10^4 cp. The dashed line indicates the values of $\bar{\upsilon}_F$ in polystyrene film at different temperatures.

blue shift is about 530cm^{-1}. At 270°K in paraffin oil the spectrum is further blue shifted by 480cm^{-1} and the half width decreases. These effects are attributed solely to viscosity changes since 3MP solution begins to exhibit a blue shift below 150°K and since the viscosity of 3MP is little affected by cooling down to 200°K. The frequency maxima $\bar{\nu}_F$ and half widths $\Gamma_{\frac{1}{2}}$ of the fluorescence band are compared under various conditions in Table I. At low viscosities, the fluorescence spectra are appreciably broader than at high viscosities. This broadening is in part due to emission from excited molecules at various states of relaxation and in part due to the detailed shapes and relative displacements of the potential surfaces involved in emission.

Samples of TPMB in plastic matrices (in polystyrene and polyvinyl acetate) were studied at different temperatures. The fluorescence frequency maximum exhibits very little change as shown in Figure 6, which further supports our earlier conclusion of temperature invariance of $\bar{\nu}_F$.

The value of $\bar{\nu}_F$ in plastic is 22,400cm^{-1} which corresponds to a macroscopic viscosity of $\eta \sim 10^6$cp. An increase in microscopic viscosity by lowering the temperature would have been observed since $\bar{\nu}_F$ in plastics is about 1,000cm^{-1} lower than its value in rigid glasses. This indicates that excited TPMB exhibits some intramolecular twisting relaxation in its plastic cage. Our observations also support the expectation that the microscopic viscosities of plastics are essentially invariant with temperature in the region of our study.

TABLE 1

Fluorescence energy maximum $\bar{\nu}_F$ and half band widths $\Gamma_{\frac{1}{2}}$ of TPMB in 3MP, paraffin oil (P.O.) and a plastic matrix at various temperatures.

	3MP/77°K	3MP/298°K	P.O./298°K	P.O./270°K	Plastic/298°K-77°K
$\bar{\nu}_F$ (cm^{-1})	23,256	20,325	20,859	21,340	22,271
$\Gamma_{\frac{1}{2}}$ (cm^{-1})	3,500	5,680	4,840	4,340	4,325

It should be noted that in the low viscosity region, $\bar{\nu}_F$ has a different value depending on the polarity of the medium. This behavior is anomalous since one would not expect polarity effects for the nearly non-polar TPMB molecule. Moreover, Figures 5 and 6 show that the fluorescence maximum in n-propanol and hydrocarbon solvents initially red shifts upon increasing the viscosity (in the range 0-100cp) before it blue shifts as the viscosity is further increased. An interpretation of these observations will be discussed later in terms of a selective red-quenching phenomenon which is more prominent in polar solvents.

Fluorescence Intensity

Fluorescence intensities in two different media, namely: MCH/IP (3:1) and n-propanol were measured as a function of viscosity. The

viscosity was changed by lowering the temperature and its value was obtained from the viscosity-temperature data discussed in the experimental section. Intensities (corrected for changes in absorption due to temperature variation) are plotted as log I_F vs. log viscosity in Figure 7 and as log I_F vs. temperature in Figure 8. The values given are relative to the fluorescence intensity at room temperature in n-propanol. It should be noted that the fluorescence intensity at room temperature in n-propanol is lower than that in hydrocarbon solvent although their viscosities are comparable. The reverse is true at lower temperatures due to the higher viscosity of n-propanol. Fluorescence intensity gradually increases as a function of viscosity, reaching a maximum value which is the same for both solvents. Since degassing does not have any affect on fluorescence intensities at any temperature, one may assume that the only radiationless process which deactivates the excited singlet occurs directly to the ground state and not via a triplet state, i.e. deactivation occurs mainly via the internal conversation process $S_1 \rightsquigarrow S_0$. The quantum yield at high viscosity is probably unity since no phosphorescence is observed under any conditions and since the intensity levels off at viscosities where some relaxation can still occur. Assuming that the quantum yield is one at high viscosity, the extrapolated room temperature quantum yields in n-propanol and MCH/IP mixture are .004 and 0.013 respectively. Measured quantum yields at room temperature in these two solvents are 0.003 and 0.009 respectively. The measured quantum yields are only

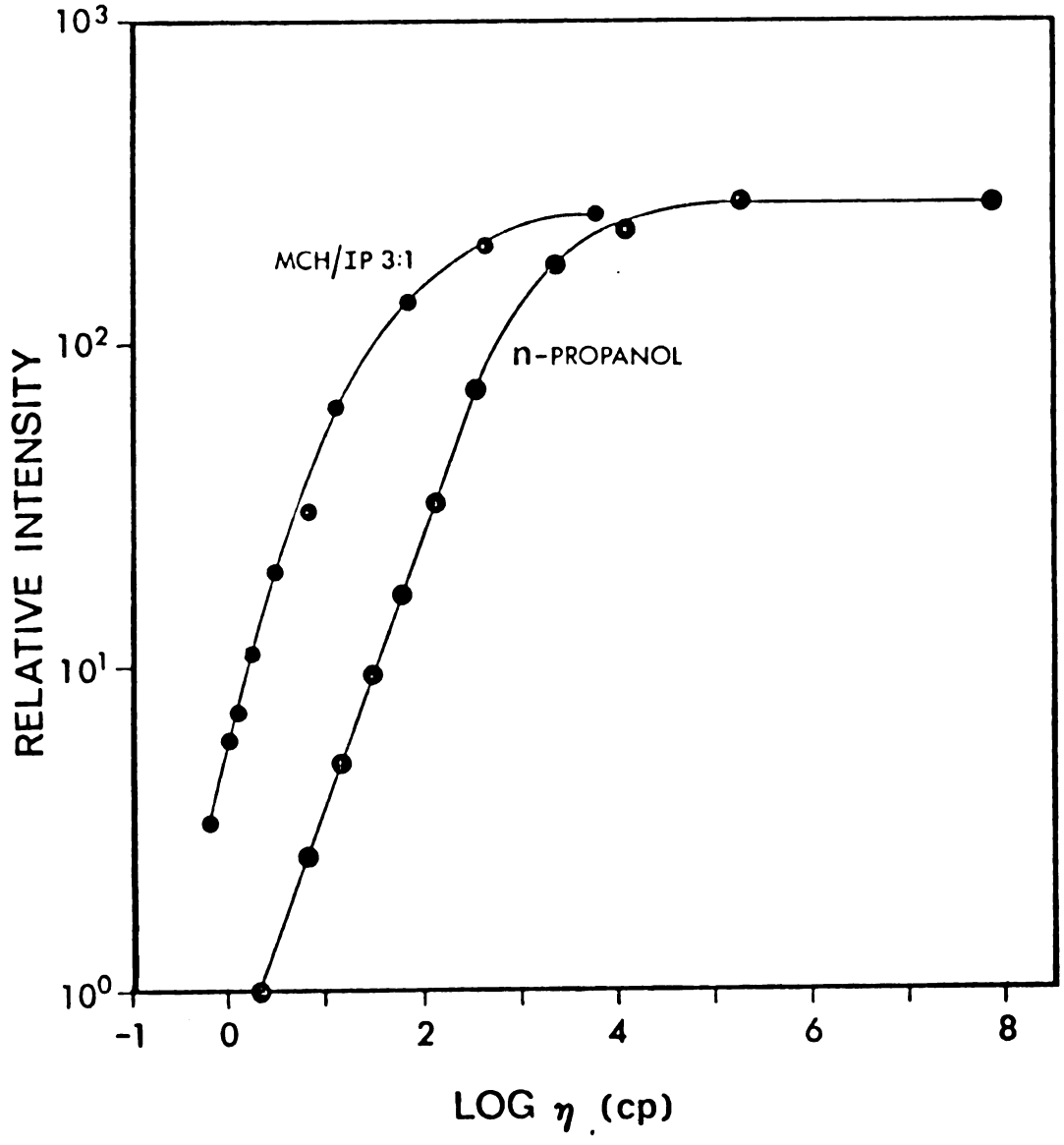

FIGURE 7. The change of fluorescence intensity of TPMB in n-propanol and in MCH/IP (3:1) as a function of viscosity. The intensity is measured relative to the value at 298°K in n-propanol.

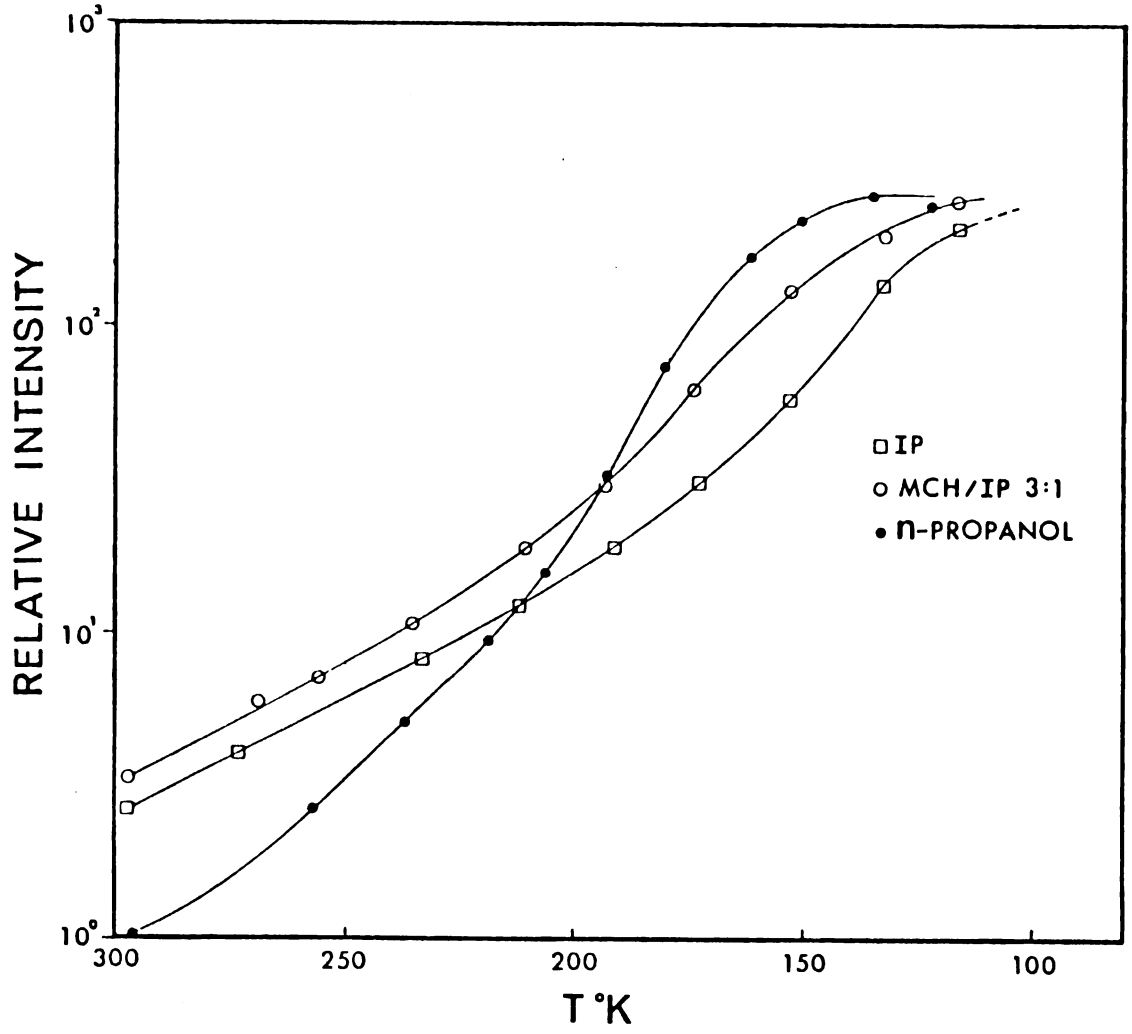

FIGURE 8. The change of fluorescence intensity of TPMB in n-propanol, MCH/IP (3:1) and IP as a function of temperature. The intensity is measured relative to the value at 298°K in n-propanol.

approximate with an error of almost 20%. The fluorescence intensity (corrected for changes of optical density with temperature) increases by a factor of 270 in n-propanol in the temperature range (298-135°K) and by a factor of 75 in MCH/IP mixture in the temperature range (298-117°K). In plastic, the corrected fluorescence intensity remains nearly constant in the same temperature rante. However, one must note that the frequency maximum in plastic corresponds to macroscopic viscosities where the quantum yield has already achieved it maximum value in the two solvent systems.

In order to separate temperature from viscosity effects, the fluorescence intensities were measured in mixtures of paraffin oil and 3MP at room temperature, where the viscosity range varied from 0.5 in 3MP to 45cp in paraffin oil/3MP mixture (9:1). The fluorescence intensity increases by a factor of 7.2 over this range in these mixtures. This change is attributed soley to viscosity. In MCH/IP mixture the fluorescence intensity increases by a factor of 33 over the same viscosity range when the temperature is lowered from 298°K down to 160°K; and therefore, one may attribute a factor of 4.5 as being due to temperature effects on luminescence yield. This value is obtained assuming a linear variation of $\log\eta$ with percent volume of paraffin oil in the mixture.

In ethanol/glycerol mixtures where the viscosity at room temperature varies from 1.2cp for ethanol to 63cp for ethanol/glycerol mixture (4:6), the intensity increases by a factor of 8.7. In an experiment where the viscosity of propanol was increased from 2.3cp

at room temperature to 63cp at 205°K, the intensity has increased by a factor of 18. Thus a factor of approximately 2 may be attributed to lowering temperature from 290 to 205°K. Here we assume that the polarity of ethanol/glycerol mixtures is invariant and is the same as that of propanol.

Selective Red-Quenching

As mentioned before the fluorescence energy maximum $\bar{\nu}_F$ decreases as the viscosity is decreased until it reaches a minimum value at viscosities around 100cp where complete relaxation occurs during the lifetime of the excited state. Further lowering of the viscosity should not have any effect on $\bar{\nu}_F$. Indeed if the viscosity is varied in the range of 1-30cp by mixing 3MP and paraffin oil in various proportions at room temperature, $\bar{\nu}_F$ remains invariant. However, in Figures 5 and 6, it is clear that as the viscosity is lowered $\bar{\nu}_F$ decreases reaching a minimum value near viscosities of about 100cp, but then it begins to increase upon further warming of the sample. This clearly demonstrates that the increase in $\bar{\nu}_F$ in a fluid medium is due to temperature and does not reflect a real change in transition energies. This behavior was also observed for arylethylenes (38) but no interpretation was given.

To interpret this behavior one should make the following points: 1.) Emission is composite in nature, i.e. it originates from excited molecules which have different geometric configurations depending on the extent of relaxation at the time of the transition. Although in

a fluid medium most of the molecules relax to the equilibrium excited state geometric configuration before emission occurs, a few molecules still emit from partially relaxed geometric configurations. This explains in part the fact that the emission in a fluid medium is broad (see Table 1), corresponding to various degrees of relaxation. In a rigid medium emission arises from one geometric configuration which corresponds approximately to that of the ground state. This accounts for the relatively small half width of the emission in a rigid medium at 77°K. 2.) The paths of energy degradation (i.e. radiative vs. radiationless transitions) will depend on the excited state geometric configuration. A Franck-Condon (unrelaxed) state is expected to have a small radiationless rate constant since the overlap factor* of the vibrationless level of the upper state with the isoenergetic levels of the ground state is small; the radiative processes will therefore dominate. Therefore in a rigid medium ($\eta > 10^8$ cp) the fluorescence is intense, the band is relatively narrow and the maximum occurs at higher energies. The equilibrium excited state geometric configuration is expected to have a large radiationless rate constant due to a large overlap factor arising from the displacement of the excited state potential surface relative to that of the ground state. Therefore in a fluid medium the fluorescence yield is low, the band is broad and the fluorescence maximum lies at lower energies. Since the fluorescence yield of completely relaxed molecules is very small compared to non-relaxed molecules, emission originating from the latter

*The overlap factor in the rate equation for radiationless transitions depends on the extent of displacement of the potential energy surfaces of the upper and lower states relative to each other and is expected to be minimum for the Franck-Condon state configuration.

will contribute more significantly than their proportions. 3.) Increasing the temperature will change the Boltzmann distribution of excited state. If radiationless decay from upper torsional modes in the excited state is more efficient (larger rate constant), one would expect fluorescence quenching of molecules emitting at longer wavelengths as the temperature is increased.

Therefore the apparent blue shift which occurs at low viscosities as a result of warming the sample is really a selective-red quenching phenomenon. Increasing the temperature leads to a preferential quenching of fluorescence of completely relaxed molecules. Partially relaxed molecules will progressively contribute more to the emission intensity as the temperature is increased. Since their emission occurs at higher energies, an apparant blue shift of the fluorescence maximum is observed as the yield decreases with temperature, giving rise to a selective red-quenching effect. This is demonstrated in Figure 9, where the fluorescence spectra in MCH/IP (3:1) at different temperatures are shown.

Medium Polarity Effects. Comparing the fluorescence spectra in n-propanol, MCH/IP (3:1) and ethanol at room temperature one notices a decrease in intensity and a blue shift of the $\bar{\upsilon}_F$ as the polarity of the medium is increased. As shown in Figure 10, the half width of the fluorescence band is larger in hydrocarbon medium. One should note that TPMB is practically non-polar and therefore the observed blue shifts in the polar media could not be attributed to energy changes of the states involved in emission.

The selective-red quenching observed when the medium is made polar is interpreted in terms of a larger solvent-solute coupling (mainly due to dipole-induced dipole interactions) in polar media. One may therefore expect a more efficient radiationless decay in polar medium particularly for completely relaxed molecules where radiationless processes are dominant.

We believe that the selective-red quenching phenomenon observed when temperature is increased or when the medium is made polar is a general one and will occur in situations where excited molecule undergo geometric relaxation during their lifetime.

Discussion

From the previous sections, it is apparent that the fluorescence intensity depends on both the temperature and the viscosity of the medium. By lowering the temperature the upper torsional modes of the equilibrium excited state become less populated and, if one makes the reasonable assumption that radiationless decay from these levels is more efficient compared to the lowest level in the excited state, one would expect the fluorescence intensity to increase as temperature is lowered.

In the viscosity range where no shifts are observed the increase in viscosity will have no effect on the excited state geometric configuration. However, the torsional oscillations will be damped and their maximum amplitudes will be smaller at higher viscosities, i.e. the shape of the potential energy surface will be dependent on the viscosity. In the excited state this will affect the overlap integrals that govern the rates of radiationless decay. At low

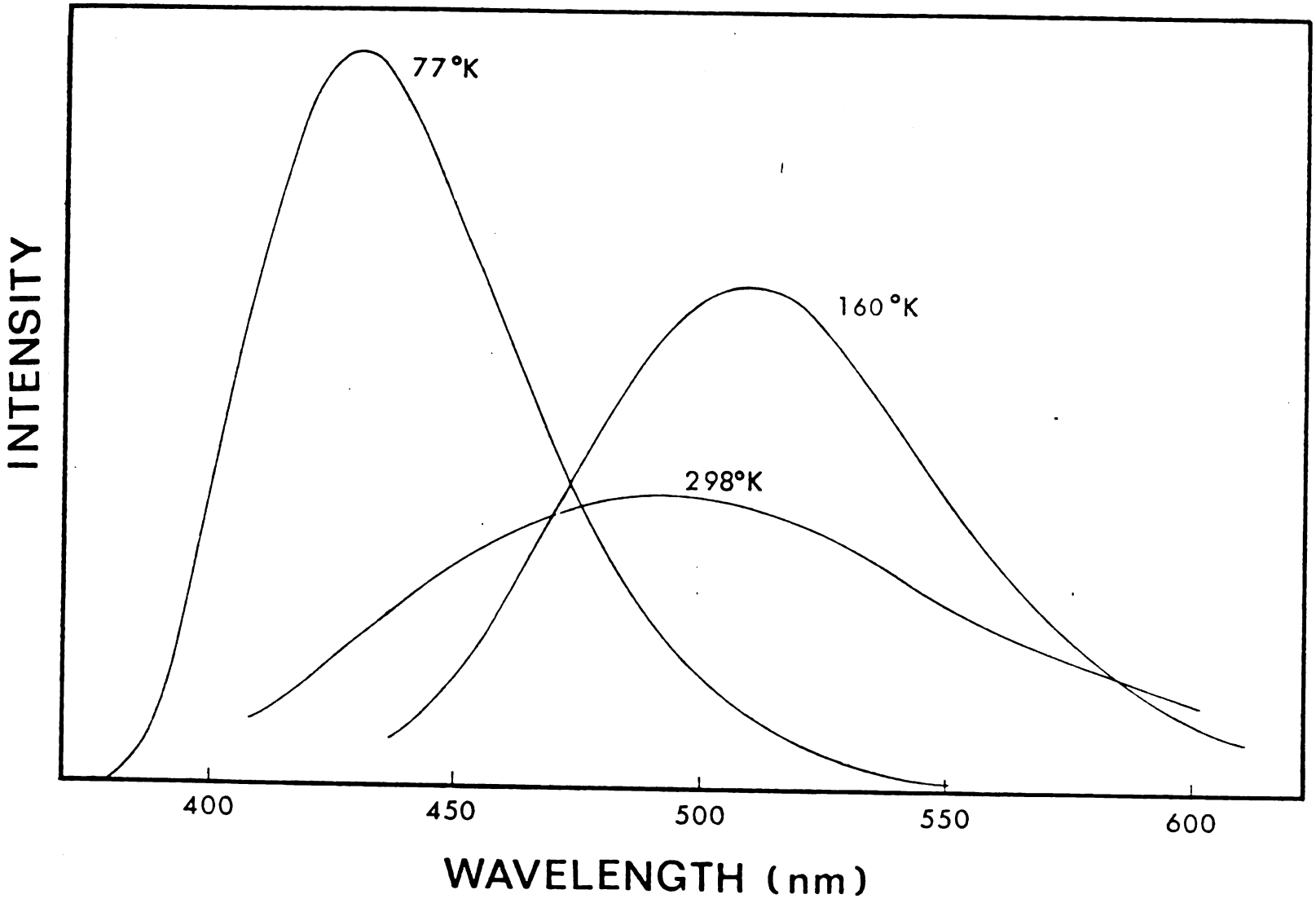

FIGURE 9. Emission spectra in MCH/IP (3:1) at three different temperatures 298°K, 160°K and 77°K. The relative sensitivities at which the spectra were recorded are X180, X7, X1, respectively.

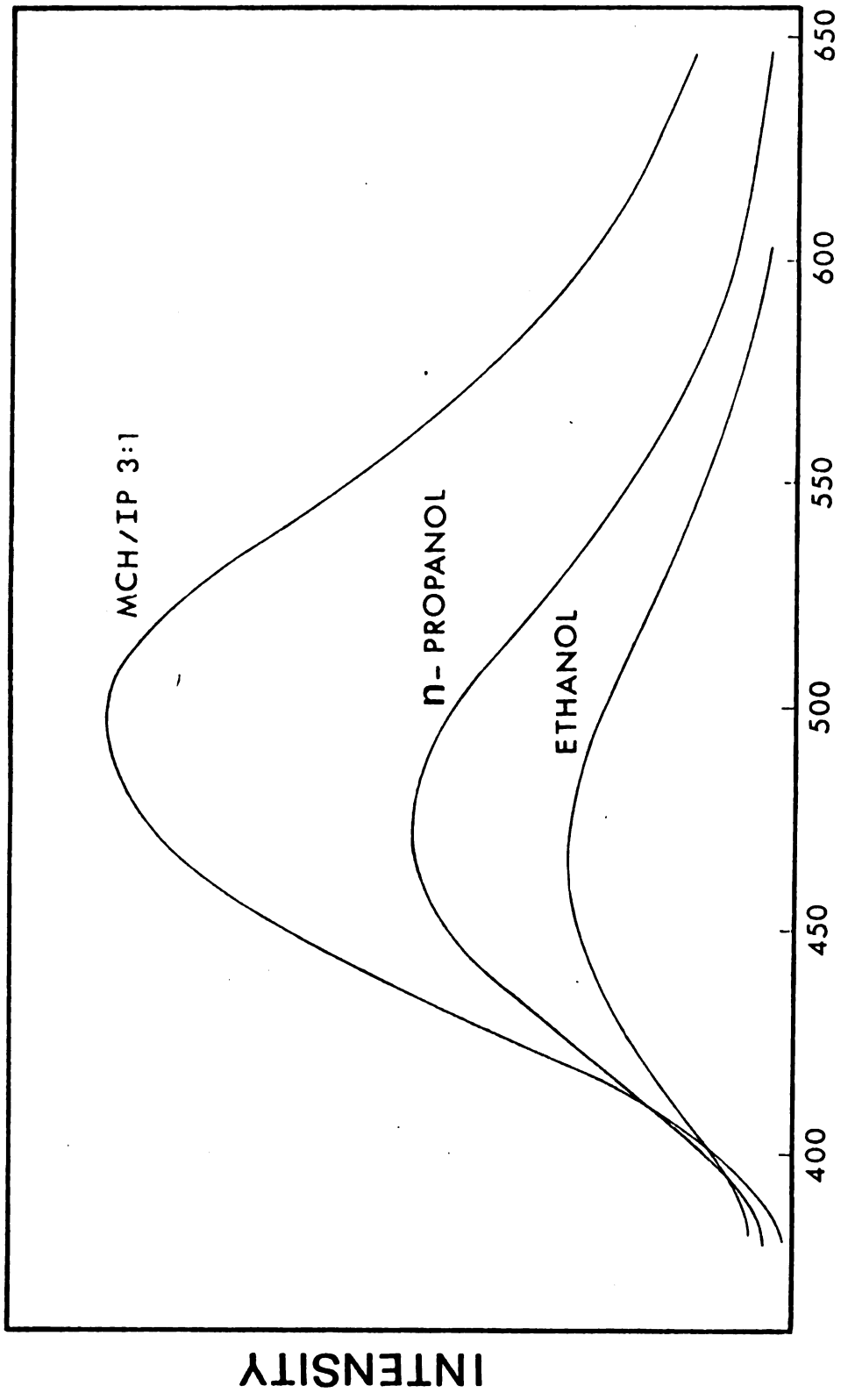

FIGURE 10. Emission spectra at 298°K in three solvents, MCH/IP (3:1), n-propanol and ethanol. The emission in n-propanol and ethanol were recorded at the same sensitivity (X1) while that in MCH/IP (3:1) was recorded at a lower sensitivity (X.5).

viscosities the excited state potential energy surface is broader and the overlap integrals larger than at relatively higher viscosities. Solute-solvent coupling may also be dependent on viscosity to some extent particularly in polar medium. Further increase in the viscosity will alter the relative positions of the upper and lower potential surfaces causing a change in the overlap integral and leading to enhance intensities as well as blue shifts of fluorescence.

In order to get some idea regarding the nature of the non-radiative process, plots of log $(\frac{1}{\phi_F} - 1)$ vs. 1/T in three different media were obtained and are shown in Figure 11. The activation energies obtained are 890cm^{-1} (MCH/IP,3:1) 710cm^{-1} (IP) and 1530cm^{-1} (n-propanol). The intensity data were obtained by changing the viscosity of the solvent by lowering temperature in a region where no shifts in $\bar{\nu}_F$ are observed, i.e. solute molecules may completely relax and equilibrate among the various excited state torsional modes. Solvent activation energies obtained from macroviscosity vs. temperature data are 1000 for MCH/IP and 1950cm^{-1} for n-propanol.

To separate the dependence of fluorescence on temperature from viscosity, it is necessary to use intensity data obtained as a function of viscosity at constant temperature. The fluorescence intensity change due to temperature alone is then plotted as log $(\frac{1}{\phi_F} - 1)$ vs. 1/T. The activation energies thus obtained are 527cm^{-1} and 700cm^{-1}, in propanol and MCH/IP respectively. Considering the

approximations involved* one may only state that the activation energy is a few hundred wavenumbers. This corresponds probably to the torsional frequency in the excited state and indicates that a bond with double-bond character is involved which is consistent with our earlier conclusion (34). Thus the variation of fluorescence intensity as the viscosity is varied by lowering the temperature is interpreted in terms of activation energies which combine a solvent (viscosity) activation energy as well as a solute activation energy, the solute activation energy being related to the torsional frequency in the excited state.

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*(Namely that the η is a linear function of percent volumes of paraffin oil and that the polarity of ethanol/glycerol mixtures is invariant and is the same as that of n-propanol).

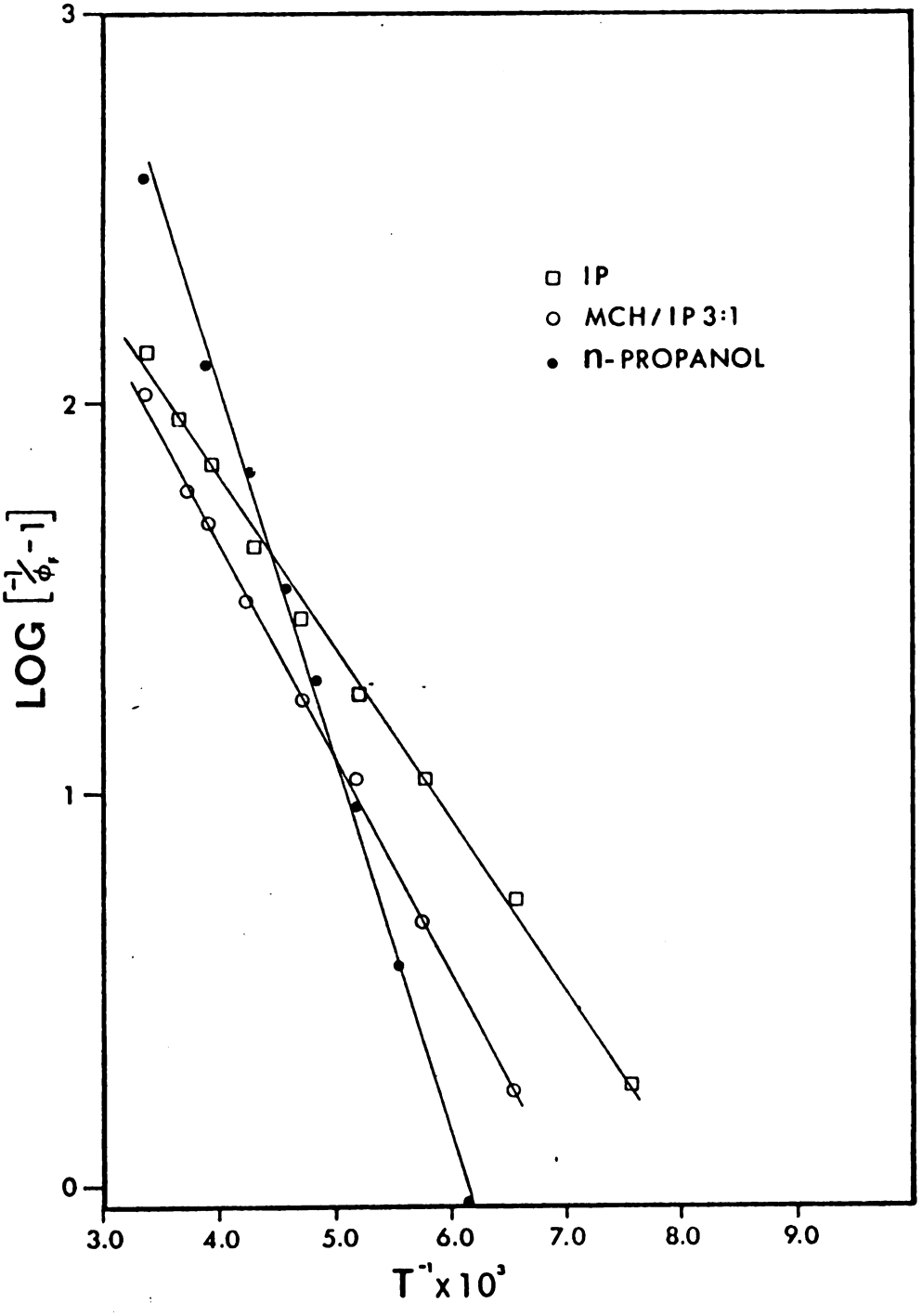

FIGURE 11. A plot of log $(\frac{1}{\phi_F} - 1)$ vs. T^{-1} for TPMB in three solvents MCH/IP (3:1), n-propanol and IP where ϕ_F is the absolute quantum yield at temperature T. The activation energies determined from the plots are 890cm^{-1}, 1530cm^{-1} and 710cm^{-1} respectively.

CHAPTER 3

CONCLUSION

Sterically crowded molecules that undergo intramolecular twisting relaxation after excitation exhibit luminescence characteristics that can be used to probe microfluidity. The fluorescence characteristics that may be used are: emission maximum $\overline{\upsilon}_F$, relative fluorescence yield ϕ_F, polarization p, the rate of fluorescence decay, k_f, as well as the rate of twisting relaxation, k_r, obtained from time resolved spectra. I have studied quantitatively the dependence of the emission maxium, $\overline{\upsilon}_F$, and the relative yield, ϕ_F, of a sterically crowded molecule on the viscosity, temperature and polarity of the medium.

I have shown in Figures 5 and 6 that $\overline{\upsilon}_F$ is sensitive only to viscosity, being completely independent of temperature. So that a simple measurement of $\overline{\upsilon}_F$ will give a good indication of the fluidity of the medium. Relative measurements of the fluidity using this technique may be more meaningful than absolute measurements because this relaxation process seems to be also sensitive to the structure of the medium. That is, it may give different measurements of η for two hydrocarbons solvents, one being cyclohexane and the other methylpentane, even though the two solvents bulk viscosities are the same. I think that the problem here is that of defining microviscosity as compared to macroviscosity. Microviscosity may include a factor reflecting

the structure of the molecules of the medium; whereas macroviscosity is just a bulk viscosity where the medium is considered isotropic and continuous.

$\overline{\upsilon}_F$ also depends on the polarity of the medium —— selective red-quenching. Normally one would expect that a non-polar molecule would be completely unaffected by the polarity of the environment, but it seems that molecules which undergo a relaxation in the excited state are affected by the polarity of the medium. Note that the selective red-quenching increases with the polarity of the solvent for both TPMB and tetraphenylethylene (TPE). We think that this is due to the fact that polar solvents couple more strongly with the excited chromophore possibly by dipole-induced dipole interactions. It would be interesting to see how general a phenomenon this selective red-quenching is. For instance, is selective red-quenching also a factor for polar molecules which undergo solvent cage relaxation in their excited state?

The relative fluorescence intensity, ϕ_F, may also be used to investigate microfluidity. Figures 7 and 8 show that ϕ_F increases by a factor of 270 in n-propanol over a viscosity range of $1\text{-}10^4$ cp. However, ϕ_F also depends on temperature. This dependence on temperature limits the usefulness of ϕ_F to comparative measurements in which the temperature is kept constant. For systems that scatter light, the fluorescence decay time is a more useful measure of η than an intensity measurement if the natural lifetime remains constant in the fluidity region studied.

Care must be exercised in utilizing complex molecules to probe microfluidity. It is necessary to study quantitatively the luminescence properties of these molecules under ideal conditions (i.e. in solution) and to examine the temperature and medium effects as well as the viscosity effects before applying them to probing membranes.

One problem with utilizing such molecules as TPMB and TPE as fluidity probes is that they are completely insoluable in water ($< 10^{-6}$). So that getting the molecule into the structure being investigated becomes a very difficult problem. In the process of inserting the TPMB into membranes, crystal particles of TPMB attach themselves to the membrane. These particles emit with a quantum yield of approximately one and with an energy similar to that of a molecule in a rigid medium. Therefore the particle emission completely masks any meaningful data. One possible way of solving the solubility problem is to introduce a substituent that enhances the molecule's solubility in water without altering the absorption and luminescence properties of the molecule. The sulfonate group seems to be a suitable substituent to achieve this purpose.

From my study of sterically crowded molecules, I would like to list some criteria for designing a fluidity probe using a sterically hindered molecule.

Criteria for a Sterically Hindered Fluidity Probe

1. The molecule must be slightly soluble in water ($>10^{-6}$M) however not so soluble that it favors the water over the hydrocarbon.

2. The rate of relaxation must be sensitive to the packing of the medium. Hopefully, it will be sensitive in a fluidity range corresponding to a macrospic viscosity of 0-100cp. All work in biological systems has fallen into this region investigated by steady state depolarization.

3. The $\overline{\nu}_F$ of emission should not depend on temperature so that it may be used to study such phenomena as membrane phase transitions.

4. The quantum yield should be great enough for easy detection.

5. It would be nice if the probe's fluorescent lifetime was long enough so that the actual rate of geometric relaxation could be used to study medium rigidity; however, this is probably impossible.

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