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Studies on the Conformation of the Anticodon Loop of Phenylalanine Transfer Ribonucleic Acid. Effect of Environment on the Fluorescence of the Y Base*

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ABSTRACT: The fluorescence intensity of the Y base of yeast and wheat germ $tRNA^{Phe}$ is enhanced two- to threefold when magnesium ion is added to a neutral aqueous solution of the tRNA. In 0.1 M NaCl, the range of magnesium concentrations used, 10^{-1} to 10^{-5} M, appears to have little effect on the overall structure of these two tRNA species. Our results suggest, however, that magnesium does have a profound effect on the conformations at or near the anticodon loop. The activity of magnesium is unlikely to be the result of direct binding near the Y base since the fluorescence of a hexanucleotide containing Y is independent of magnesium. The fluorescence lifetime and intensity in this oligomer are considerably less than what was found for magnesium-free tRNA. The fluorescence of $tRNA^{Phe}$ in the absence of magnesium is enhanced by D_2O. A similar enhancement occurs in the hexanucleotide but virtually no effect is observed with intact $tRNA^{Phe}$ when magnesium is present at high concentrations. Studies of the nanosecond depolarization of Y in $tRNA^{Phe}$ show that the fluorescent base is not rigidly attached to the bulk of the tRNA. The fluorescence is partially depolarized both in the presence and absence of magnesium. All of these results are consistent with the following picture. The Y base is quenched by contact with water. When magnesium is present, the conformation of $tRNA^{Phe}$ changes in such a way that the Y base is shielded from the solvent.

Most physical techniques that have been used to study the conformation of tRNA provide information about the average secondary structure or overall shape (Cantor *et al.*, 1966; Englander and Englander, 1965; Adams *et al.*, 1967; Lake and Beeman, 1968). To construct an accurate picture of the structure of tRNA in aqueous solution it is necessary to obtain detailed information about the relative stability and proximity of a number of important regions of the

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molecule. Previous approaches have yielded information about the CCA terminus (Hoffman *et al.*, 1969; Millar and Steiner, 1966), regions involving ψ residues (Millar, 1969), and the proximity of the thio bases in *Escherichia coli* RNA (Lipsett and Doctor, 1967; Yaniv *et al.*, 1969). Chemical modification studies have indicated that some of the bases on the anticodon loop are susceptible to attack by guanine- (Litt, 1969) and adenine- (Cramer *et al.*, 1968) specific reagents. Little else is known about the structure of the anticodon loop although some detailed structures have been suggested on the basis of model building (Fuller and Hodgson, 1967).

The presence of a naturally fluorescent base, Y, in the anticodon loop of several species (RajBhandary *et al.*, 1967; Dudock *et al.*, 1969; Fink *et al.*, 1968) of tRNA^{Phe} permits the conformation of this region of a tRNA molecule to be examined selectively. There are at least three different ways that fluorescent chromophores attached to a macromolecule can yield structural information (Stryer, 1968). These are quantum yield or lifetime measurements, fluorescence depolarization, and energy-transfer experiments. Applications of the third technique using the Y fluorescence have been described elsewhere (Beardsley and Cantor, 1970; Tao *et al.*, 1970). In the present work a number of studies employing the first two techniques will be described.

From these preliminary studies it appears that the Y base is a very sensitive indicator of the structure of the anticodon loop.

Materials and Methods

tRNA. Some of the yeast tRNA^{Phe} used was prepared and assayed as described previously (Beardsley and Cantor, 1969). Various samples had a purity between 67 and 90%. Additional yeast tRNA^{Phe} was purchased from Boehringer-Mannheim. As assayed in our laboratory it was 68% pure. Wheat germ tRNA^{Phe}, highly purified, was a gift from Professor Bernard Dudock, State University of New York at Stony Brook.

Solutions Used for Optical Studies. All solutions except those used for nanosecond depolarization studies contained 0.1 M NaCl and 0.01–0.001 M phosphate buffer (pH 6.6). Magnesium was removed from tRNA^{Phe} as described by Reeves *et al.* (1970). To prepare solutions containing magnesium, $MgCl_2$ was added to the magnesium-free solutions. Using the conditions described by Reeves *et al.*, we found the optical changes accompanying magnesium removal to be completely reversible. This is true, in fact, even if more drastic conditions, higher EDTA concentrations and temperatures (R. Langlois, unpublished results), are used. The solutions used for depolarization measurements contained 0.02 M Tris (pH 7.8) and either no magnesium or 0.0026 M magnesium.

D_2O solutions of tRNA were prepared either by lyophilizing an aqueous solution to dryness and adding an appropriate amount of D_2O, or by dilution. Lyophilization itself caused very small changes in the fluorescence of Y which were not very reproducible. This may be due to slight losses of material. Some samples of D_2O were redistilled to make sure that no artifacts were introduced by impurities in the D_2O. No differences were observed whether redistilled or ordinary commercial D_2O were used. The changes in fluorescence caused by D_2O are qualitatively the same when different tRNA samples were used. However, the exact quantitative results often changed about 25% from sample to sample. We have no explanation for this observation.

Static Fluorescence Measurements. The shape of the emission spectrum of Y and the relative fluorescence efficiencies were obtained on an Aminco-Bowman spectrofluorimeter equipped with a Beckman 10-in. recorder and a thermostatted sample compartment. All emission and excitation spectra shown are uncorrected. Unless specifically mentioned, the temperature was 24°.

Fluorescence Lifetime and Nanosecond Depolarization. The instrument used for these measurements employs the single photon-counting technique. The details of its construction have been described elsewhere (Tao, 1969). Because the lifetime of Y is not significantly longer than the decay time of the excitation, a curve-fitting technique outlined by Hundley *et al.* (1967) was used to analyze the data. Assuming that the fluorescence decays with a single lifetime, τ, the observed decay curve $R(t)$ would be a convolution integral

$$R(t) = \int_0^t E(t')e^{-(t-t')/\tau}dt' \qquad (1)$$

where $E(t')$ is the time profile of the excitation. $R(t)$ and $E(t)$ are experimentally determined. A computer program generates a grid of τ values in steps of 0.01 nsec, computes by numerical integration the calculated decay curve $R_c(t)$ at each point t_i, and then takes the sum of the residues

$$X^2 = \sum_i [R_c(t_i) - R(t_i)]^2 \qquad (2)$$

The value of τ that gives rise to the smallest X^2 is taken to be the desired lifetime. Using this method of analysis, the lifetime of 10^{-6} M fluorescein in 0.1 N NaOH was found to be 4.8 nsec. Duplicated measurements yielded a standard deviation of 0.07 nsec. The quality of the fits can be judged from two standpoints: (1) the overlap between the experimental decay curve and the calculated decay curve; (2) the small X^2 values. Thus for good exponential decays like that of fluorescein, the experimental points fall around the calculated convolution integral for at least 2.5 decades. It is rather easy to recognize nonexponential decays, for although X^2 is minimized, this minimal value would be significantly larger than that due to only statistical scatter. In addition, one would notice that one portion of the experimental curve might entirely fall beneath (or above) the calculated curve, while another portion might fall entirely above (or beneath). Thus although we cannot resolve multiple exponential decays, any deviation from simple exponential decay can readily be detected.

To minimize detection of scattered light, we have employed a combination of Corning C57-60 and C57-54 glass filters on the excitation beam. The emission was observed at 440 nm through a Jarrell-Ash 0.25-m monochromator (5-mm slits). The degree of scattering was deemed insignificant after the following tests were made. (1) An additional C57-60 filter was inserted; data collection took more than twice as long, but the shape of the decay curve was unchanged. (2) The emission wavelength was moved further into the visible,

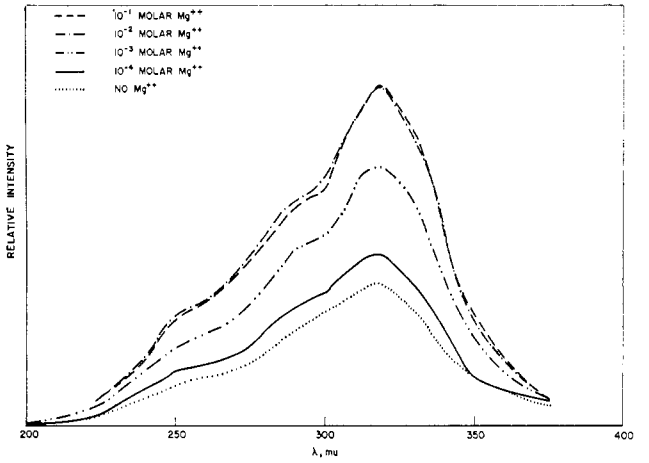

FIGURE 1: Effect of magnesium ion on the uncorrected excitation spectrum of yeast $tRNA^{Phe}$. The fluorescence intensity at 450 nm is shown as a function of the wavelength of the exciting light.

namely 500 nm, again there was no change in the shape of the decay curve.

Circular Dichroism and Absorption Spectra. These measurements were made, respectively, using a Cary 6001 circular dichroism attachment to the Cary 60 spectropolarimeter and using a Cary 15 spectrophotometer. Temperature-dependent spectral measurements employed a jacketed 10-mm diameter silica cell for circular dichroism measurements, and a thermostatted cell holder for absorbance measurements. All results described in this paper are given in units of molar ellipticity or molar extinction per residue. Unless specifically mentioned, the temperature was 26°.

Hydrolysis of $tRNA^{Phe}$. Pancreatic ribonuclease was used to hydrolyze $tRNA^{Phe}$ to a mixture of oligonucleotides. The hydrolysis was carried out in a cuvet by the addition of 5 μg of enzyme to a solution containing between 200 and 700 μg of tRNA in 0.01 M phosphate buffer (pH 6.6) and no magnesium. Changes in absorbance were monitored to follow the reaction to completion. Under our conditions, this took less than 1 min. The hyperchromicity accompanying enzyme digestion was 30.3%. Cleavage of yeast $tRNA^{Phe}$ to yield the fragment Y^+ was carried out by treatment at pH 2.8 for 3 hr at 37° as described by Thiebe and Zachau (1968). Y^+ fluorescence was examined after neutralization. In one case, the Y^+ was isolated from the reaction mixture by $CHCl_3$ extraction.

Results

Static Fluorescence of $tRNA^{Phe}$. The Y base of yeast (RajBhandary *et al.*, 1967), rat liver (Fink *et al.*, 1968), and wheat germ (Dudock *et al.*, 1969) tRNA is located on the anticodon loop, immediately adjacent to the 3′ end of the anticodon. The structure of this base is still unknown, although bases in the analogous position of all other tRNAs of known sequence have been shown to be modified adenines. The Y base differs from all of these bases because its absorption maximum occurs at about 315 mμ (Thiebe and Zachau, 1968). This is considerably to the red of the maxima of most known nucleic acid bases. More remarkable is the fact that the Y base in the tRNA is moderately fluorescent. The

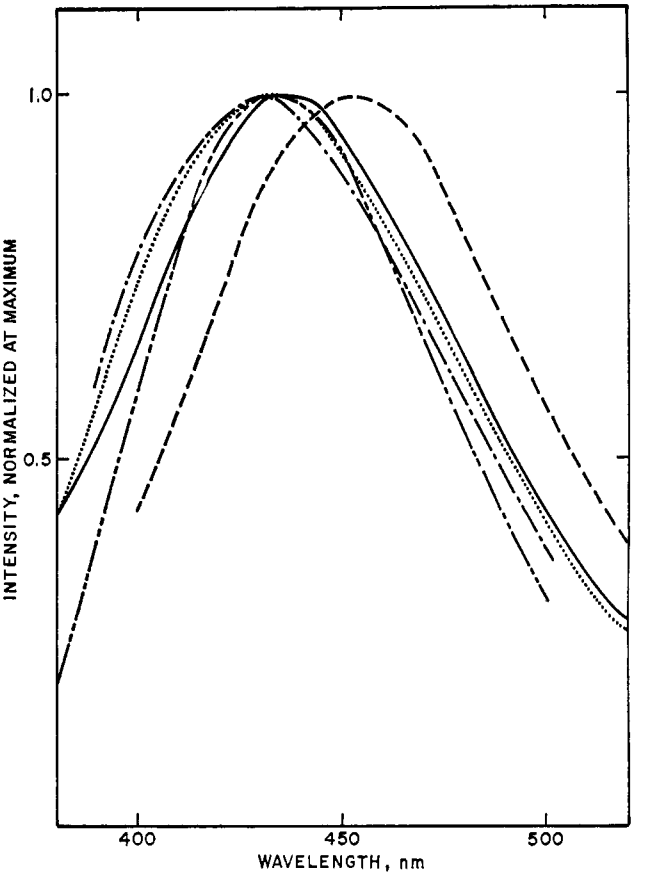

FIGURE 2: Emission spectrum of the Y base in various environments. All samples were excited at 320 nm. (·····) $tRNA^{Phe}$ in 0.01 M Mg^{2+}; (—·—·—) $tRNA^{Phe}$ in no Mg^{2+}; (————) RNase digested $tRNA^{Phe}$ in no Mg^{2+}; (—··—··—) Y^+ in $CHCl_3$; (------) Y^+ in H_2O.

uncorrected excitation spectrum of yeast $tRNA^{Phe}$ is shown in Figure 1 as a function of magnesium concentration. In addition to magnesium ion, all of these solutions contain 0.1 M sodium chloride. The fluorescence of Y is strongly enhanced as magnesium is added to a tRNA solution. The increase in relative quantum efficiency occurs with virtually no change in the shape of the excitation spectrum and only a small shift in frequency. Under the conditions we have used, the response of the system to magnesium ion occurs very rapidly. If magnesium is added to a tRNA sample in a cuvet and the fluorescence is monitored, no time dependence is observed.

The emission spectrum of yeast $tRNA^{Phe}$ is plotted in Figure 2 for samples which contain either no magnesium or 0.01 M magnesium ion. The data in Figure 2 have been adjusted such that all curves shown have the same relative intensity at the emission maximum. This permits changes in the shape of the emission spectrum to be visualized clearly. The true relative emission intensities are shown in Table I. It is apparent from the data in Figure 2 that less than a 5-nm shift in fluorescence accompanies the addition of magnesium.

The sensitivity of the Y base of yeast $tRNA^{Phe}$ to magnesium extends over a wide range of magnesium ion concentration. A 250% increase in fluorescence intensity occurs gradually as the magnesium ion concentration is raised

TABLE I: Fluorescence from the Y Base of tRNA^{Phe}

Sample	Rel Static Fluorescence[a]	Decay Time (nsec)[b]	X^2/Channel[c]
Fluorescein in 0.1 N NaOH		4.8	3.0×10^{-4}
$tRNA^{Phe}_{yeast}$, 0.01 M Mg^{2+}	243	6.3	4.3×10^{-3}
$tRNA^{Phe}_{yeast}$, no Mg^{2+}	100	4.3	8.6×10^{-3}
$tRNA^{Phe}_{yeast}$, in D_2O, 0.01 M Mg^{2+}	267		
$tRNA^{Phe}_{yeast}$, in D_2O, no Mg^{2+}	153	7.3	2.2×10^{-3}
$tRNA^{Phe}_{yeast}$, RNase treated	45	1.8	1.0×10^{-2}
$tRNA^{Phe}_{yeast}$, RNase treated, in D_2O	95		
$tRNA^{Phe}_{wheat\ germ}$, 0.01 M Mg^{2+}	111	6.6	5.9×10^{-4}
$tRNA^{Phe}_{wheat\ germ}$, no Mg^{2+}	36	4.2	4.0×10^{-3}
Excised Y^+, in chloroform	703		
Excised Y^+, in H_2O	110		

[a] Emission at 430 nm, excitation at 320 nm. All at the same concentration except for excised Y^+. The relative intensities for those samples were corrected for this concentration difference. [b] All decay curves were fitted to one exponential. See text for further details. [c] All decay curves were normalized so that the areas under the curves were equal to 100. Approximately 70 of the initial channels (or 1.5 decades of decay) were fitted.

from $<10^{-5}$ to 0.1 M. At both high and low magnesium concentrations the effect appears to saturate. These emission changes are accompanied by a small decrease in the molar extinction at 257.5 nm of the tRNA as magnesium is added. This closely parallels the fluorescence changes as shown in Figure 3. Very similar results on the dependence of Y fluorescence in tRNA^{Phe} on magnesium concentration have been obtained by J. Eisinger, B. Feuer, and T. Yamane (manuscript in preparation).

It was of interest to see whether the variations in magnesium concentrations employed for the fluorescence studies had a drastic effect on the overall conformation of tRNA^{Phe}. For this purpose, circular dichroism measurements were made on two of the samples used for fluorescence studies. These results are shown in Figure 4. Addition of magnesium causes a small increase in the magnitude of the 267-nm positive circular dichroism band of tRNA. There is no evidence of a shift of the maximum of this band; some small changes were observed in the intensity of the long-wavelength negative dichroic band at 295 nm. This suggests that only little increase in secondary structure has occurred upon addition of magnesium ion.

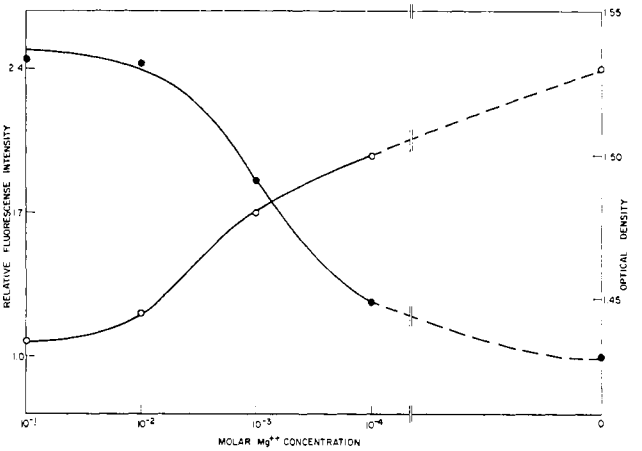

FIGURE 3: Fluorescence intensity of Y base at 450 nm (excited at 320 nm) and absorbance of tRNA^{Phe} at 257.5 nm as a function of magnesium ion concentration.

Similar studies have been performed using wheat germ tRNA^{Phe}. While these have not been quite as comprehensive, the results on this tRNA are quite similar to what is found with yeast tRNA^{Phe}. In fact, the enhancement of Y fluorescence by magnesium is slightly larger. A 300% enhancement is observed as magnesium is added to a previously dialyzed sample.

Flourescence of tRNA^{Phe} Fragments. The findings detailed thus far indicate that either magnesium causes a conformational change which indirectly affects the fluorescence of the Y base or that magnesium can in some way bind near or to the Y base and directly alter its emission properties. To distinguish between these two hypotheses, studies were made on the fluorescence of fragments of tRNA containing the Y base. A sample of yeast tRNA^{Phe} containing no magnesium was hydrolyzed in a fluorescence cuvet by the addition of extremely small amounts of pancreatic RNase. This produces a set of oligonucleotide fragments including the hexanucleotide $G_{2'\text{-}OMe}AAYA\psi$. The reaction was followed to comple-

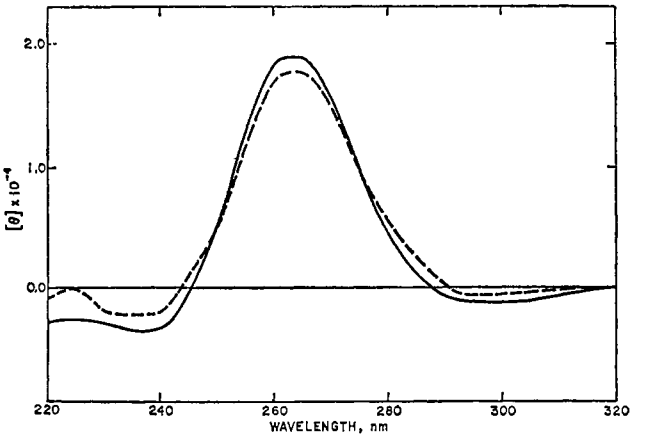

FIGURE 4: Circular dichroism of yeast tRNA^{Phe} in the presence and absence of magnesium ion. Both solutions contained 0.1 M NaCl. Very similar results are observed for wheat germ tRNA^{Phe}: (——) 0.01 M Mg^{2+}; (------) no Mg^{2+}.

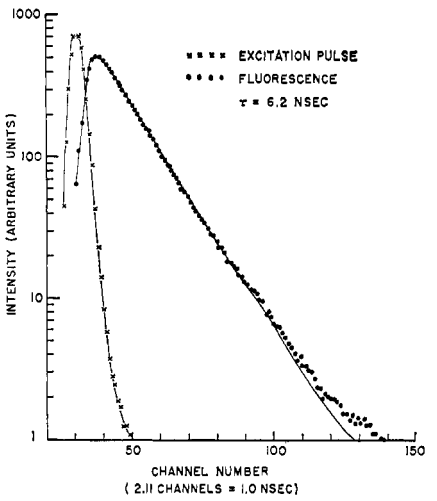

FIGURE 5: Fluorescence decay of the Y base of $tRNA^{Phe}$ in 2.6 $\times 10^{-3}$ M Mg^{2+}. The solid line passing through the filled circles is a best one exponential fit representing a decay time 6.2 nsec.

tion by monitoring the absorption spectrum. The digestion, of a sample which originally contained no magnesium, is accompanied by more than a 2-fold decrease in the fluorescence of the Y base but no substantial change in the shape of the emission spectrum. These experiments are summarized in Table I and Figure 2. Addition of magnesium to the mixture of oligonucleotides resulting from pancreatic RNase hydrolysis caused no change in the fluorescence. This demonstrates that in the absence of an intact anticodon loop, magnesium cannot affect the emission spectra of Y.

To further explore this point, yeast $tRNA^{Phe}$ was treated at pH 2.8 for 3 hr. Under these conditions, Thiebe and Zachau (1968) have shown that the Y base is specifically cleaved from the tRNA liberating a fluorescent fragment, Y^+, with very similar emission properties. We found that the fluorescence of Y^+ is also independent of magnesium ion. These experiments ruled out a direct interaction between magnesium ion and the Y base unless the specific structure of the anticodon loop provides a binding site for magnesium immediately adjacent to the strange base. If this possibility is excluded, one must then explain the effect of magnesium in terms of a conformational change in the tRNA.

The experiments reported thus far raised the suspicion that the Y base was, like ethidium bromide and ANS,[1] a chromophore which fluoresced intensely in nonpolar solvents and was strongly quenched by interaction with polar solvents. To examine this point, the relative efficiency of Y^+ fluorescence was measured in H_2O and $CHCl_3$. The results, shown in Table I indicate that Y^+ is more than 6 times as fluorescent in a relatively nonpolar solvent than it is in water. The emission maximum of Y^+ in water occurs at least 20 nm to the red of the maximum in $CHCl_3$. In $CHCl_3$ the emission of Y^+ closely resembles the emission of Y in intact $tRNA^{Phe}$ as shown in Figure 2. It is indeed fortunate to have a naturally occurring solvent-sensitive fluorescent probe like Y next to the anticodon loop of $tRNA^{Phe}$.

[1] Abbreviation used is: ANS, 1-analino-8-naphthalenesulfonate.

Fluorescence Lifetime Measurements. As described earlier, the magnesium-dependent fluorescence changes appear to level off at both high and low magnesium concentrations. It was of interest to see whether, at the end points, a single conformation was present in solution. This question was examined by measurements of the fluorescence lifetime of the Y base in $tRNA^{Phe}$. The fluorescence decay of Y in a typical sample of yeast $tRNA^{Phe}$ is shown in Figure 5. Additional results are summarized in Table I. Also included in the figure is the shape of the pulse of exciting light, and the calculated curve, using eq 1. In the presence of magnesium, the emission of Y can be fitted reasonably well by a single exponential convoluted with the excitation pulse. Best fits were obtained with a lifetime of 6.6 nsec for wheat germ $tRNA^{Phe}$, and 6.3 nsec for yeast $tRNA^{Phe}$. For samples with no magnesium, on the other hand, the decay kinetics are more complex. If the short time portion of the decay curve is fitted to a single convoluted exponential, the resulting lifetime is considerably decreased when compared with magnesium samples. The fits are quite poor, however, as shown by the X^2 values in Table I. This indicates that at least two exponentials are needed to fit the observed nanosecond fluorescence decay.

Since the data obtained for these very short lifetimes is very inaccurate, the parameters derived from a two exponential fit would not be very meaningful. One can conclude, however, from the lack of a single exponential decay that the magnesium-free $tRNA^{Phe}$ samples are a mixture of more than one emitting component. This suggests that under our conditions complete conversion into a conformation stable in the absence of magnesium has not occurred.

The lifetime of Y fluorescence in the oligonucleotide $G_{2'\text{-}OMe}AAYA\psi$ was also measured. The experimental data is given in Table I. The actual value is not terribly significant since the lifetime is so short. However, it is completely safe to conclude that the Y in an oligonucleotide has even a shorter lifetime than the magnesium-free form. The progressive decrease in lifetime as Y is examined in $tRNA^{Phe}$ with magnesium, without and in a single-strand oligomer is consistent with the idea mentioned earlier that these changes expose the Y to the solvent which quenches the fluorescence.

Effect of D_2O. The effect of D_2O on the emission properties of the Y base of $tRNA^{Phe}$ was examined to obtain further information about the effect of environment on Y fluorescence. It is known that in the presence of D_2O the fluorescence of many molecules is enhanced. This can be explained by either of two mechanisms. If the excited state is quenched by a competing protonation or deprotonation reaction, substitution of D_2O for H_2O should slow this process and thus enhance the fluorescence (Stryer, 1966). Alternatively, the quenching may be the result of solvent reorientation about the excited state (Eisinger and Navon, 1969). This will also be diminished in the presence of D_2O. Regardless of the mechanism, the presence of a D_2O effect on emission would be strong circumstantial evidence for the ability of an excited Y base to interact with solvent. It seems quite unlikely that D_2O could cause substantial conformational changes in the tRNA (Bock and Hoffman, 1968). The effect of D_2O on the fluorescence of Y in $tRNA^{Phe}$ and fragments is illustrated by the data in Table I. In the absence of magnesium, D_2O causes an approximately 50% enhancement of the

fluorescence of Y. No shift in spectrum is observed. A somewhat larger effect is observed if ribonuclease-digested yeast tRNA^{Phe} is dissolved in D_2O. In contrast, virtually no enhancement is observed when D_2O is added to a sample of intact yeast tRNA^{Phe} when magnesium is present. The precise amount of enhancement differed somewhat when different tRNA^{Phe} samples were used. This is not well understood and further experiments are in progress. However, results obtained thus far suggest that in magnesium-free samples of intact tRNA^{Phe} the Y base is exposed to the solvent. Adding magnesium changes the environment of the Y base in such a way that the solvent quenching is strongly diminished.

A few preliminary measurements of the fluorescence decay of Y in D_2O solutions of tRNA^{Phe} in the absence of magnesium have been carried out. These indicate, as shown in Table I, that in the presence of D_2O, the complex decay kinetics at low magnesium concentration are suppressed. The emission can now be fit to a single exponential with a lifetime quite similar to samples containing magnesium.

Temperature Dependence of Optical Properties. The results described thus far indicate that a conformational difference exists between tRNA^{Phe} in the presence and absence of magnesium ion which strongly affects the fluorescence of Y. At high temperature (greater than 75°) the secondary structure of most tRNAs is virtually completely unfolded. One should then expect the fluorescence of Y to be very similar in the absence and presence of magnesium since perturbation of its environment by tRNA conformation should largely be eliminated. The fluorescence of Y in tRNA^{Phe} is shown in Figure 6 as a function of temperature. Also included is the ultraviolet absorption at 257.5 nm which provides an estimate of the amount of secondary structure (base pairing and base stacking) remaining in the tRNA. The melting curve for yeast tRNA^{Phe} in the absence of magnesium afforded by the ultraviolet spectrum is in very good agreement with the previously published results of Cramer *et al.* (1968). The melting in the presence of magnesium is qualitatively quite similar except that it occurs at a considerably higher temperature.

For both samples at low temperature, the secondary structure is fairly independent of temperature. In contrast, the fluorescence of the Y base either in the presence or absence of magnesium diminishes rapidly as the tRNA^{Phe} is warmed above room temperature. The temperature dependence of the fluorescence is very complex since it can arise both from conformational changes and from the intrinsic temperature dependence of various possible quenching processes. At the high-temperature limit of our experiments, however, the magnesium effect appears to be disappearing. This is the result that would be expected. Further confirmation comes from the temperature dependence of the fluorescence of the RNase digest. This is also shown in Figure 6. At high temperatures, the fluorescence of the digest appears to be approaching that of the intact tRNA.

Nanosecond Fluorescence Depolarization. The fluorescence lifetime of the Y base of tRNA^{Phe} is sufficiently long to permit measurements of the decay of the fluorescence antisotropy $r(t)$

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} = \frac{d(t)}{s(t)} = e^{-t/\tau_D} \qquad (3)$$

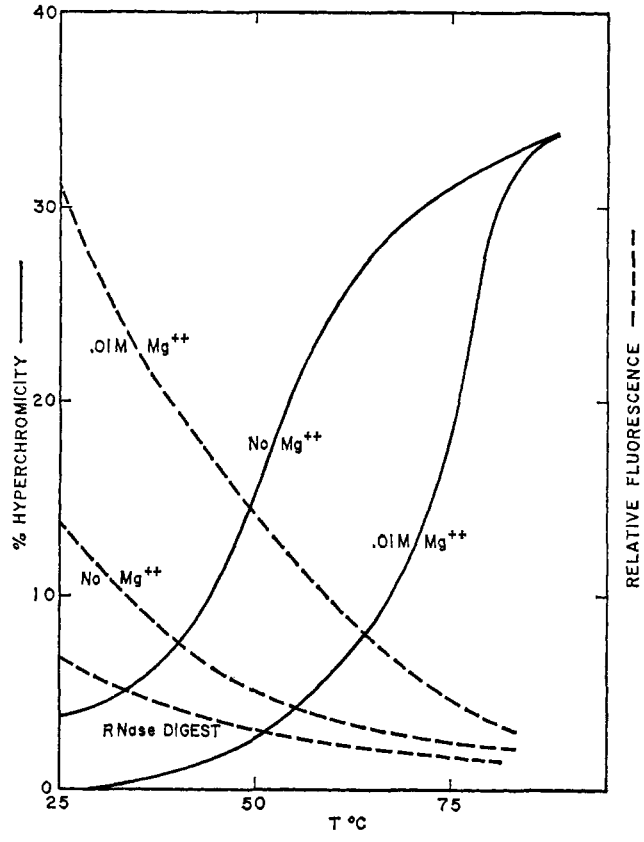

FIGURE 6: Temperature dependence of Y fluorescence in various environments. Also shown is the 257.5-nm absorbance of yeast tRNA^{Phe} as a function of temperature.

In this expression $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the emitted light intensities polarized parallel and perpendicular to the direction of polarization of the exciting beam. For simplicity, we have assumed to a first approximation that the tRNA^{Phe} molecules were spherical, such that $r(t)$ is a single exponential. The apparent relaxation time of a sphere, τ_D, can be calculated from the Einstein equation $1/\tau_D = kT/V\eta$, where T is the temperature, η the viscosity, and V the volume. The sum, $s(t)$, is proportional to the fluorescence decay and can be measured independently as described elsewhere (Tao *et al.*, 1970). The difference, $d(t)$, was obtained directly from $I_{\parallel}(t)$ and $I_{\perp}(t)$. Once again, because the lifetime of Y is not significantly longer than the decay of the excitation, we could obtain $d(t)$ and $s(t)$ convoluted with the time profile of the excitation, but not $r(t)$ directly by point by point division. Instead, we have shown the data in Figures 7 and 8 in the form of $d(t)$ for two samples, one containing no magnesium, the other containing 0.0026 M Mg^{2+}. This low magnesium concentration was used to eliminate the risk of aggregation at the relatively high sample concentrations needed for anisotropy measurements (Tao *et al.*, 1970; Millar and Steiner, 1966).

As described earlier, $s(t)$ for the sample with magnesium could be fitted with a single convoluted exponential of lifetime $\tau = 6.3$ nsec. Thus $d(t) = e^{-t/\tau}e^{-t/\tau_D}$ is also a single exponential under these circumstances, and could be fitted to a single exponential of decay time $\tau' = 3.8$ nsec. Together with the decay time, τ, of $s(t)$, this yields an apparent relaxa-

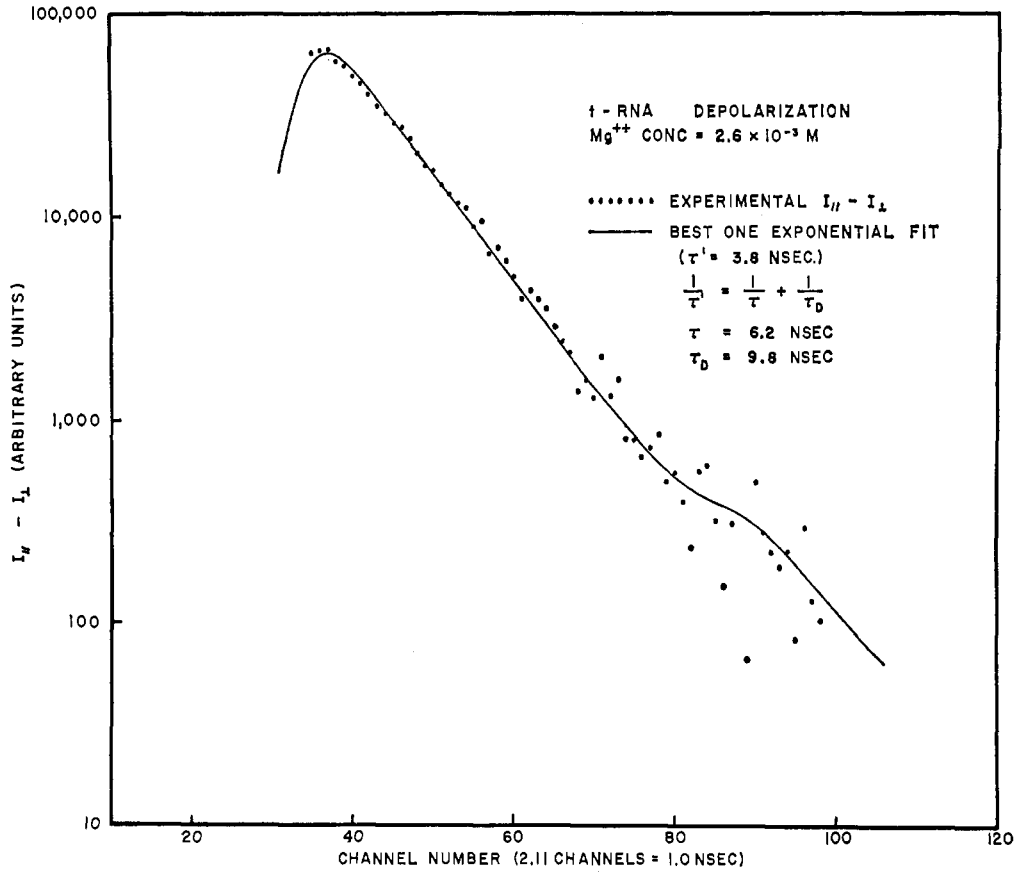

FIGURE 7: Nanosecond fluorescence depolarization of the Y base of yeast $tRNA^{Phe}$ in 0.0026 M magnesium ion. τ is the fluorescence lifetime, τ_D is the rotational relaxation time.

tion time of $\tau_D \simeq 9.8$ nsec. A similar analysis of the data in the absence of magnesium yielded $\tau_D = 9.2$ nsec. In comparison, measurements on ethidium bromide (presumably rigidly attached) complexed $tRNA^{Phe}$ yielded apparent relaxation times of $\tau_D = 25$ nsec both in the presence and absence of magnesium (Tao *et al.*, 1970). If the Y base were rigidly attached to the $tRNA^{Phe}$ molecule, the apparent relaxation time could be calculated from the size of the molecule. The shortest possible relaxation time corresponds to motion of a sphere of the same hydrated volume as the ellipsoidal tRNA. At 25° and aqueous solution, this would be 15 nsec. Of course, most possible orientations of Y would give a relaxation time considerably longer than this value. Our observation of measurably shorter relaxation times suggests some degree of flexibility on the part of the Y base with respect to the $tRNA^{Phe}$ molecule. Whatever the nature of this flexibility may be, it must be quite similar both in the presence and the absence of magnesium ions.

Discussion

As magnesium ion is added to $tRNA^{Phe}$ there is a gradual but dramatic change in the fluorescence properties of the base Y. What is responsible for this change? Other optical techniques suggest that a small conformational change has taken place in the tRNA. As the fluorescence of Y increases, the extinction coefficient of the tRNA drops gradually by about 5%. There is a similar increase in the magnitude of the principle positive circular dichroism band. This increases by 8% with no perceptible shift or change in the 295-nm negative band. These changes are unlikely to be a result of any direct influence of magnesium on Y. Rather, a small net change in secondary structure seems likely. If base-paired regions of a tRNA are disrupted to form unstacked bases rather than stacked bases the net result will be a diminution in circular dichroism without a large shift. This is the sort of effect that has been observed.

Magnesium-dependent conformational changes are well known for other tRNA species but these are usually accompanied by much more dramatic changes in the ultraviolet and circular dichroism spectra (Adams *et al.*, 1967; Reeves *et al.*, 1970). In the case of yeast $tRNA_{Iab}^{Ala}$ and $tRNA_{II}^{Ala}$ the changes occurred over a very narrow range of magnesium concentration, quite unlike what has been observed here. Additional evidence that the conformational changes induced in $tRNA^{Phe}$ is quite different from typical interconversions of "native" and "denatured" forms comes from studies on the size and shape of the tRNA molecule. For unfractionated yeast tRNA samples (Tao *et al.*, 1970) or purified *E. coli* $tRNA^{Leu}$ (Adams *et al.*, 1967), magnesium removal was accompanied by a considerable change in the shape of the tRNA. At very low magnesium concentration, the tRNA samples resembled a long prolate ellipsoid. In the presence of magnesium the axial ratio was much smaller, probably

only about 2.5. However, when such studies were carried out on yeast tRNA^{Phe} using the same conditions for magnesium removal as those in the present study, no change in the overall size and shape of the tRNA^{Phe} could be observed (Tao *et al.*, 1970). This does not mean that tRNA^{Phe} cannot be denatured by magnesium removal. The presence of 0.1 M NaCl, however, has enabled a less dramatic conformational change to be observed.

It is clear that the magnesium-induced conformational change in tRNA^{Phe} has a drastic effect on the fluorescence of the Y base. As the magnesium is removed, the structure of the tRNA alters in such a way that the Y base becomes exposed to the solvent and is less sheltered by adjacent secondary or tertiary structure. This causes a diminution in the relative quantum yield of Y and a shortening of the fluorescence lifetime. Under our conditions it is not possible to fully convert the tRNA^{Phe} sample into the conformation in which Y is exposed. This is demonstrated by the lack of a single exponential fluorescence decay at low magnesium concentrations. Furthermore, even in samples to which no magnesium is added, the fluorescence of Y in the intact tRNA is still two times more intense than it is in a single-strand oligonucleotide fragment containing the Y. This may be due either to the presence of a large amount of tRNA^{Phe} still existing in a conformation in which the Y base is hidden from the solvent. A second possible explanation is that even in the absence of magnesium, the presence of an intact sequence near the anticodon loop results in a conformation near the Y different from an ordinary stacked single strand. Present experimental limitations prevent these two alternative hypotheses from being unequivocally distinguished.

The conformational change induced by magnesium removal is most likely the destruction of tertiary structure accompanied by the breaking of a few base pairs with little or no change in overall shape of the molecule. This could occur gradually as the magnesium concentration is lowered. At least some, but not necessarily all, of this change probably involves regions of the tRNA near or at the anticodon loop. While the present results do not exclude a more drastic conformation rearrangement whose final result is a structure similar in overall shape but different in the details of base pairing, this seems quite unlikely because of the rapid rate at which the influence of magnesium is felt (see Experimental Section). The schematic picture of the magnesium-induced conformational change in yeast tRNA^{Phe} described above is consistent with all of the results presented here. It also agrees very well with findings on the binding of ethidium bromide to yeast tRNA^{Phe} (Tao *et al.*, 1970). These indicated that in the absence of magnesium one molecule of ethidium bromide can bind very strongly to a site which is in the immediate vicinity of Y. If magnesium is present, however, this site is unavailable, and the dye binds to a site quite far away from Y. This again supports the idea of a conformational change near the Y base.

The one observation which remains to be accounted for is the nanosecond depolarization results. Recall that these indicated that a considerable amount of motion of Y relative to the bulk of the tRNA is occurring. This is, at least qualitatively, unchanged when magnesium is added. The motion is still restricted, however, since much greater fluorescence anisotropy is observed than would be expected for a typical

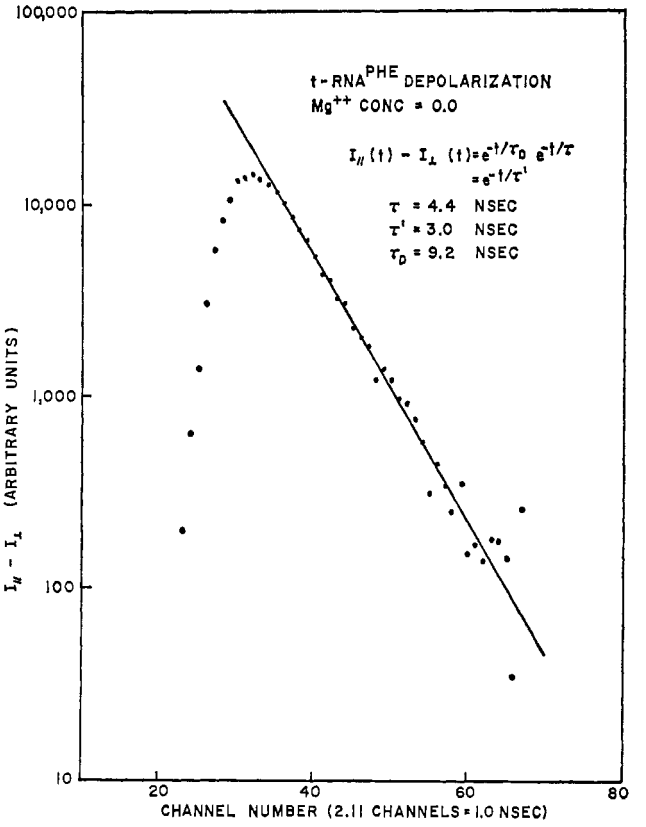

FIGURE 8: Nanosecond fluorescence depolarization of the Y base of yeast tRNA^{Phe} in the absence of magnesium ion.

freely dangling or rotating chromophore (Wallach, 1967). The partial depolarization of Y fluorescence could arise either from rotation about single bonds, from torsional motions similar to those suggested for stacked bases (Glaubiger *et al.*, 1968), or from flexibility of attachment of the anticodon loop to the rest of the tRNA. A quantitative distinction among these three hypotheses will probably not be possible until the structure of Y is known. However, the fact that the depolarization remains unaltered during a local conformational change near the Y base suggests that the first alternative is unlikely. The last would make similar fluorescence depolarization results for ethidium bromide bound to two different sites very difficult to explain. Thus one is left, tentatively, by default, with the notion that the torsional motion of the base in the anticodon loop may be responsible for partial depolarization of the Y fluorescence.

Yoshikami *et al.* have suggested that the tRNA^{Phe} can be assayed by fluorescence, a procedure considerably less time consuming than enzymic loading with labeled amino acids (1968). Our findings indicate that for such assays to be meaningful the magnesium ion concentration must be known.

The experimental observations on Y fluorescence will probably be of considerable help in correlating the solid-state structure of tRNA^{Phe}, once it is known, with structures in solution. It is less useful at the moment, in evaluating some of the specific suggestions that have been made for the structure of tRNA^{Phe} or other tRNAs since most of these have been concerned with aspects of the gross folding of the secondary structure of the molecule rather than the particu-

lar kinds of detailed structure probably responsible for the sensitivity of Y fluorescence to magnesium ion concentration.

The experiments discussed above indicate that the fluorescence of the Y base is very strongly affected by the environment. Striking changes in the relative fluorescence efficiency of this base are observed as a result of rather mild perturbations of the tRNA structure. This suggests that studies of Y fluorescence may yield important information about subtle conformational changes which may occur at the anticodon loop as a result of tRNA interaction with activating enzymes, ribosomal factors, ribosomal subunits, whole ribosomes, and enzymes involved in aromatic amino acid metabolism (Duda *et al.*, 1968). The finding that the Y fluorescence is relatively depolarized suggests that studies of the fluorescence depolarization of tRNA complexes with other macromolecules could help to decide whether this part of the tRNA is involved in a specific interaction.

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Addendum

Recent work by Eisinger *et al.* (1970) has confirmed a number of observations reported in this paper.

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