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Studies of Transfer RNA Tertiary Structure by Singlet-Singlet Energy Transfer*

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Abstract. The distance between a base next to the anticodon of tRNA and the 3' CpCpA terminus of the molecule has been estimated by singlet-singlet energy transfer experiments. The energy donor was the Y base of unknown structure found in yeast tRNA\textsuperscript{phe}. Three different energy acceptors were used: acriflavine, proflavinylic acid hydrazide, and 9-hydrazino acridine. These were attached to the periodate-oxidized 3' end of the tRNA. \( R_s \)'s between 24 and 30 Å were calculated for the three chromophore couples by assuming that the relative orientation of donor and acceptor is random. This assumption is supported by the consistency of the experimental results with all three acceptors and by studies of the fluorescence depolarization of Y. The energy transfer observed both by quenching of Y and enhanced activation of the acceptors is quite small, indicating that the anticodon is more than 40 Å away from the amino acid accepting terminus. This places severe restrictions on the type of tertiary structure possible for tRNA.

Little is known about the detailed tertiary structure of transfer RNA. All of the tRNA's of known sequence can be assembled into the cloverleaf pattern of secondary structure.\textsuperscript{1, 2} A variety of physical and chemical evidence suggests that the cloverleaf is folded into a compact three-dimensional structure which may contain additional regions of base pairing.\textsuperscript{3–9} There are relatively few types of measurements which can directly yield information about the details of this folding.

Recently work from several laboratories has shown that intramolecular singlet-singlet energy transfer can be used to measure the distance between two fluorescent groups attached to a large molecule.\textsuperscript{10–12} The dependence of the efficiency of this transfer on the distance between the energy donor and acceptor is accurately predicted by the Förster theory.\textsuperscript{13} The finding that yeast tRNA\textsuperscript{phe} contains a strongly fluorescent nucleotide, Y, \textsuperscript{14, 15} adjacent to the anticodon immediately suggested that this technique could be profitably applied to studies of the conformation of tRNA. All that was needed was to attach a suitable energy acceptor at some defined site in the tRNA.

Materials and Methods. Preparation of yeast tRNA\textsuperscript{phe}: Several different samples of purified yeast tRNA\textsuperscript{phe} were used. These were purified from unfractionated yeast tRNA by BDC or BDSC chromatography using either the method of Litt\textsuperscript{16} or Wimmer \textit{et al.}\textsuperscript{17} Our samples showed loading of between 1200 and 1600 \( \mu \)moles of \(^{14}\text{C} \) phenylalanine per \( A_{260} \) unit indicating purities ranging from 67 to 90%.

Fluorescent labels: Acriflavine and proflavine were obtained from Aldrich Chemi-
cal Co., 9-hydrazone acridine from Eastman, respectively. These were used without further purification. Proflavinylic acid hydrazide (PAH) was prepared by reacting proflavine with acetic anhydride and sodium acetate. The resulting diamide was suspended in ethyl iodoacetate and heated at 140°C for 16 hr. This afforded an almost quantitative yield of the iodide salt of N,N-diacetyl proflavinylic ethyl acetate. Treatment of this compound with 40% hydrazine hydrate and then dilute HCl yielded PAH.

**Coupling of fluorescent labels to tRNA:** Yeast tRNA<sub>phe</sub> in 1–5 mg batches was oxidized with periodic acid as described by Zamecnik et al.\(^\text{18}\) The extent of oxidation was measured by reactivity with 2,4-dinitrophenyl hydrazine. The oxidized tRNA was reacted with either of the three dyes essentially as described by Millar and Steiner.\(^\text{19}\) It was found that this procedure is not very reproducible because, at the large dye concentrations needed to achieve a reasonable extent of loading, the dye precipitates most of the tRNA out of solution. In our hands the optimal conditions for loading were either 5–15 mg/ml tRNA, 0.2–1.0 mg/ml PAH or acriflavine, pH 4.3, 5 hr at 37°C; or 10<sup>−4</sup> M 9-hydrazone acridine, 2 × 10<sup>−3</sup> M tRNA, pH 4.3, 2 hr at 25°C. Noncovalently attached dye was removed by repeated ethanol precipitation of the tRNA at 0°C. Controls with unoxidized tRNA showed that a minimum of five precipitations was needed to remove all of the intercalated dye. The extent of reaction of PAH and acriflavine with unoxidized tRNA was less than 2% of the reaction with oxidized material. This is strong evidence that the dye is attached specifically to the 3′ terminus. Concentrations of dye attached to the tRNA were determined spectrophotometrically using a Cary 15 and the extinction coefficients shown in Table 1. These extinction coefficients for bound dye

**Table 1. Energy acceptors.**

<table>
<thead>
<tr>
<th>Acriflavine</th>
<th>PAH</th>
<th>9-Hydrazone acridine</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="attachment" alt="Chemical Structure" /></td>
<td><img src="attachment" alt="Chemical Structure" /></td>
<td><img src="attachment" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>ε&lt;sub&gt;max&lt;/sub&gt; (×10&lt;sup&gt;−4&lt;/sup&gt;)</td>
<td>3.31</td>
<td>3.31</td>
</tr>
<tr>
<td>Absorption λ&lt;sub&gt;max&lt;/sub&gt;, nm</td>
<td>463</td>
<td>470</td>
</tr>
<tr>
<td>Emission λ&lt;sub&gt;max&lt;/sub&gt;, nm</td>
<td>510</td>
<td>505</td>
</tr>
</tbody>
</table>

were determined either by quantitatively removing the bound dye or by fitting the spectrum of the reaction mixture to a sum of components.

**Fluorescence measurements:** All measurements were made on an Aminco Bowman fluorescence spectrometer equipped with a Beckman 10-in. recorder. This yielded a considerable improvement in the signal-to-noise of the instrument compared with the X-Y recorder supplied by Aminco. Emission spectra were corrected by comparing the spectra of four compounds with published corrected spectra.\(^\text{20}\) For most measurements the temperature was controlled to ±0.1°C by using an external thermostatic bath. Freshly precipitated tRNA or samples freshly eluted from a G-25 Sephadex column were used for all measurements. All solutions contained 0.01 M MgCl<sub>2</sub>, 0.1 M Na<sup>+</sup>, and either 0.01 M phosphate buffer, pH 6.6 or 0.01 M acetate buffer, pH 6.2. Concentrations of tRNA were usually about 4 × 10<sup>−4</sup> M.

**Calculations of R<sub>0</sub>:** The terms needed to evaluate R<sub>0</sub> using equation (2) were computed as follows. The spectral overlap of absorption and emission was determined by numerical integration. The index of refraction of the medium through which energy is transferred was estimated to be 1.4. This is a weighted average of the values for water, sucrose, magnesium phosphate, and pyridine. The quantum yield of Y was estimated by two methods. The integrated intensity of the emission of Y was compared with that of quinine sulfate at equal optical densities.\(^\text{20}\) This yielded a value of 0.07 for the quantum yield of Y in 0.01 M Mg<sup>2+</sup>. This is the value used in our calculations. It is very sensitive to magnesium concentration and temperature.\(^\text{21}\) A second estimate
was made from the ratio of the measured fluorescent lifetime\textsuperscript{22} (6 × 10\textsuperscript{-9} sec) to the radiative lifetime calculated from the integrated absorption intensity of the 320 m\textmu\textsuperscript{a} band of Y.\textsuperscript{25} This value is 0.08 in reasonable agreement with the first result.

**Calculation of transfer efficiency:** For energy transfer experiments, three different systems are required: a macromolecule containing only the donor, both donor and acceptor, and only the acceptor. Purified yeast tRNA\textsuperscript{phe} is obviously the first of these. Yeast tRNA\textsuperscript{phe} containing a covalently attached dye is the second system. For the third system it proved convenient to use a dye coupled to a sample of unfractionated yeast tRNA where the amount of Y is negligible. This is a reasonable system because of the extreme similarity of the 3' ends of all tRNA's of known sequence.\textsuperscript{1,2}

(a) **Quenching of donor.** The efficiency of energy transfer, as defined in equation (1) is related to the observed fluorescence of the donor, Y, as follows:

\[ f_{Y:a} = f_{Y}(1 - E). \]

Here \( f_{Y:a} \) is the fluorescence of Y on tRNA\textsuperscript{phe} coupled with an energy acceptor; \( f_{Y} \) is the fluorescence of tRNA\textsuperscript{phe} in the absence of an acceptor. If, as in some of our cases, there is more or less than 1 mole of acceptor coupled per mole of tRNA, appropriate corrections must be made. The above equation is useful only if a wavelength exists where the observed fluorescence is due solely to the donor. For most of our samples a suitable wavelength is 430 m\textmu. Since the dyes used as acceptors absorb at this wavelength, one must be sure that proper corrections are made for reabsorption of emitted light. These corrections are, fortunately, extremely small at the very low concentrations of dye (absorbance less than 0.02) in the solutions.

(b) **Sensitized fluorescence of acceptor.** No wavelength exists which permits accurate measurements of the emission of the acridine labels without some interference from Y emission. Thus a slightly complicated approach must be used to estimate the efficiency of singlet-singlet energy transfer. The fluorescence of a labeled tRNA\textsuperscript{phe}, \( f_{1:a} \), at 500 m\textmu after excitation anywhere in the range of 300–340 m\textmu will arise from three possible sources: fluorescence of the Y group at 500 m\textmu, fluorescence of acceptor due to direct excitation by the ultraviolet radiation, and fluorescence of the acceptor due to energy transfer from Y. Since the quantum yield of acriflavine or PAH is an order of magnitude larger than that of Y, a high efficiency of energy transfer will result in a large increase in fluorescence at 500 m\textmu. To identify the fluorescence due to transfer, \( f_{0} \), the two other contributions must be measured separately. This is done as follows: (1) The fluorescence due to Y at 500 m\textmu, \( f_{Y:a} \), is calculated from the fluorescence of Y in the labeled tRNA at 430 m\textmu where only Y fluoresces. This assumes that the shape of the emission spectrum of Y is not altered by transfer. (2) The fluorescence of the dye at 500 m\textmu is calculated from the fluorescence of the label attached to a sample of unfractionated yeast tRNA, \( f_{1:c} \). This must be corrected for the difference in concentration of the label in the two samples. Experimentally, this difference is made as small as possible. The procedure outlined above is summarized in the following equation:

\[ f_{1:a} = f_{1:n}C_{1:a}C_{1:a} + f_{Y:a} + f_{0}. \]

The concentrations of dye on labeled unfractionated tRNA and tRNA\textsuperscript{phe} are respectively \( C_{1:a} \) and \( C_{1:a} \). The fluorescence due to transfer can easily be related to the efficiency of transfer, once corrections have been made for differences in concentration.

\[ f_{0}/f_{1:a} = E_{p}/E_{a}. \]

Here \( E_{p} \) and \( E_{a} \) are the extinction coefficients of Y and the dye at exciting wavelength.

**Results.** The dipole-dipole coupling theory of singlet-singlet energy transfer developed by Förster demonstrates that the efficiency of transfer depends on the sixth power of the distance, R, between the donor and the acceptor.\textsuperscript{11}

\[ E = R_{0}^{6}/(R_{0}^{6} + R^{6}) \]
$R_0$ can be calculated from the following expression:\textsuperscript{24}

$$R_0 = 9.79 \times 10^4 (J n^{-4} K^{2} Q)^{1/6}. \quad (2)$$

$J$ is the spectral overlap of the normalized emission spectrum of the donor and the absorption spectrum of the acceptor, $n$ is the index of refraction, $Q$ is the quantum yield of the donor, and $K^2$ is the square of the geometric part of the dipole-dipole interaction tensor averaged over the distribution of orientations of the transition dipoles of the donor and the acceptor. $K^2$ can vary between 0 and 4. We shall assume that there is no correlation between the orientation of the donor and the acceptor. Then $K^2$ becomes equal to $2/3$. This assumption will be justified in detail later.

In the present experiments, the donor is the $Y$ base of yeast tRNA\textsuperscript{phe}. This base of unknown structure shows an absorption maximum at 320 m\textmu and an emission maximum at 460 m\textmu. The excitation and corrected emission spectra of $Y$ are shown in Figure 1. Three different acridine dyes, acriflavine, proflaviny1 acetic acid hydrazide (PAH), and 9-hydrazino acridine were used as energy acceptors. The structures of these dyes and a summary of their optical properties are given in Table 1. All react specifically with the periodate oxidized 3' end of tRNA. Acriflavine has been used before as a tRNA label for fluorescence polarization studies.\textsuperscript{19, 25}

The absorption and corrected emission spectra of acriflavine attached to tRNA are shown in Figure 1. The $R_0$ for the acriflavine $Y$ couple is calculated

![Figure 1](image-url)

**Fig. 1.**—Optical properties of $Y$ and acriflavine. Similar results are obtained for the other two energy acceptors used. These are summarized in Tables 1 and 2. --- --- excitation spectrum of $Y$ base of tRNA\textsuperscript{phe} $\times 5$; --- ---, corrected emission spectrum of tRNA\textsuperscript{phe} $\times 5$; ..., absorption spectrum of tRNA-bound acriflavine; ---, emission spectrum of tRNA-bound acriflavine.
to be 30 Å. Acriflavine would be an ideal energy acceptor save for the fact that the Schiff's base link which bonds it to the tRNA is not especially stable. The other two dyes form a quite stable link with the periodate-oxidized 3' end of tRNA, but they are optically not as ideal acceptors as acriflavine. The $R_0$ for $Y$ transfer to 9-hydrazino acridine is only 24 Å. The absorbance of PAH and 9-hydrazino acridine is appreciable at the absorption maximum of $Y$. This makes sensitized fluorescence difficult to observe.

The results of the energy transfer experiments are given in Table 2. A number of different samples have been used for several of the labeled tRNA's. The extent of loading is shown in the first column. This was not very reproducible for reasons explained earlier. The most convenient way to summarize the fluorescence results is to compare the observed fluorescence with that expected if no energy transfer had occurred. The data in the fourth column of Table 2 show the change in the fluorescence of the dye acceptors at 500 m$m$ due to apparent transfer after the appropriate corrections have been made for the emission of $Y$ at this wavelength. Similar data on the quenching of $Y$ fluorescence at 430 m$m$ by the dye labels are shown in the fifth column of the table. To place these results in a meaningful perspective we have calculated the results that would be expected if the distance between donor and acceptor were equal to $R_0$. These are shown in column six. It is immediately apparent that we are observing far smaller changes in fluorescence than these calculated results. This demonstrates that the donor and acceptor are considerably further apart than $R_0$.

Before discussing the quantitative aspects of the results shown in Table 2, we should note some of the limitations of the present approach. Except for the data obtained with acriflavine, the observed changes in fluorescence due to apparent transfer are very small. The results are reproducible to the extent shown in Table 2. Systematic errors, while difficult to estimate, may not be

<table>
<thead>
<tr>
<th>Dye, loading</th>
<th>$R_0$ (Å)</th>
<th>Exciting wavelength</th>
<th>Per cent change of dye due to apparent transfer</th>
<th>Per cent change of $Y$ due to apparent transfer</th>
<th>Calculated per cent change $^*$ for $R = R_0$ (Å)</th>
<th>Distance</th>
</tr>
</thead>
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<td>Acriflavine</td>
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<td>315</td>
<td>+30</td>
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<td>9-Hydrazino acridine</td>
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<tr>
<td>Loaded 0.7:1</td>
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</tbody>
</table>

* Corrected for purity of tRNA $^{54}$A samples.
† Corrected for 2:1 loading.

Table 2. Summary of energy transfer experiments.
insignificant. An additional source of uncertainty is introduced in the calculation of \( R_0 \). The values used for the quantum yield of \( Y \), the refractive index, \( K^2 \), and for the extinction coefficient of the bound dye are only approximate. The true value of \( R_0 \) could probably be 20 per cent larger or smaller than the values used here. A third source of error may arise from the use of dye labeled crude tRNA to estimate the fluorescence of energy acceptors attached to yeast tRNA\(^{a,b,e}\). Finally one must ask whether the presence of a fluorescent label perturbs the tertiary structure it is being used to measure. The work of Yarus and Berg suggest that periodate oxidation does not change the conformation of tRNA.\(^{26}\) Adding a dye is, however, a more drastic change, and there is some indication that dyes bound to the 3' end of tRNA can intercalate into a nearby double helix.

In spite of the difficulties just discussed, there is surprising agreement among the ten estimates that can be made of the distance between the dye acceptors and \( Y \). These are shown in the last column of Table 2. The mean value of the distance is 49 Å, with a range from 45 to 59 Å. The most reliable experimental data points (those for acriflavine) are, however, slightly shorter than the mean. From these considerations it seems likely that the true distance between base \( Y \) and a dye coupled to the 3' terminus of tRNA is somewhere in the range 40–60 Å.

Studies of the fluorescence depolarization of \( Y \) provide justification for using a value of \( K^2 \) of \( 2/3 \) to calculate \( R_0 \). The rotational relaxation time of \( Y \) is \( 10 \times 10^{-9} \) sec compared with the value of \( 25 \times 10^{-9} \) sec found for the tRNA itself by nanosecond depolarization measurements on intercalated ethidium bromide.\(^{22}\) These results show that the orientation of the \( Y \) group is not rigidly fixed to the macromolecule. Motion of \( Y \) will average the dipole-dipole interaction between donor and acceptor. The value of \( K^2 \) will tend toward \( 2/3 \). Additional justification for the use of this value comes from the excellent agreement among results with three different acceptors. The fluorescence depolarization studies of Millar and Steiner showed that acriflavine coupled to the periodate oxidized 5' end of tRNA is rigidly fixed to the macromolecule.\(^{19}\) The three dyes shown in Table 1 are essentially the same chromophore attached at three different orientations. If \( Y \) were rigidly oriented relative to the dyes, it is likely that \( K^2 \) for each of the three systems would be quite different. Thus one should expect large discrepancies in the calculated values of \( R \), contrary to what is observed.

**Discussion.** The value for the distance between \( Y \) and the 3' end of tRNA calculated above is far from precise. Even with the present level of uncertainty, the results presented here place a strong constraint on the possible tertiary structure of tRNA. By way of illustration, consider the set of possible structures shown very schematically in Figure 2. This is by no means an exhaustive list of possible tRNA conformations. It merely indicates some of the obvious possible ways of folding a cloverleaf plus one additional structure which has been suggested from chemical evidence.\(^{27}\) Four of the eight structures shown in Figure 2 have values for the distance between \( Y \) and 3' terminus which fall outside the range of our experimental measurements. The hairpin model\(^{28}\) is
Fig. 2.—Highly schematic representation of several classes of plausible tRNA conformations showing which are consistent with the energy transfer results.

also excluded by our results. Of those models which remain, one, in which the dihydro $U$ and $GT\psi C$ loops are folded down next to the stem of the tRNA, has been favored by Cramer and his co-workers on the basis of data on the specificity of performic acid oxidations of tRNA$^{\text{phe}}$. In his model, the distance between $Y$ and the $3'$ end is 59 Å. All things considered, this is consistent with our results. At present, however, our experiments are not really accurate enough to permit a number of possible other models to be excluded. Further experiments are in progress to introduce fluorescent labels into other specific places in the tRNA molecule. If several additional intramolecular distances can be measured using techniques analogous to those described here, it should be possible to begin to make fairly precise statements about the conformations of tRNA in aqueous solution. The principal power of the fluorescence techniques described here, however, is that they are applicable, in principle, not only to isolated tRNA but also to complexes between this molecule and activating enzymes, ribosomal factors, and perhaps even whole ribosomes.

We are very grateful to Mr. Avi Snyder for planning and executing the synthesis of PAH and to Professor Lubert Stryer for carefully and critically reading this manuscript. Discussions with Dr. Terence Tao and Professor Richard Bersohn were extremely helpful. We are indebted to Professor Robert Chambers for considerable assistance in purifying tRNA$^{\text{phe}}$ and for several gifts of partially fractionated yeast tRNA.

**Abbreviations:** PAH, proflavinylic acid hydrazide; BDC, benzoylated diethyl amino ethyl cellulose; BDSC, BDC adsorbed onto silicic acid; $Y$, unknown base present in yeast tRNA$^{\text{phe}}$.

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23 Beardsley, K. P., T. Tao, and C. R. Cantor, manuscript in preparation.


31 Cramer, F., private communication.