

## Reduced 8–13 Link, a Viscosity-Dependent Fluorescent Probe of Transfer RNA Tertiary Structure<sup>†</sup>

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**ABSTRACT:** The mechanism of enhancement of the fluorescent yield of the “reduced 8–13 link” in the tRNA tertiary structure has been investigated using different synthetic N(1),N'(1)-substituted derivatives of Pyo(4'–5)hCyt. From an examination of their emission quantum yields at 300 K in 20 pure solvents, as well as at different temperatures in a few solvents, it is concluded that local viscosity is the most important parameter in the emission mechanism. The quantum yield ranges from 10<sup>–4</sup> in water at 300 K to a value close to unity in a rigid medium at 77 K. Proton-transfer reactions are relatively unimportant, as judged by the small isotopic deuterium stimulation, the values of ground and first-excited singlet state pKs,

and the effect of substitution of exchangeable protons. Furthermore, the yield is independent of solvent polarity. We propose that viscosity hinders an efficient nonradiative deactivation path by slowing down the rotation of one heteroaromatic cycle with respect to the other around the C(4')–C(5) bond. Direct support for this mechanism is provided by the effect on fluorescence yield of substituents of varying steric hindrance or oligonucleotides of varying length. The data obtained in monoalcohols and in glycol are well accounted for by a mechanism involving rotational relaxation in the first-excited singlet state.

Fluorescence spectroscopy is a method of choice in exploring the interior of macromolecules. In the field of nucleic acids, however, this technique has been little used because of the low yield of emission of the common bases under physiological conditions (Ballini et al., 1976). Even in transfer RNA, where the largest variety of naturally modified bases is found, only two, the Y base and 4-thiouracil, meet the following necessary requirements; i.e., (1) they can be selectively excited and (2) their emission is easily detected (Tao et al., 1970; Shalitin and Feitelson, 1976). Alternatively, extrinsic fluorochromes, either intercalated or covalently bound, have been used. In these cases, accurate information can be gained if the dye is linked to (a single) well-defined site(s) and if it is known how it affects the macromolecular conformation and biological activity. In addition, one should know the dependence of the dye emission upon solvent parameters such as polarity, viscosity, etc. (as has been achieved in a few cases).

In many *Escherichia coli* tRNAs, a specific intramolecular photoreaction occurs upon 335-nm irradiation between 4-thiouracil in the 8th position and cytosine in the 13th position (Favre et al., 1969, 1971; Yaniv et al., 1969; Carré et al., 1974). The tRNA photoadduct can be reduced by sodium borohydride to give a highly fluorescent compound when in tRNA (Favre and Yaniv, 1971), namely, 5-( $\alpha'$ -oxopyrimidin-4'-yl)-3,6-dihydrocytidine (7), which is referred to as Pyo(4'–5)hCyt<sup>1</sup> (Bergström and Leonard, 1972; Favre et al., 1972). By this procedure one obtains in tRNA a fluorescent sensor which fulfills the conditions defined above. Firstly, the fluorescence characteristics are appropriate ( $\lambda_{\text{max}}^{\text{ex}}$  386 nm;  $\lambda_{\text{max}}^{\text{em}}$  440 nm; quantum yield close to 0.22 at 25 °C). Secondly, the probe is located in an interesting part of the mole-

cule, in a known position in the tertiary structure. Third, it leaves the tRNA<sup>Val</sup> molecules biologically active (Carré et al., 1974). In addition, it can be introduced in all the tRNA species which have the 4-thiouridine in position 8 and the cytidine in position 13 in common, thus allowing detailed comparison. Until now, the fluorescent emission of Pyo(4'–5)hCyt 7 has been used mostly in order to follow the 8–13 cross-linking reaction in tRNAs. Thus it was valuable in identifying cross-linkable tRNA species (Carré et al., 1974) and in revealing fine differences in the region of tertiary structure (Delaney et al., 1974; Favre et al., 1975).

Our knowledge of the fluorescence mechanism has been limited to a striking observation: the quantum yield of emission decreases 400-fold when the tRNA structure is completely disrupted by enzymatic hydrolysis (Favre and Yaniv, 1971). Hydrophobicity has been proposed as the main factor responsible for this effect (Favre and Yaniv, 1971), a view which has been generally accepted (Delaney et al., 1974; Bergström and Leonard, 1972).

Here we present data extending previous observations (Favre and Yaniv, 1971; Ofengand and Bierbaum, 1973) on the emission yield of the probe in tRNAs and well-defined oligonucleotides. The yield appears to be related to the size of the oligonucleotides, an effect difficult to explain on the basis of hydrophobicity. To elucidate further the mechanism of emission, we examined in aqueous solvent the possible role of proton transfer reactions. Our main approach, however, was to look at the absorption and emission characteristics, including quantum yield, in as large a variety of solvents as possible and in a few cases at different temperatures. The poor solubility of the diriboside derivatives in most organic media led us to synthesize the corresponding N(1),N'(1)-dimethyl and dicyclohexyl derivatives. In addition, substitutions of exchangeable protons by methyl groups revealed useful information.

In contrast to previous assumptions, it is shown here that solvent polarity plays at most a minor role. The emission yield is mainly determined by solvent viscosity. An emission mechanism is discussed which relates the bicyclic nature of the probe to the dependence on viscosity of its emission.

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<sup>1</sup> For the sake of clarity and convenience, we use throughout this paper the dipyrimidine abbreviations as recommended by Cohn et al. (1976).

<sup>2</sup> Abbreviations used: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

Under these conditions the final yield of 8-13 link in cross-linkable tRNAs is close to 1 and is not further improved by deaeration. After irradiation, samples were generally diluted five times in the same buffer (without magnesium) and reduction was allowed to proceed according to Favre and Yaniv (1971). In all cases during reduction at pH 9.7, the absorption spectra of irradiated tRNAs exhibit an isobestic point at 357 nm. The reduction kinetics vary with the tRNA species, suggesting, in agreement with previous results (Favre and Yaniv, 1971; Ofengand and Bierbaum, 1973), that the diffusion of the reducing agent is controlled by the tRNA structure in the vicinity of the 8-13 positions. In every case, the reduction was quantitative (90-100%). After completion of the reaction, the tRNAs were extensively dialyzed against standard buffer and kept frozen at  $-20^{\circ}\text{C}$  until use.

### Techniques and Measurements

Absorption measurements were performed with a Cary 15 spectrophotometer; room temperature fluorescence was studied with a thermostated Jobin Yvon "Bearn" spectrofluorimeter. Measurements in different solvents were performed as follows: the adducts were first dissolved in methanol (final concentration 0.1 mM) and 100  $\mu\text{L}$  of this stock solution was carefully evaporated in a glass tube. It was then redissolved by shaking with 1 mL of the appropriate solvent and transferred into a cuvette. The absorption spectrum was recorded, taking pure solvent as a blank. The same cuvette was used for the 300 K fluorescence measurements. In each case, the instrumental  $\lambda_{\text{max}}$  of excitation (with  $\lambda_{\text{em}}$  450 nm) was determined. We also verified that the emission spectrum of the Pyo(4'-5)hCyt 7 derivatives remains practically invariant in the different solvents. In every case, the wavelength of maximum emission was determined and in a few cases the entire spectrum recorded. The relative quantum yield  $\Phi$  was computed from the emission signal ( $\lambda_{\text{ex}}$  380 nm;  $\lambda_{\text{em}}$  450 nm) divided by the probe absorbance at 380 nm. We confirmed that  $\Phi$  was independent of concentration in the range from 0.05 to 0.5  $A_{380}$ . With the effective light path being 1 mm, no inner-filter corrections were necessary. Most organic solvents were spectroscopic grade. When necessary, their background emission (at most 20% of the signal) was subtracted.

Quinine bisulfate was used for emission spectra corrections and absolute quantum yield determination. A quantum yield of 0.56 at  $22^{\circ}\text{C}$  in 0.1 M  $\text{H}_2\text{SO}_4$  was used (Gill, 1969).

For polarization measurements the fluorimeter was equipped with two Polaroid HNP'B filters, and polarization was computed using the relation

$$P = \frac{I_{VV}I_{HH} - I_{VH}I_{HV}}{I_{VV}I_{HH} + I_{VH}I_{HV}}$$

were  $I_{VV}$ ,  $I_{VH}$ ,  $I_{HH}$ , and  $I_{HV}$  are the intensities of the four components of the fluorescent light. The first subscript indicates the plane of polarization (vertical or horizontal) of the exciting light and the second that of the emitted light.

The Jobin Yvon apparatus was used for low-temperature fluorescence measurements. In this case, the thermostated cell holder was replaced by a quartz Dewar. For 77 K measurements the sample was dissolved in 50% ethylene glycol-water solution placed in a quartz tube and frozen in liquid nitrogen. For measurements at 201 K, the sample was dissolved either in methanol or glycerol, and the quartz tube was placed in a cooling mixture of ethanol and solid  $\text{CO}_2$ . The relative error on the measurements was 3% at room temperature and 20% at low temperature.

pH titrations were performed by stepwise addition of HCl or NaOH to the solution in the cuvette. pH was measured with

a micro glass electrode. Aqueous solutions were used for absorption titration. A 40% sucrose solution was used for fluorescence titration in order to increase the quantum yield.

### Results

**Absorption and Fluorescence of Pyo(4'-5)hCyt 7 in tRNAs and Oligonucleotides.** In order to evaluate the influence of tRNA sequence and structure on the properties of Pyo(4'-5)hCyt 7, the spectroscopic features of the fluorescence were examined in four different purified tRNA species as well as in bulk tRNA, where 50% of the molecules can be cross-linked (Carré et al., 1974). The Pyo(4'-5)hCyt 7 probe was introduced in the different samples by irradiation and reduction as described under Materials and Methods. In the final stage of the preparation, the tRNAs were dialyzed against standard buffer, where they assume their functional structure.

After modification, all samples present at 300 K have the characteristic properties of Pyo(4'-5)hCyt 7, i.e., a long-wavelength absorption band ( $\lambda_{\text{max}}$  386 nm) and a fluorescence emission very similar to that originally described in tRNA<sup>Val</sup> (Favre and Yaniv, 1971). The emission spectrum ( $\lambda_{\text{max}}$  440 nm) is independent of the excitation spectrum. The excitation spectrum, on the other hand, presents two peaks at 260 and 386 nm. The former is unexpected and has been attributed to energy transfer from the common bases (Favre and Yaniv, 1971). The near-ultraviolet peak corresponds to the absorption band. Polarization measurements were performed at  $5^{\circ}\text{C}$  in buffered 40% sucrose solution. The observed value  $p = 0.42$  ( $\lambda_{\text{ex}}$  390 nm;  $\lambda_{\text{em}}$  440 nm) can be considered close to the fundamental polarization. It does not vary with the excitation wavelength in the range of 340-400 nm ( $\lambda_{\text{em}}$  440 nm). Reciprocally, upon 390-nm excitation, polarization is invariant over the emission spectrum (410-470 nm). In contrast to these similarities, the quantum yields of emission ( $\Phi$ ) are significantly different in the various tRNA species (Table I).

Digestion of purified tRNAs with specific RNases allows the study of Pyo(4'-5)hCyt 7 in well-defined oligonucleotides.<sup>3</sup> Our results confirm and extend previous ones (Favre and Yaniv, 1971; Ofengand and Bierbaum, 1973). On hydrolysis, the 386-nm absorption peak shifts to the blue, but the emission spectrum remains practically unaltered. The quantum yield shows much more striking variations. Table I shows that its value correlates well with the oligonucleotide length. This observation appears to challenge the previous assumption that hydrophobicity was the main factor eliciting high values of  $\Phi$ . This would have been achieved in the oligonucleotides by stacking of the residues proximal to the probe. One would have expected, therefore,  $\Phi$  to be strongly dependent on the four nearest-neighbor residues and little dependent on the more distant ones. Clearly this is not the case. T<sub>1</sub> oligo<sup>Val</sup> or T<sub>1</sub> oligo<sup>Phe</sup> has a smaller  $\Phi$  compared to pancreatic oligo<sup>Met</sup>, though the former has four nearest neighbors and the latter only two.

**Absorption and Fluorescence in Organic Solvents.** Our initial goal was to attempt to promote with pure solvents the fluorescence level observed in the interior of tRNA. This study was initiated with the "natural" compound N(1),N'(1)-diribosyl-Pyo(4'-5)hCyt 7 (R = ribosyl) purified from bulk cross-linked *E. coli* tRNA or its synthetic analogue (Favre et al., 1972). The two samples behaved identically, but their poor solubility in most solvents made the conclusions uncertain. We

<sup>3</sup> The reduced 8-13 link containing oligonucleotides obtained after digestion of tRNA<sup>Val</sup> with a specific endonuclease such as T<sub>1</sub> RNase is abbreviated to T<sub>1</sub> oligo<sup>Val</sup>. Similar abbreviations are used for bulk or the other pure tRNA species cited in this work.

TABLE I: Summary of the Spectral Properties of Pyo(4'-5)hCyt in Different tRNAs and Oligonucleotides.<sup>a</sup>

	no. of nucleotides	sequence	rel quantum yield	rel absorp at 386 nm
tRNA <sub>3</sub> <sup>Arg</sup>	76	U-C-C-G-Pyo-A-G   D-C-G-A-hC-U-C   G-G-G-G-Pyo-G-G   C-C-G-A-hC-G-A   U-G-A-U-Pyo-A-G   hU-C-G-A-hC-U-C   C-G-G-A-Pyo-A-G   C-D-G-A-hC-U-C   G-G-G-G-Pyo-   hC-G-A-G-G	135	76
tRNA <sub>f</sub> <sup>Met</sup>	77		120	76
tRNA <sub>i</sub> <sup>Val</sup>	76		100	84
tRNA <sup>Phe</sup>	76		99	88
panc oligo <sub>f</sub> <sup>Met</sup>	10		20	20
T <sub>1</sub> oligo <sub>i</sub> <sup>Val</sup>	10	A-U-Pyo-A-G   G-A-hC-U-C   A-Pyo-A-C   G-A-hC-U-C   Pyo-G   hC-A-G   G-G-A-Pyo-   hC-	18	79.5
T <sub>1</sub> oligo <sup>Phe</sup>	9		11	80
T <sub>1</sub> oligo <sub>f</sub> <sup>Met</sup>	5		7	84
panc oligo <sup>Phe</sup>	5		4.5	92
panc oligo <sup>Arg</sup>	3	G-Pyo   hC   Pyo   hC   Pyo   hC	4	91
panc oligo <sup>Val</sup>	2		1	99
diriboside-Pyo(4'-5)-hCyt	2		0.25	100

<sup>a</sup> Preparation of the samples and determination of the spectral parameters are described under Materials and Methods. The nucleotide sequence is represented going from the 5' to the 3' side on the upper line and from the 3' to 5' side on the lower line. Notice the small discrepancy between the quantum yield of pancreatic oligo<sub>f</sub><sup>Val</sup> and the synthetic diriboside, presumably due to incomplete hydrolysis. For bulk tRNA and its pancreatic and T<sub>1</sub> hydrolysate, the relative values of  $\Phi$  are, respectively, 102, 3, and 7.5.

were, therefore, led to synthesize *N*(1),*N'*(1)-Me<sub>2</sub>Pyo(4'-5)hCyt **7** and the analogous dicyclohexyl derivatives. More than 30 pure solvents were tested amongst which 20 (legend of Figure 4) were found to be of sufficient spectroscopic quality. The dimethyl derivative, which is quite soluble in water and weakly in pyridine, was found to be insoluble in a strictly apolar solvent. The dicyclohexyl compound, on the other hand, is no more soluble in water but can be solubilized in benzonitrile (an aprotic solvent) and even in xylene and cyclohexane in the presence of a trace amount of methanol.

As solvents are changed, the quantum yield exhibits striking variations. In the different solvents for the dimethyl derivatives, it varies from  $2.4 \times 10^{-4}$  in methanol to  $7.5 \times 10^{-3}$  in glycerol, i.e., over a 30-fold range. We tried to link the level of  $\Phi$  with a physical parameter of the solvent. No correlation with the dipolar moment is evident. For the same dipolar value, (1.7 D),  $\Phi$  varies over 25-fold. Conversely,  $\Phi$  is almost identical in 1-propanol and benzonitrile, whose dipolar moments are 1.7 and 4 D, respectively. Also, the *Z* value, an empirical measure of solvent polarity (Kosower, 1958), does not correlate with  $\Phi$ .

TABLE II: Effect of the Substituents at the N(1),N'(1) Positions of Pyo(4'-5)hCyt.

solvent	quantum yield of Me <sub>2</sub> Pyo(4'-5)-hCyt	quantum yield ratios	
		dicyclohexyl dimethyl	diribosyl dimethyl
water	$2.0 \times 10^{-4}$		3.5
methanol	$2.4 \times 10^{-4}$	2.6	3.6
ethanol	$7 \times 10^{-4}$	1.7	3.5
1-butanol	$1.8 \times 10^{-3}$	1.8	
hexanol	$3 \times 10^{-3}$	1.8	
<i>tert</i> -butyl alcohol	$3.2 \times 10^{-3}$	1.6	
ethylene glycol	$3.1 \times 10^{-3}$	1.8	3.6
glycerol	$7.3 \times 10^{-3}$	1.7	2.7

TABLE III: Quantum Yield of the Fluorescence Emission at 300 K in Methanol and Glycerol.<sup>a</sup>

	methanol	glycerol
<i>N</i> (1), <i>N'</i> (1)-Me <sub>2</sub> Pyo(4'-5)hCyt	$2.4 \times 10^{-4}$	$7.3 \times 10^{-3}$
<i>N</i> (1), <i>N'</i> (1), <i>N</i> (3)-Me <sub>3</sub> Pyo(4'-5)hCyt	$1.9 \times 10^{-4}$	$6.0 \times 10^{-3}$
<i>N</i> (1), <i>N'</i> (1), <i>N</i> (4)-Me <sub>3</sub> Pyo(4'-5)hCyt	$1.8 \times 10^{-4}$	$9.6 \times 10^{-3}$
<i>N</i> (1), <i>N'</i> (1)-Me <sub>2</sub> Pyo(4'-5)hUra	$\leq 0.8 \times 10^{-4}$	$\leq 0.5 \times 10^{-3}$

<sup>a</sup> In the case of *N*(1),*N'*(1)-Me<sub>2</sub>Pyo(4'-5)hUra, the fluorescence level was about twice the background emission.

In contrast, there is a clear correlation between  $\Phi$  and the macroscopic viscosity of the solvent (Figure 2). However, the dispersion of the points is larger than expected from experimental errors. A better fit would probably be seen if  $\Phi$  were plotted as a function of the local viscosity. This can be illustrated in the extreme cases of aqueous gels where the microviscosity is that of free water. Thus, when included in gels of DNA, GMP, or indubiose, the emission yield of Me<sub>2</sub>Pyo(4'-5)hCyt **7** remains close to that observed in water. The different behavior of the drug in the pure solvents and in the water-glycerol mixtures (Figure 2) or water-sucrose mixtures (data not shown) can be understood on the same basis. The water molecules would have a tendency to condense around the probe, thus decreasing local viscosity. Even in the case of the pure solvents, we were unable to extract from the data a secondary macroscopic parameter influencing  $\Phi$ .

More rewarding is the fact that in a given solvent  $\Phi$  is dependent on the substituents at the N(1),N'(1) positions: the bulkier the substituent, the higher the yield. To a first approximation the ratios of the different  $\Phi$  values are independent of the solvent considered (Table II). The spectral properties of the probe are much less sensitive to solvents effects. In a given solvent they are identical irrespective of the nature of the substituents at the N(1),N'(1) positions. Slight spectral shifts can be observed that appear again to correlate with the solvent viscosity. In water or methanol, the *N*(1),*N'*(1)-Me<sub>2</sub>Pyo(4'-5)hCyt **7** exhibits a prominent absorption band at 375 nm. As the solvent viscosity increases, the absorption peak shifts to the red, with an unchanged width,  $\lambda_{\max}$  reaching 380 nm in glycerol. In all solvents the excitation spectrum ( $\lambda_{\text{em}}$  450 nm) corresponds well to the absorption band. The  $\lambda_{\max}$  of emission remains unchanged, at 450 nm, but the width of the emission band decreases with increasing viscosities (Figure 3).

Our data indicate that solvent viscosity is a predominant factor in eliciting a high yield of emission in the Pyo(4'-5)hCyt **7** series. Can this be extended to the closely related Pyo(4'-5)hUra adduct? The answer is clearly negative, since even in glycol or glycerol its emission yield remains quite low, less than  $5 \times 10^{-4}$  (Table III). Obviously, the electronic configuration

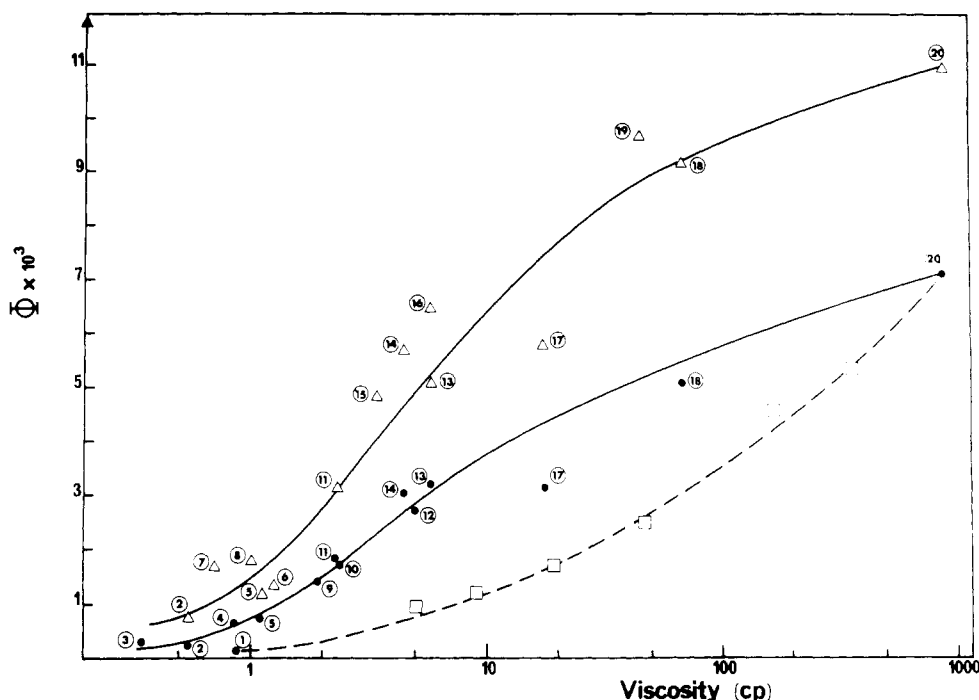


FIGURE 2: Correlation between quantum yield and solvent viscosity at 300 K for the *N*(1),*N*(1')-dicyclohexyl ( $\Delta$ ) and the *N*(1),*N*(1')-dimethyl ( $\bullet$ ) derivatives of Pyo(4'-5)hCyt. The solvent numbering is as follows: (1) water, (2) methanol, (3) acetonitrile, (4) pyridine, (5) ethanol, (6) benzonitrile, (7) xylene, (8) cyclohexane, (9) 1-propanol, (10) isopropyl alcohol, (11) 1-butanol, (12) isoamyl alcohol (13) *tert*-butyl alcohol (14) hexanol, (15) *tert*-amyl alcohol, (16) benzyl alcohol, (17) ethylene glycol, (18) cyclohexanol, (19) 1,2-propanediol, (20) glycerol. Solubilization in 7 and 8 was only obtained in the presence of a trace amount of methanol: ( $\square$ ) *N*(1),*N*(1')-Me<sub>2</sub>Pyo(4'-5)hCyt dissolved in various water-glycerol mixtures. Viscosities are taken from the Handbook of Chemistry and Physics, and from the International Critical Tables.

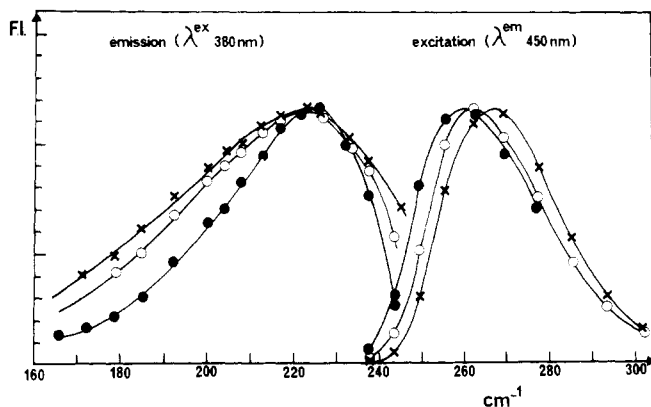


FIGURE 3: Corrected excitation and emission spectra of the *N*(1),*N*(1')-Me<sub>2</sub>Pyo(4'-5)hCyt in methanol (X) or in glycerol (O) and of the Pyo(4'-5)hCyt probe in native *E. coli* bulk tRNA ( $\bullet$ ).

of the latter compound promotes very efficient nonradiative deactivation path(s) in the viscosity range investigated here at 300 K.

**Proton Transfer.** Considering the chemical structure 7 of our probe (Figure 4), one may expect proton transfer reactions to play an important role in the emission mechanism and thus to strongly influence  $\Phi$ . Proton exchange can occur at position N(3),N'(3) and at the exocyclic amino group. More specifically, proton exchange could result from ionization or from tautomerization either in the ground or in the excited state. The proton transfer reaction should be independent of the nature of the N(1),N'(1) substituents. All the following experiments were performed with the dimethyl derivative.

Our first approach was to examine the deuterium isotopic effect. The small enhancement of  $\Phi$  observed in D<sub>2</sub>O (stimulation 5%), as compared to H<sub>2</sub>O or in CD<sub>3</sub>OD (stimulation 8%), and CH<sub>3</sub>OD (stimulation 8%), as compared to CH<sub>3</sub>OH,

argues against these possibilities (Stryer, 1966). We then compared in aqueous medium the spectrophotometric and fluorimetric pKs. The former titration indicates two transitions, one basic ( $pK_b = 11.7$ ) and one acidic ( $pK_a = 3.5$ ) (Figure 6), which are in agreement with those already reported for the diribosyl derivative (Favre et al., 1972). In the acidic pH range, at pH lower than 1, small reversible changes in absorption indicate a second pK which should lie in the vicinity of pH 0. The fluorimetric titration was performed in the presence of 40% sucrose in order to increase the emission yield. No acidic or basic transitions were observed in the pH range of 4 to 10, thus ruling out excited-state ionization as an important step in the emission mechanism.

The importance of tautomerization was checked by blocking one of the tautomeric forms. This was achieved by methylation at the N(3) position of the cytosine moiety of *N*(1),*N*'(1)-Me<sub>2</sub>Pyo(4'-5)hCyt 3 ( $R = CH_3$ ) followed by reduction. The methylation reaction is described under Materials and Methods, and the nature of the product is unambiguously established as the *N*(1),*N*'(1),N(3)-Me<sub>3</sub>Pyo(4'-5)Cyt 4 ( $R = CH_3$ ). At pH 7 this compound exhibits spectroscopic properties quite similar to those of the acidic form of the *N*(1),*N*'(1)-Me<sub>2</sub>Pyo(4'-5)Cyt 3 ( $R = CH_3$ ) and in addition is shown by electrophoresis to be positively charged (Table IV). As expected, its susceptibility to NaBH<sub>4</sub> reduction is quite high, and the reaction is immediate upon the stoichiometric addition of the reducing agent. The reduced product *N*(1),*N*'(1),N(3)-Me<sub>3</sub>Pyo(4'-5)hCyt 10 ( $R = CH_3$ ) exhibits in water an absorption spectrum similar to that of the *N*(1),*N*'(1)-Me derivative 7 ( $R = CH_3$ ), except for a 5-nm red shift. Spectrophotometric titration indicates an acidic transition ( $pK_a = 4.4$ ) but no basic pK at a pH lower than 13 (Figure 5). Its acidic spectrum is close to the acidic spectrum of compound 7 ( $R = CH_3$ ). Finally, electrophoresis at pH 7 indicates that the N(3) methylated product 10 ( $R = CH_3$ ) is no longer electrically

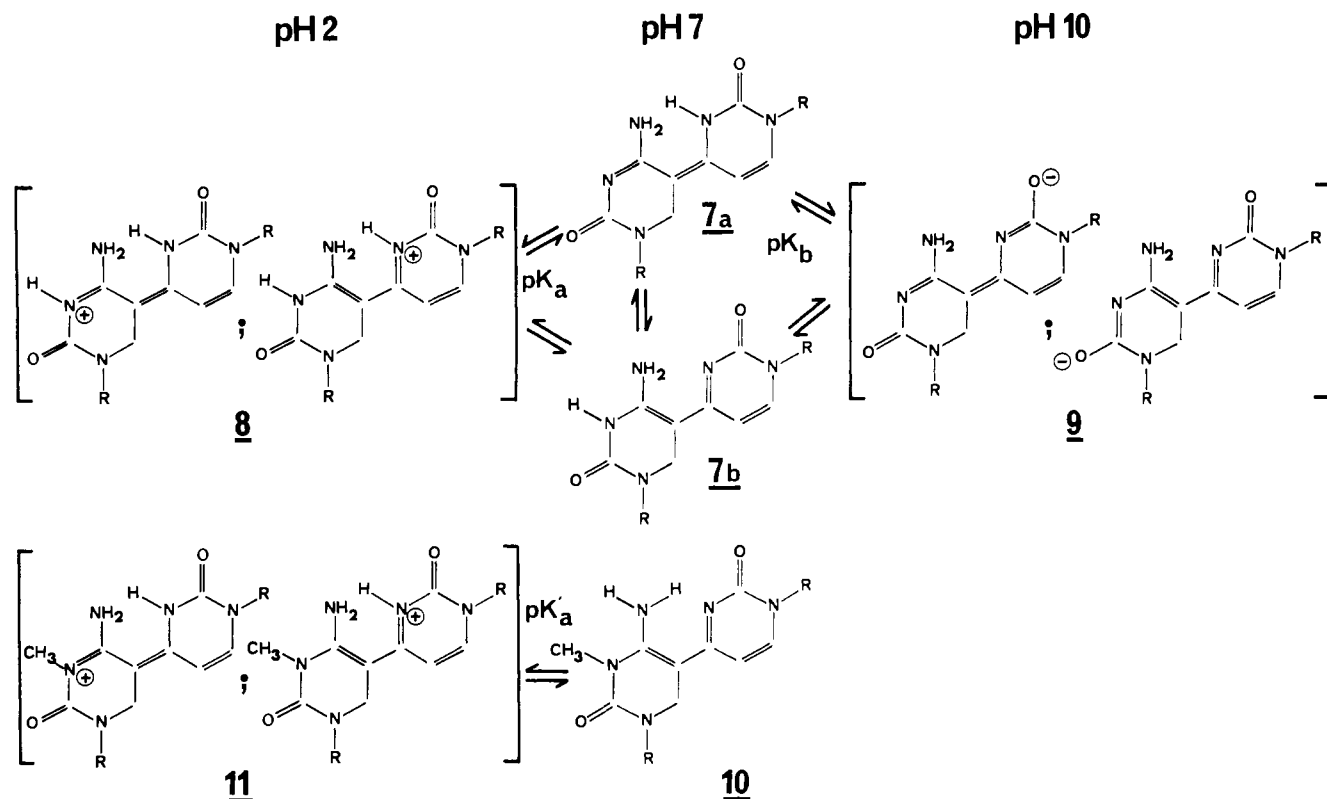


FIGURE 4: Tautomeric forms at neutral, acidic, and basic pH of the  $N(1),N(1')$ -substituted Pyo(4'-5)hCyt and of the  $N(3)$ -methylated derivative.

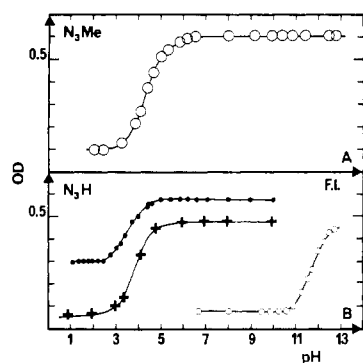


FIGURE 5: (A) Spectrophotometric titration of  $N(1),N(1'),N(3)$ -Me<sub>3</sub>Pyo(4'-5)hCyt **10** ( $R = \text{CH}_3$ ). The transition was monitored by the absorption at 380 nm. No transitions were observed at basic pH at any wavelengths between 350 and 450 nm. (B) Spectrophotometric and spectrofluorimetric titration of  $N(1),N(1')$ -Me<sub>2</sub>Pyo(4'-5)hCyt **7** ( $R = \text{CH}_3$ ). Absorption: (●)  $\lambda$  375 nm, (○)  $\lambda$  410 nm. Fluorescence: (+)  $\lambda_{\text{ex}}$  380 nm,  $\lambda_{\text{em}}$  450 nm. The fluorimetric titration was performed in the presence of 40% sucrose.

charged (Table IV). At pH 7 this product has, therefore, the electronic configuration shown on structure **10** (Figure 4). The spectral properties of this product were examined in a few organic solvents and were found to behave similarly to those of the dimethyl parent compound **7** ( $R = \text{CH}_3$ ), the excitation  $\lambda_{\text{max}}$  being shifted to the red by 5 nm and the emission  $\lambda_{\text{max}}$  by 10 nm. Again, on going from methanol to glycerol,  $\Phi$  increases from  $1.9 \times 10^{-4}$  to  $6 \times 10^{-3}$  (Table III), and the  $\lambda_{\text{max}}^{\text{ex}}$  shifts to the blue by 5 nm, the  $\lambda_{\text{max}}^{\text{em}}$  remains constant at 460 nm, but the emission band becomes narrower. In conclusion, the tautomeric form **7b** shows all the required properties. We do not know, however, whether form **7a** does exist and how it contributes to the fluorescence emission.

The only remaining possibility for proton transfer reactions lies on the amino group. We were able to substitute one or two

TABLE IV: Electric Charge Determination by Electrophoresis.<sup>a</sup>

product	$R_f$	charge
$N(1),N(1')$ -Me <sub>2</sub> Pyo(4-5)hCyt	0.26	0
$N(1),N(1'),N(3)$ -Me <sub>3</sub> Pyo(4-5)hCyt	0.97	+1
$N(1),N(1')$ -Me <sub>2</sub> Pyo(4-5)hCyt	0.25	0
$N(1),N(1'),N(3)$ -Me <sub>3</sub> Pyo(4'-5)hCyt	0.38	

<sup>a</sup> Electrophoretic migrations were performed on TLC cellulose plates in Tris-HCl,  $3 \times 10^{-2}$  M (pH 7.5) buffer.  $R_f$  values are given taking methyl green as reference.

amino protons of  $N(1),N'(1)$ -Me<sub>2</sub>Pyo(4'-5)Cyt by methyl groups (see Materials and Methods). The dimethyl amino substitution strongly affects the NaBH<sub>4</sub> reduction reaction, leading to a very low yield of 380-nm absorbing product. It was not further considered. Reduction of the monomethylamino derivative is similar to that of the parent compound. The spectral properties of this reduced adduct are almost identical to those of the parent compound. In the different solvents their quantum yields are quite close (Table III).

**Temperature.** For most fluorescent substances in solution, temperature is an important factor affecting the emission yield. In the case of Pyo(4'-5)hCyt **7** one would like to know whether temperature acts directly on the nonradiative deactivation rate or indirectly through the viscosity variation of the medium. For this purpose, a detailed investigation was performed in five solvents by continuously increasing temperature from 6 to 72 °C, at a rate of 1 °C/min. The solvents (methanol, ethanol, 1-butanol, glycol, and glycerol) were selected because the temperature dependence of their viscosity ranges in a large domain. Furthermore, their spectroscopic properties allow accurate measurement of the fluorescence emission. In every solvent, the absorption and emission spectra remained invariant. The quantum yield, on the other hand, decreases with increasing temperature in a solvent-dependent fashion (Figure

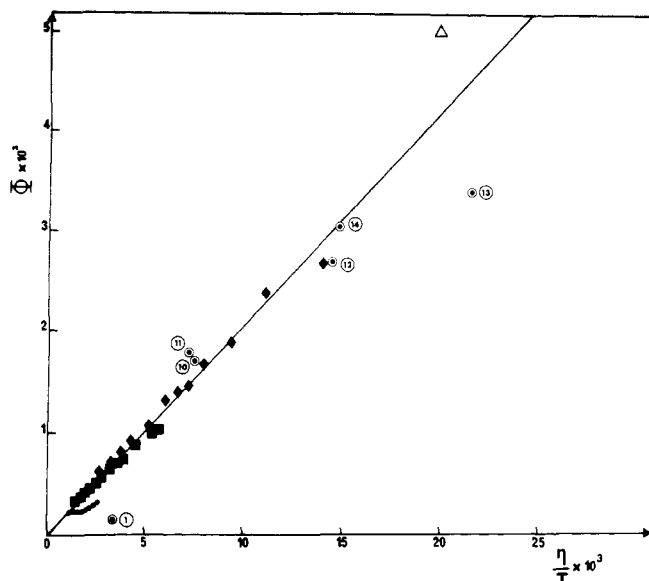


FIGURE 6: Effect of solvent viscosity and temperature on the quantum yield of emission of  $N(1),N(1')$ -Me<sub>2</sub>Pyo(4'-5)hCyt in the monoalcohol series.  $\Phi$  is represented as a function of  $\eta/T$  ( $\eta$  in centipoises,  $T$  in K). The numbered points referred to the measurements done at 300 K in the solvents listed in Figure 2. The triangle corresponds to the measurements done in methanol at 201 K. Other symbols correspond to temperature variations in the range of 6 to 72 °C in methanol (●), ethanol (■), and 1-butanol (◆).

6). In order to further analyze the data, the corresponding apparent enthalpies were determined. Since  $\Phi$  remains small,  $\Phi \approx k_f/k_d$ , where  $k_f$  and  $k_d$  are, respectively, the radiative and nonradiative deactivation rates, the former being temperature independent, the latter of the form  $k_d = k_0 \exp(-\Delta H/RT)$ . Plots of  $\ln \Phi$  against  $1/T$  yield straight lines, allowing the determination of  $\Delta H$  values. In the temperature range studied, the variation of viscosity follows, to a first approximation, the De Guzman empirical equation  $\eta = A \exp(B/RT)$  (Molwyn-Hughes, 1965). The striking finding here is that the absolute values (in kcal/mol) of  $B$  are quite close to the  $\Delta H$  values obtained in the different solvents. In methanol,  $\Delta H = 2.1$  and  $B = 2.45$ . These values are, respectively, 3.5 and 3.4 in ethanol, 4.8 and 4.6 in 1-butanol, 6.7 and 6.4 in ethylene glycol, and 16 and 14 in glycerol. Assuming now that  $B = \Delta H$ , the temperature effect on  $\Phi$  can be represented in this range by the approximate equation.

$$\Phi = \left(\frac{k_f}{k_0}\right) \left(\frac{\eta}{A}\right) \quad (1)$$

However, when  $\Phi$  is plotted as a function of  $\eta/T$ , a similar quasilinear dependence is obtained, since over this temperature range  $T$  varies much less than  $\eta$  (Figure 6). We do not know yet which of the parameters  $\eta$  or  $\eta/T$  is the more relevant to the fluorescence mechanism. In order to extend the data to a higher viscosity range, two experiments were performed at 201 K. In methanol,  $\Phi$  remains low ( $\Phi = 5 \times 10^{-3}$ ) and the emission maximum is at 450 nm. In the much more viscous glycerol at the same temperature, these values are, respectively, 0.4 and 420 nm, again supporting our conclusion that viscosity is a parameter of major importance. Finally, it was of interest to characterize the emission in a rigid medium. We have been unsuccessful with plastic matrices and, therefore, made observations at 77 K in a 50:50 water-glycol glass. The emission band becomes narrower, its  $\lambda_{\max}$  being now at 415 nm. The lifetime is 6.3 ns, a value close to the theoretical intrinsic lifetime (Favre and Yaniv, 1971), and, as expected, the quantum yield becomes close to unity.

## Discussion

Pyo(4'-5)hCyt **7**, the so-called "reduced cross-link", is a particularly attractive fluorescent label, since it can be introduced in the tRNA structure with a minimum of distortion. Our purpose here is to discuss its emission mechanism on the basis of its molecular structure **7**. The present investigation is limited to excitation in the absorption band (320–400 nm) that allows selective excitation in the presence of other nucleic acid residues. The events occurring at shorter wavelengths will be described elsewhere. Our polarization data concerning the probe both when free and in tRNA and the fit between the absorption and excitation spectra argue that the excitation band is due to a single  $\pi\pi^*$  transition.

Since the quantum yield of emission  $\Phi$  is highly sensitive to the probe environment, our discussion will focus on this observation. Some molecular processes which might have been related to the emission mechanism can be neglected. Proton transfer reactions in the excited state appear not to play a significant role in the deactivation process. Thus, the deuterium effect is very small, no excited state pKs can be detected (Figure 5), and substitution of exchangeable hydrogens by methyl groups hardly affects the emission (Table III). This conclusion is in full agreement with the lack of dependence of  $\Phi$  on solvent polarity. Static quenching would be expected if the two tautomeric structures **7a** and **7b** (Figure 4) of the probe proposed by Bergström and Leonard (1972) were present in comparable amounts, but with distinct quantum yields. The close correspondence of the absorption and excitation spectra under different conditions argues against this possibility. This is reinforced by the very similar behavior of the N(3) methylated probe **10** and its parent compound **7b**. On the other hand, the data obtained in a large number of fluid media clearly indicate that viscosity plays a major role in the emission mechanism. However, the dependence of  $\Phi$  on the solvent viscosity  $\eta$  is clearly distinct in the pure liquids and in the water-sucrose or water-glycerol binary mixtures (Figure 2). This is exactly the behavior one would predict if the probe was sensitive to the viscosity of its immediate environment, which is referred to here as the local viscosity. In pure solvents,  $\eta$  and local viscosities are close if not identical. In binary mixtures, this is no longer true. Since the  $N(1),N'(1)$ -dimethyl adduct is approximately one order of magnitude more soluble in water than in pure glycerol (as measured by the 385-nm absorbance of saturated solutions), it is likely that in the binary mixture the immediate environment of the probe is enriched in water molecules. Hence, the macroviscosity  $\eta$  should be higher than the local viscosity and the observed  $\Phi$  lower than expected on the basis of  $\eta$ . The extreme situation is encountered in aqueous gels where  $\Phi$  is low, though the macroviscosity is extremely large. Although we have not been able to ascertain whether  $\Phi$  was linearly dependent on  $\eta$  or  $\eta/T$  in pure solvents, most of the temperature effect can also be explained by the variation of viscosity (see Results).

The viscosity dependence of the fluorescence yield is indicative of a molecular rearrangement taking place after absorption of a photon. This is corroborated by the narrowing of the emission spectrum, together with a blue shift of its maximum and an improved mirror-image relationship (Franck-Condon emission) observed in highly viscous media. Is it possible to define the site and nature of this molecular change in view of the chemical structure of our probe? Many dyes exhibiting similar behavior contain two or more unfused aromatic rings. In the case of auramine O (Oster and Nishijima, 1956), crystal violet (Förster and Hoffman, 1971), tetraphenylbutadiene (Kordas and El-Bayoumi, 1974), and stilbene (Birch

and Birks, 1970), the best studied cases, intramolecular twisting around the covalent bond(s) linking the phenyl groups has been proposed as the main radiationless deactivation path. For Pyo(4'-5)hCyt 7 one should, therefore, consider the internal rotation around the C(5)-C(4') bond. Indeed, we have direct experimental evidence supporting the involvement of this rotation between the two rings in the quenching mechanism. Substitutions at the N(1),N'(1) positions by identical chemical groups increase  $\Phi$  according to their bulkiness and irrespective of their polar (or apolar) character (Table I and Figure 2). This can be understood, since increasing the substituents volume increases the frictional coefficients of the two moieties of the probe and, therefore, hinders their rotational motion. The two substituents, however, should play a different role in the emission mechanism. The N(1) substituent directly acts on the frictional coefficient of the dihydrocytosine ring. On the other hand, owing to the internal rotation around the R-N'(1) bond, the N'(1) substituent can only affect the frictional coefficient of the 2'-oxypyrimidin-4'-yl ring through a weak coupling. The fact that  $\Phi$  increases with the number of nucleotides in the oligonucleotides series (Table I) can be interpreted along similar lines. The other mobile group that should be considered is the exocyclic amino group, which can rotate around the C(4)-N bond. In anthranilic acid the rotational motion of the amino group has been proposed to take part in the quenching mechanism (Tramer, 1970). In Pyo(4'-5)hCyt 7, the role of the amino group rotation appears, however, at most, of secondary importance, since its methylation hardly affects the quantum yield (Table III).

A high yield of emission is observed when the probe is blocked in its ground-state conformation (in a glass at 77 K, in the highly viscous solvent glycerol, or when stacked in tRNA). The probe should be close to planarity, i.e.,  $\theta$  close to  $0^\circ$ . On the contrary a low yield is observed in a medium of low viscosity when the probe is allowed to twist freely around the C(5)-C(4') bond. From the linear dependence of  $\Phi$  with  $\eta/T$  in the monoalcohols and in glycol (Figures 6 and 7), one could suggest that the nonradiative deactivation occurs via internal rotational diffusion (rate controlled by viscosity) (Oster and Nishijima, 1956). This would be expected if the potential energy curves as a function of  $\theta$  were shallower in  $S_1$  than in  $S_0$ . From our present data, however, it is not clear whether  $\eta/T$  or  $\eta$  is the parameter which is more relevant to the emission mechanism. The apparent fit of the 201 K emission yield with those obtained in the range of temperature 0 to  $60^\circ\text{C}$  in the  $\eta/T$  representation (Figure 7) can be misleading due to the possible large error in the estimation of the 201 K viscosities (by the De Guzman equation).

Alternatively, at least for the monoalcohols and glycol,  $\Phi$  is, to a first approximation, proportional to viscosity in the temperature range 0 to  $60^\circ\text{C}$  (eq 1). This can be accounted for by a simple mechanism derived from the proposal of Förster and Hoffman (1971) for crystal violet. In this model, it is assumed that the equilibrium conformation of the ground singlet state  $S_0$  and that of the first excited state  $S_1$  lies at a different value of  $\theta$ . Suppose that for  $S_0$  the molecule is planar ( $\theta = 0$ ) and that for  $S_1$  the stable position is at  $\theta = \theta_m$ . After absorption of a photon, the molecule in the  $S_1$  excited state would relax by twisting around the C(5)-C(4') bond, and the angle  $\theta$  as a function of time is given by:

$$\theta = \theta_m \left[ 1 - \exp\left(-\frac{c}{f}t\right) \right] \quad (2)$$

where  $f$  is the frictional coefficient of the rotating group and is proportional to  $\eta$ ,  $f = a\eta$ , and where  $c$  is the momentum constant associated with the rotation.

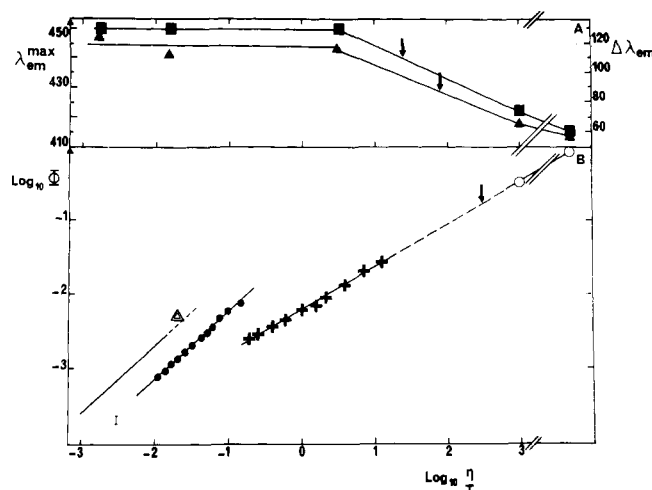


FIGURE 7: A summary of the spectral data obtained with  $N(1),N'(1)$ - $\text{Me}_2\text{Pyo}(4'-5)\text{hCyt}$  as a function of  $\ln \eta/T$ . (A) Wavelength of the maximum of emission (■) and width at half-height of the emission band (▲). (B) Variation on a logarithmic scale of the quantum yield: the straight line of the left side corresponds to the data of Figure 6, and the triangle again corresponds to methanol at 201 K. The symbol I corresponds to water at 300 K. The lines (●-●) and (+ + +) are the data obtained by variation of temperature in glycol and glycerol, respectively. At  $\log \eta/T = 2.8$  are the 201 K data in glycerol and on the right side the 77 K data in a 50:50 glycol-water glass. The vertical arrow indicates the values for  $\text{Pyo}(4'-5)\text{hCyt}$  in native tRNA at  $25^\circ\text{C}$ .

In order to obtain a linear dependence of  $\Phi$  upon viscosity, we supposed in the following oversimplified model that quenching occurs only when  $\theta$  is larger than a critical angle  $\theta_0$ .  $\theta_0$  could represent the angle where the potential energy curve of  $S_1$  crosses that of another excited electronic state or that of the ground state itself. Emission occurs (with a radiative rate constant  $k_f$ ) only when  $\theta$  is inbetween 0 and  $\theta_0$ . The time interval required for the molecule to reach the critical angle  $\theta_0$  can be derived from:

$$t_0 = \frac{a}{c} \eta \ln \frac{\theta_m}{\theta_m - \theta_0} \quad (3)$$

and the emission yield becomes:

$$\Phi = 1 - \exp(-k_f t_0) \quad (4)$$

which for the low values of  $\Phi$  simplifies down to

$$\Phi = k_f t_0 = \frac{t_0}{\tau_0} \quad (5)$$

$$\Phi = \frac{a}{\tau_0 c} \eta \ln \frac{\theta_m}{\theta_m - \theta_0}$$

where  $\tau_0$  is the intrinsic lifetime.  $\Phi$  is, therefore, simply related to geometric parameters ( $a$ ,  $\theta_m$ ,  $\theta_0$ ). It is clear that substitutions at the N(1),N'(1) positions only affect the  $a$  constant. Thus, eq 5 accounts satisfactorily for the data of Table II. It provides also a simple interpretation for the different proportionality constant found in the monoalcohols and in glycol. The solvent should slightly affect the potential-energy curves. In the ground state this is illustrated by the displacement of the excitation  $\lambda_{\text{max}}$  in the different solvents. Thus, the angle  $\theta_0$  and, to a lesser extent,  $\theta_m$  could possibly be solvent dependent.

In order to check the validity of the relaxation model ( $\Phi$  proportional to  $\eta$ ) or of the diffusion model ( $\Phi$  proportional to  $\eta/T$ ), three approaches can be used: (1) by studying the fluorescence dependence on viscosity as pressure is varied at constant temperature (Brey et al., 1977); (2) by freezing the probe in different twisted conformations (for instance, increasing the



bulkiness of substituents at position 5' of **7** should allow an experimental measure of  $\theta_0$ ; and (3) by studying the effect of substituents at the N'(1) and N(1) positions on  $\Phi$ . As already discussed,  $\Phi$  should be more sensitive to a change at the latter position.

Measurements of local viscosity are generally performed using fluorescence anisotropy techniques for which a large body of theoretical work has been developed (Belford et al., 1972). Its determination using the fluorescence yield of viscosity-sensitive fluophores provides an alternative approach which is well illustrated by the recent studies of lipid vesicles fluidity with *trans*-stilbene (Geiger and Turro, 1977). With appropriate substituents, Pyo(4'-5)hCyt **7** fluorescence should allow the monitoring of local viscosity in either polar or apolar media and should be particularly useful in following relaxation processes.

The two techniques yield, however, complementary pieces of information when the probe is attached to a macromolecule. For example, nanosecond fluorescence anisotropy studies of tRNA containing the reduced 8-13 link allow the determination of the rotational motion of the whole tRNA molecule. The quantum yield of emission of Pyo(4'-5)hCyt, on the other hand, is related to the local viscosity inside the tRNA tertiary structure. Further data and their analysis in terms of the internal Brownian motion of tRNA will be reported elsewhere. The use of Pyo(4'-5)hCyt **7** is not limited to tRNA. It has been chemically introduced in synthetic polynucleotides (Favre, 1972; Vigny and Favre, 1974; Favre and Fourrey, 1974) and this has recently been extended to natural nucleic acids.

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