Circular Dichroism Study of Nine Species of Transfer Ribonucleic Acid†

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ABSTRACT: A detailed circular dichroism (CD) study of nine species of tRNA has been done to obtain as much information about the structure of these molecules as possible. CD spectra are calculated from sums of mononucleotide, dinucleoside monophosphate, and double-strand polynucleotide spectra and compared with experimental CD spectra. Absorbance–temperature profiles show that tRNA in less than 10^{-5} M magnesium is single stranded at 40° while tRNA in the presence of 1 mM magnesium is native. Comparison of the CD spectrum of the single-stranded tRNA with appropriate sums of dinucleoside monophosphate spectra shows that the CD of the dinucleoside monophosphates is not a good model for the CD of single-stranded tRNA. The CD of native tRNA at 40° may be calculated using the experimental single-strand spectrum to represent the CD of the single-strand regions of the tRNA, and double-strand pairing interaction spectra to represent the double-strand regions. Any unique contribution of tertiary structure of the tRNA to the CD is thus shown to be small. For most tRNAs, two to four base pairs in addition to those due to the commonly accepted cloverleaf secondary structure improve the agreement between calculated and experimental spectra. Comparison of the difference between the CD of native and denatured $tRNA_3^{Leu}$ (yeast) with sums of double-strand pairing interactions suggests that the native to denatured transition for $tRNA^{Leu}$ involves the loss of 4–5 base pairs. On the other hand, the native to denatured CD difference spectrum for $tRNA^{Trp}$ could not be fit by any combination of double-strand pairing-interaction spectra.

Circular dichroism (CD) spectroscopy is a useful technique for obtaining structural information of simple nucleic acids. Only small amounts of material are needed and solution parameters such as pH, temperature, and salt concentration can easily be varied. Since the optical rotatory dispersion (ORD) spectra of small oligomers depend strongly on the base sequence and structure (Warshaw and Tinoco, 1966), these methods should also be useful for examining larger RNA molecules, such as the tRNAs. Indeed, considerable work in this direction has already been accomplished. Optical rotatory dispersion (ORD) spectra have been reported for $tRNA^{Ala}$, $tRNA^{Tyr}$, $tRNA^{Asp}$, $tRNA^{Gly}$, and $tRNA^{Lys}$ from yeast (Vournakis and Scheraga, 1966; Sarin *et al.*, 1966). CD spectra are available for two species of yeast $tRNA^{Ala}$ (Reeves *et al.*, 1970) as well as $tRNA^{Val}$, $tRNA^{fMet}$, $tRNA^{Phe}$, $tRNA^{Arg}$, and $tRNA^{Gly}$ from *Escherichia coli* (Adler and Fasman, 1970; Robison and Zimmerman, 1971; Willick and Kay, 1971). ORD spectra may be converted to CD using the Kronig Kramers transform (MacDonald and Brackmen, 1956), which should allow qualitative comparison of all these spectra. However, it is difficult to analyze these spectra carefully and compare different tRNA molecules with each other for two major reasons. First of all, measurements were made on a variety of instruments, and the spectra were measured in various buffers and at various temperatures. Secondly, the molar extinction coefficients for purified tRNAs were often not determined; so, although qualitative comparisons could be made, quantitative analysis was difficult. For these reasons analysis of the CD spectra of a large number of tRNAs with known sequence under standard conditions should (1) allow evaluation of CD spectroscopy as a tool for obtaining information on the structure of large RNA molecules and (2) perhaps add to the available knowledge of tRNA structure.

This work is directed toward answering three related questions. First, are the CD spectra of different native tRNAs distinguishable in a well-defined solvent? This question is answered by obtaining computer-smoothed CD spectra between 210 and 310 mμ of nine species of tRNA. The sequences and probable secondary structures of the nine tRNAs are shown in Figure 1. These structures are versions of the cloverleaf model modified to maximize the length of the helical regions and to agree with the suggestion that these regions are roughly parallel (Doctor *et al.*, 1969). These structures lead to the second question. Can the CD spectra of the tRNAs be understood in terms of the CD spectra of simple single- and double-stranded model compounds? In other words, does the tertiary structure of tRNA contribute a unique CD spectrum? This question is answered by attempting to calculate the spectrum of a tRNA by adding spectra of single-stranded dimers and double-stranded regular polymers in relative amounts dictated by the proposed structure (Cantor *et al.*, 1966).

One application of the above addition techniques is the subject of the last question posed in this paper. Two of the tRNAs in Figure 1 have a second stable conformation, called the denatured form, which cannot accept an amino acid (Ishida and Sueoka, 1967; Gartland and Sueoka, 1966; Henley *et al.*, 1966; Adams *et al.*, 1967; Lindahl *et al.*, 1967). From the increase in ultraviolet absorption in the denatured molecule relative to the native, it has been suggested that the structural change between the two forms involves a loss of about four base pairs in the denatured molecule. A similar experiment is thus suggested with circular dichroism. Can the difference between native and denatured tRNA be described by a combination of single- and double-strand interactions?

Materials and Methods

Sources of tRNAs. The nine tRNAs studied in this work are listed in Table I. $tRNA_F^{Met}$ and $tRNA^{Val}$ were obtained from

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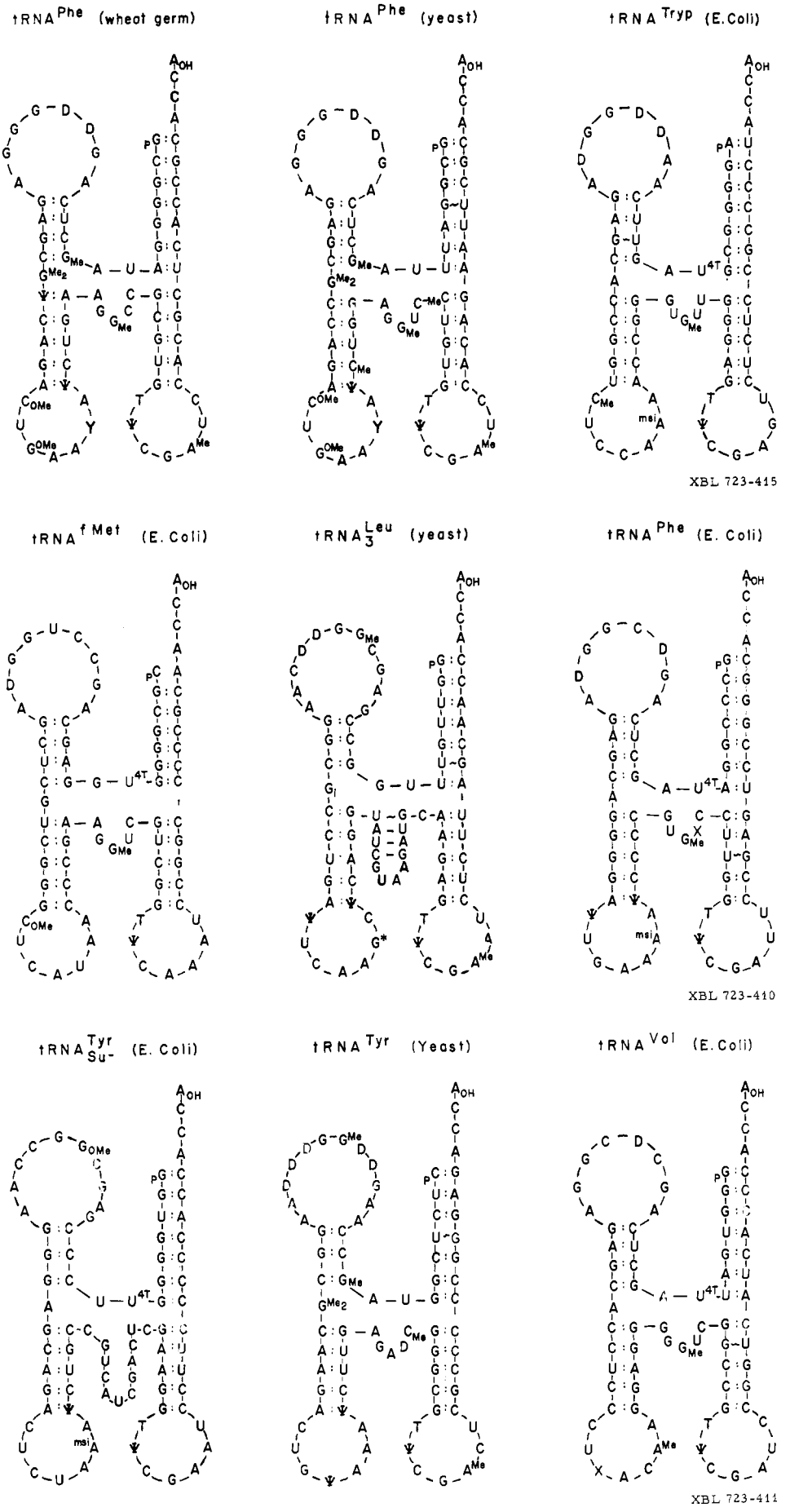

FIGURE 1: The nucleotide sequence and likely secondary structure of nine tRNAs. References to the sequences are given in Table I.

TABLE I: Extinction Coefficients per Mole of Residue for the Nine tRNAs in 10 mM Tris-HCl–1 mM $MgCl_2$ Buffer (pH 7.8).

tRNA	$\epsilon_{258} \times 10^3$	Sequence Reference
fMet (*E. coli*)	7.1	Dube *et al.* (1968)
Leu (yeast)	7.4	Kowalski *et al.* (1971)
Phe (*E. coli*)	7.2	Barrell and Sanger (1969)
Phe (wheat)	7.4	Dudock *et al.* (1969)
Phe (yeast)	6.6	RajBhandary *et al.* (1967)
Trp (*E. coli*)	6.7	Hirsh (1970)
Tyr (*E. coli*)	7.4	Goodman *et al.* (1968)
Tyr (yeast)	7.1	Madison *et al.* (1966)
Val (*E. coli*)	7.5	Yaniv and Barrell (1969)

Oak Ridge National Laboratory. Yeast $tRNA^{Leu}$ was a gift from Professor J. Fresco and wheat $tRNA^{Phe}$ was a gift from Professor B. S. Dudock. The other five tRNAs all are specific for aromatic amino acids and were purified by the method developed by Maxwell *et al.* (1968). In each case, the difference in mobility of the tRNA with and without its amino acid on a benzoylated DEAE-cellulose column is sufficient to purify the tRNA to greater than 95% purity. Unfractionated tRNA (Schwartz) is applied to a BD column in 0.4 M NaCl–0.01 M $MgCl_2$–0.01 M sodium acetate, (pH 4.5) and is eluted first with a linear gradient of 0.4–1.0 M NaCl and then with a linear gradient of 0–15% ethanol in 1.0 M NaCl, all in the same magnesium and buffer concentrations. The activity peak of the aminoacyl tRNA of interest was pooled, the enriched fraction was aminoacylated with crude activating enzyme, and, after deproteinization, the tRNA was rechromatographed in the ethanol gradient. In every case, a tRNA aminoacylated with an aromatic amino acid was eluted from the column in the ethanol gradient in a position well removed from the uncharged counterpart. In some cases rechromatography was needed to obtain an activity peak which corresponded to an absorption peak. When the amino acid was removed, the activity peak returned to the original position in the gradient. More detailed descriptions of this technique of tRNA purification have been reported elsewhere (Uhlenbeck, 1972; Litt, 1968).

Extinction Coefficients. Extinction coefficients for all nine tRNAs were measured in collaboration with Dr. Marcos Maestre (Space Sciences Laboratory, Berkeley, Calif.). The concentration of a tRNA solution of measured absorbance was determined by hydrolyzing the tRNA in 0.3 M NaOH at 37° for 30 hr. After neutralization, the absorbance of the hydrolyzed tRNA was measured. From the extinction coefficients of the nucleotides,[1] their concentration and that of the tRNA could be determined. Agreement between duplicate samples was within 1%. The extinction coefficients of the nine tRNAs in 10 mM Tris-HCl–1 mM $MgCl_2$ (pH 7.8) are listed in Table I.

Desalting Procedures. Extensive dialysis was used to remove Mg^{2+} and other salts from tRNA solutions. The following buffers were prepared using twice-distilled water: (a) 0.5 M NaCl–10 mM EDTA (pH 7.5); (b) 0.2 M NaCl–1 mM EDTA (pH 7.5); (c) 1 mM EDTA (pH 7.5); (d) 0.01 mM EDTA (pH 8.5). tRNA solutions were dialyzed at 4° against four changes at 6-hr intervals of each of buffers a, b, and c, and then against 8 changes of buffer d. The volume of each change was at least 100 times as great as the volume of the sample. After desalting, the concentration of Mg^{2+} was measured by atomic absorption, and found to be less than 1 Mg^{2+} per tRNA molecule. The sodium ion concentration is less than 90 Na^+ per tRNA molecule.

Optical Measurements. Ultraviolet (uv) absorption spectra were measured at room temperature (25°) on a Cary 15 spectrophotometer. The change in absorption with temperature at 260 mμ was recorded between 10 and 95° using a modified Beckman DU spectrophotometer with a Gilford Model 2000 multiple sample absorbance recorder. CD measurements were made using a Cary Model 60 spectropolarimeter equipped with a circular dichroism attachment (Model 6001). Temperature was controlled using a calibrated thermoelectrically controlled cell block designed by Professor Donald Gray (Allen *et al.*, 1972).

"Native" tRNA spectra were measured in solutions of 10 mM Tris-HCl (pH 7.8)–1 mM $MgCl_2$. There was found to be no change in the CD or uv spectra of the tRNAs between pH 7.0 and 9.0. Spectra of "single-stranded" tRNA were measured in 10^{-5} M EDTA adjusted to pH 8.5. No additional buffer was added to avoid divalent cation impurities. The pH of these solutions was checked after optical measurements with a Beckman Expandomatic pH meter to verify that it had not dropped below 7.0. The A_{260} of solutions used in optical studies was between 0.5 and 1.2. Base-line spectra of the solvent in the same cell were obtained before and after each set of CD spectra.

CD spectra between 350 and 205 mμ were recorded on paper tape by a Digital Equipment Corp. PDP 8/S computer. A program written by Dr. B. L. Tomlinson and Dr. Martin Itzkowitz was used to obtain the CD data points. A point is recorded at every millimicron as the average of the pen position over a 0.5 mμ range. The program substracts the base line from the spectrum and uses the extinction coefficient and absorbance to calculate the molar ellipticity per residue. All CD spectra discussed refer to the CD or molar ellipticity per residue. Data are smoothed by fitting every overlapping set of 13 points to a cubic equation (Tinoco and Cantor, 1970).

Data Analysis. The CD spectrum of any single-stranded RNA of known sequence may be calculated by adding the effects of nearest neighbors (Cantor *et al.*, 1966). In a

$$(\mathrm{RNA}) = \sum_{x=1}^{N}\sum_{y=1}^{N} F_{xy}[\mathrm{XY}] + {}^1/_2(\mathrm{E}) + {}^1/_2(\mathrm{E}') \qquad (1)$$

tRNA with N different types of bases, F_{xy} is the number of times the dimer XY occurs in the sequence divided by the total number of bases in the sequence. [XY] is a single-strand basis spectrum corresponding to the contribution of the CD of XY to the CD of the tRNA. In this work, we shall follow Cantor *et al.* (1966) and assume [XY] can be expressed by eq 2 where (XY), (X), and (Y) are the CD spectra of the di-

$$[\mathrm{XY}] = 2(\mathrm{XY}) - {}^1/_2(\mathrm{X}) - {}^1/_2(\mathrm{Y}) \qquad (2)$$

nucleoside monophosphate XpY and the nucleosides X and Y. Finally, (E) and (E′) are the CD spectra of the nucleosides on each end of the RNA chain divided by the total number of bases in the RNA.

The 16 single-strand basis spectra of the four common bases at 25° were obtained from 16 dimer and 4 monomer spectra

[1] PL Biochemicals, in Circular OR-10. Values used for ϵ_{258} were 15,400 for AMP, 9700 for UMP, 7000 for CMP, and 12,700 for GMP.

measured by Cantor and Warshaw (1970). The CD spectra of the 16 dimers and 4 monomers were also measured at 40° in 1 mM $MgCl_2$–10 mM Tris-HCl (pH 7.8), and used to obtain single-strand basis spectra at 40°. CD spectra of four dimers containing dihydrouridine and the monomer dihydrouridine had been measured by Dr. C. J. Formoso at 25 and 40° (Formoso and Tinoco, 1971). It was assumed that the CD of the other interactions involving minor nucleosides approximated those of the unmodified analogs.

The CD of double-strand RNA may similarly be calculated from spectra corresponding to the ten possible double-strand polymer first-neighbor interactions.

$$(1)\ \begin{matrix}AA\\UU\end{matrix},\ (2)\ \begin{matrix}GG\\CC\end{matrix},\ (3)\ \begin{matrix}AU\\UA\end{matrix},\ (4)\ \begin{matrix}UA\\AU\end{matrix},\ (5)\ \begin{matrix}GC\\CG\end{matrix},$$
$$(6)\ \begin{matrix}CG\\GC\end{matrix},\ (7)\ \begin{matrix}AC\\UG\end{matrix},\ (8)\ \begin{matrix}CA\\GU\end{matrix},\ (9)\ \begin{matrix}UC\\AG\end{matrix},\ (10)\ \begin{matrix}CU\\GA\end{matrix}$$

Then, at each wavelength, the CD of a double-strand RNA is given by eq 3 where F_P is the fraction of the interaction P

$$(\mathrm{RNA}) = \sum_{P=1}^{10} F_P(\mathrm{P}) \tag{3}$$

and the sum is taken over 10 interactions listed above.

Work is in progress in this laboratory to obtain the optical properties of all 10 of these interactions from a set of double-strand RNA oligomers (Borer *et al.*, in preparation). However, this information is not yet available, and the calculations in this work are based on the spectra of five RNA double-strand polymers and several approximations. The CD of poly(A):poly(U), poly(G):poly(C), poly(AU):poly(AU), poly(GC):poly(GC), and poly(CA):poly(GU) have been measured in this laboratory (Gray *et al.*, 1972). The CD spectrum of the double-strand ribopolymer poly(GA):poly(CU) has not been measured and was approximated from the 5 known double-strand polymer spectra. It is then assumed that the CD of the following pairs of interactions are the same.

$$\begin{matrix}AU\\UA\end{matrix} = \begin{matrix}UA\\AU\end{matrix};\ \begin{matrix}GC\\CG\end{matrix} = \begin{matrix}CG\\GC\end{matrix};\ \begin{matrix}AC\\UG\end{matrix} = \begin{matrix}CA\\GU\end{matrix};\ \begin{matrix}UC\\AG\end{matrix} = \begin{matrix}CU\\GA\end{matrix} \tag{4}$$

Thus, each interaction pair (1–10) may be represented by one of the six double-stranded polymers. This assumption is valid if either the spectra of the paired interactions in eq 4 are similar or if there are nearly the same number of each of a pair of interactions in the double-stranded RNA. Finally, it was assumed that interactions involving the G:U base pairs that are occasionally found in tRNA were equal to the average of the corresponding interactions involving G:C and A:U base pairs.

The CD spectrum of a native tRNA at 25° can now be considered as the sum of contributions from the single-strand parts of the molecule (eq 1), the double-strand parts (eq 3), and the tertiary structure (T). The summations

$$(\mathrm{tRNA}) = \sum_{x=1}^{5}\sum_{y=1}^{5} F_{xy}[\mathrm{XY}] + {}^1/_2(\mathrm{E}) + {}^1/_2(\mathrm{E}') + \sum_{P=1}^{6} F_P(\mathrm{P}) + (\mathrm{T}) \tag{5}$$

x and y are taken over the bases A, U, C, G, and D and the summation P is taken over the six double-strand polymers.

An additional assumption must be made. The CD of the bases in a loop are approximated by dimer CD spectra just as single-stranded RNA is approximated. This includes the two unpaired bases at the juncture of the double-strand region; they are assumed to have single-stranded interactions with the first base pair of each stem.

An alternative method for approximating the CD of a tRNA is to use the measured single-stranded RNA spectra to approximate the spectra of loop residues instead of calculating the contribution from dimer spectra. Since it will be shown that it is not possible to obtain single-stranded RNA spectra below 40°, measurements are made at that temperature (eq 6), where $(\mathrm{tRNA, N})_{40}$ is the calculated native

$$(\mathrm{tRNA, N})_{40} = (\mathrm{tRNA, SS})_{40} + \sum_{P=1}^{6} F_P\{\mathrm{P}\} + (\mathrm{T}) \tag{6}$$

tRNA spectrum at 40°, $(\mathrm{tRNA, SS})_{40}$ is the single-strand spectrum measured at 40°, and very low ionic strength, and $\{\mathrm{P}\}$ are a new set of double-strand pairing basis spectra corresponding to the formation of double-strand regions at 40°. Thus, the experimental polymer spectra (P) must have the contribution of the single strand removed to obtain $\{\mathrm{P}\}$.

$$\left\{\begin{matrix}\leftarrow\\X\ Y\\X'Y'\\\rightarrow\end{matrix}\right\} = \left(\begin{matrix}\leftarrow\\X\ Y\\X'Y'\\\leftarrow\end{matrix}\right) - \tfrac{1}{4}[\mathrm{XY}] - \tfrac{1}{4}[\mathrm{YX}] - \tfrac{1}{4}[\mathrm{X'Y'}] - \tfrac{1}{4}[\mathrm{Y'X'}] \tag{7}$$

Calculated and experimental spectra were compared by a computer program which determined the mean-square deviation between the two spectra and normalized by dividing by the square of the experimental values (eq 8), where E_i is

$$\mathrm{fit} = \left(\frac{\sum_{i=1}^{N}(E_i - C_i)^2}{\sum_{i=1}^{N}(E_i)^2}\right)^{1/2} \tag{8}$$

the value of the experimental curve at the ith wavelength, C_i is the value of the calculated CD curve at this same wavelength, and the sum is taken over N wavelengths. Fit is thus an average fractional deviation between measured and calculated curves. Perfect correspondence of experimental and calculated values would have fit = 0. Comparison of repeated runs of the same sample have fit < 0.05. Differerent samples of the same tRNA differ by no more than fit = 0.10.

Results

Native tRNA at 25°. The CD spectra of nine native tRNAs in 10 mM Tris-HCl–1 mM $MgCl_2$ (pH 7.5) are shown in Figure 2. Although the general characteristics of all these spectra are similar, different species of tRNA have distinct CD spectra. For example, the position of the maximum of the large band varies from 262 to 267 mμ and six of the tRNAs have a small negative band at about 295 mμ.

The spectrum of each of the nine tRNAs was calculated from the structures shown in Figure 1 and the dimer and polymer spectra using eq 5. In general, agreement between calculated and experimental spectra was rather poor, with fit values varying from 0.29 to 0.54 (Table II, column A). Figure 3 shows the two spectra for yeast $\mathrm{tRNA^{Tyr}}$ which has fit = 0.49. The main peak and crossover of the calculated spectra are shifted about 5 mμ to higher wavelengths than the experimental spectra for all nine species of tRNA. The poor

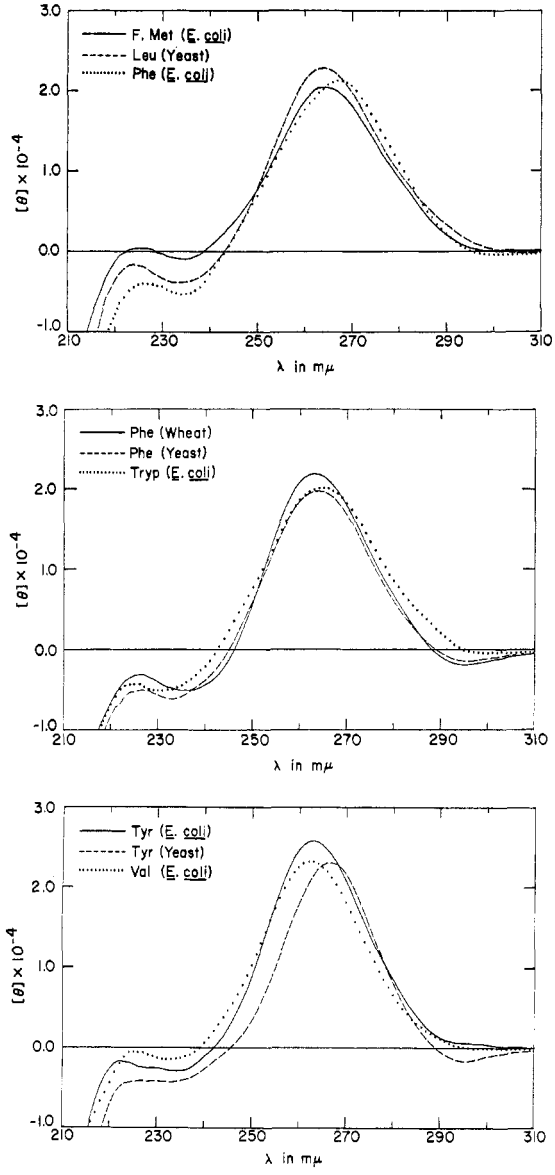

FIGURE 2: The CD spectra of the nine tRNAs in 10 mM Tris-HCl (pH 7.5)–1 mM $MgCl_2$ at 25°. These are native tRNAs.

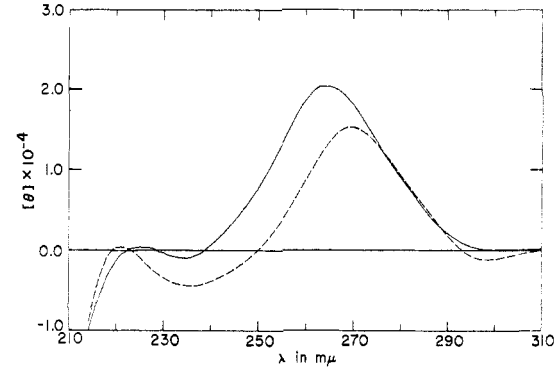

FIGURE 3: Measured (——) CD spectrum of native yeast $tRNA^{Tyr}$ at 25° and a spectrum calculated (-----) by eq 5 assuming the structure in Figure 1.

TABLE II: Comparison of Calculated and Experimental CD Spectra. The Average Fractional Deviation (Fit) Is Given.

tRNA	A Native tRNA (25°) with Sum of Dimers and Double-Strand Polymers (Eq 5)	B Single-Stranded tRNA (40°) with Sum of Dimers (Eq 1)	C Native tRNA (40°) with Single-Stranded tRNA (40°) Plus Double-Strand Pairing (Eq 6)
fMet (*E. coli*)	0.40	0.37	0.18
Leu (yeast)	0.47	0.58	0.18
Phe (*E. coli*)	0.54	0.64	0.25
Phe (wheat)	0.41	0.61	0.16
Phe (yeast)	0.40	0.57	0.25
Trp (*E. coli*)	0.41	0.51	0.15
Tyr (*E. coli*)	0.45	0.56	0.21
Tyr (yeast)	0.49	0.64	0.11
Val (*E. coli*)	0.29	0.60	0.23

agreement suggests that the single-strand basis spectra, the double-strand basis spectra, or both are not good enough models for the nucleotide residues in tRNA. To investigate this, we will first consider how well the single-stranded basis spectra can fit the experimental spectra of the single-stranded tRNAs.

Single-Strand tRNA at 40°. In an attempt to prepare tRNA without secondary or tertiary structure, samples were dialyzed into 10^{-5} M EDTA as described in Methods. However, even at these very low salt concentrations, the absorbance temperature profiles suggest that considerable structure remains at 25°. In Figure 4, a cooperative structure → coil transition in 10^{-5} M EDTA is shown for yeast $tRNA^{Tyr}$, a typical example. At 25° approximately 30% of the cooperative hypochromicity remains, suggesting residual helix at that temperature. At 40°, very little cooperative hypochromicity remains; the remaining gradual change in A_{260} with temperature can be attributed to unstacking the single-stranded RNA. The per cent change in absorbance with temperature is less than that found in single-strand polynucleotides such as poly(A) and poly(C). However, tRNA in 1 mM $MgCl_2$–10 mM Tris-HCl is still entirely native at 40°.

Single-strand spectra of the nine tRNAs were calculated using eq 1, the 20 dimer basis spectra at 40°, and the nearest-neighbor frequencies of the sequences shown in Figure 1. The experimental and calculated single-strand spectra of $tRNA^{Tyr}$ at 40° are compared in Figure 5. Agreement is not good. Fit = 0.64 for $tRNA^{Tyr}$ and the other 8 tRNAs vary between fit = 0.37 and 0.65 (Table II, column B). In general, the calculated spectra are lower in magnitude and shifted to the red relative to the experimental spectra. Comparison of calculated ORD of homopolymers with experimental spectra shows a similar red shift of the calculated ORD (Cantor *et al.*, 1966). This lack of agreement was thought to be due to long-range effects resulting from the regular structure of homopolymers (Cantor *et al.*, 1966). Observation of a similar shift for all nine tRNAs which have considerably different sequences suggests that the homopolymers are not an exception. Either the assumption that nearest-neighbor interactions are sufficient to describe the spectral properties of polymers is incorrect or dimers are only qualitative models for the cor-

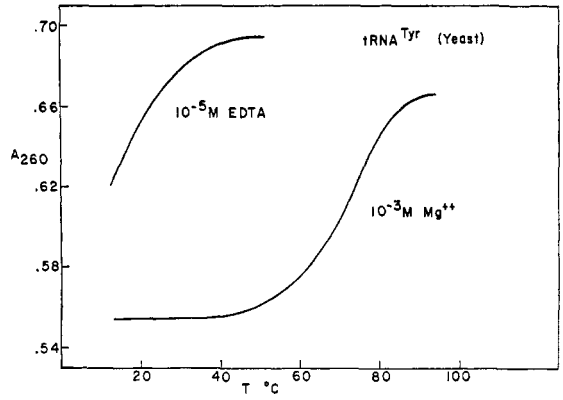

FIGURE 4: Absorbance temperature profiles of the same concentration of yeast $tRNA^{Tyr}$ in 10^{-5} M $Na_{2.5}EDTA$ and in 1 mM $MgCl_2$–10 mM Tris-HCl (pH 7.5).

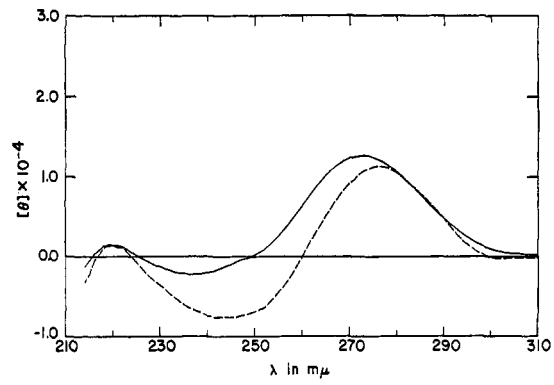

FIGURE 5: Measured (——) CD spectrum of single-stranded yeast $tRNA^{Tyr}$ at 40° in 10^{-5} M $Na_{2.5}EDTA$ and a spectrum calculated (-----) by eq 1.

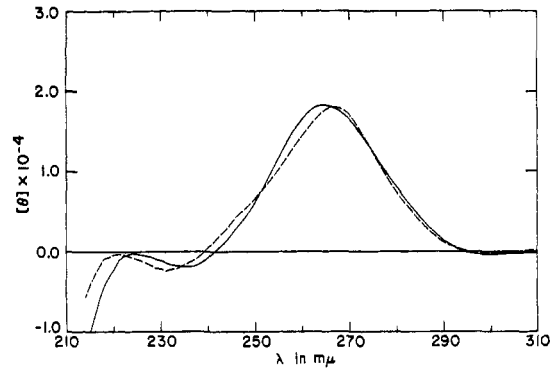

FIGURE 6: Measured (——) CD spectrum of native yeast $tRNA^{Tyr}$ at 40° in 1 mM $MgCl_2$–10 mM Tris-HCl (pH 7.5) and a spectrum calculated (-----) by eq 6.

responding residues in an RNA molecule. In either case, a major source of the error in fitting the CD spectrum of native tRNA lies in the poor fit of the single-stranded portion of the molecule. To avoid this difficulty, the next section considers an attempt to fit the native CD spectrum with the experimental single-stranded spectrum in combination with the double-stranded polymer spectra.

Native tRNA at 40°. The large difference between the CD of native and single-stranded tRNA at 40° may be seen by comparing the spectra of yeast $tRNA^{Tyr}$ shown in Figures 5 and 6. In the native tRNA the maximum is about 10 mμ lower in wavelength and much increased in magnitude relative to the single-stranded tRNA. The small negative band at 295 mμ is only observed in the native tRNA. These large changes parallel the 20% decrease in absorption shown in Figure 4. The CD difference spectrum between native and single-stranded tRNA should correspond to the formation of the secondary and tertiary structure of the tRNA. The secondary structure may be approximated with a sum of the polymer pairing basis spectra defined in eq 7 corresponding to the formation of the base-paired regions shown in Figure 1. If we assume that the contribution of the tertiary structure (T) = 0, then the CD of a native tRNA can be calculated by eq 6 and compared to the experimental native tRNA spectrum at 40°. Figure 6 shows an example of the much improved agreement for yeast $tRNA^{Tyr}$. The value of fit = 0.11 which is within the experimental error of the CD measurement. The calculated and experimental extrema of CD spectra for the nine different tRNAs are given in Table III. The values of fit vary from 0.11 to 0.30.

Since the secondary structure (helix) contribution comes close to fitting the native CD spectrum from the single-strand spectrum in spite of the many approximations previously discussed, the contribution of the tertiary structure of tRNA to the CD spectrum is not large. Although usually the magnitude of the experimental curve is slightly greater than that of the calculated curve, the difference varies considerably among the tRNA species. A contribution of (T) which is the same for all tRNAs is not supported by the data. Slightly improved values of fit can be obtained if two or three additional pairing interactions are added to the structures in Figure 1 (Table II, column C). This observation is consistent with several proposed models for the structure of tRNA (Arnott, 1971).

Native and Denatured tRNAs. Since two of the tRNAs ($tRNA_3^{Leu}$, $tRNA^{Trp}$) included in this study have been found to have a second "denatured" conformation which is stable at 25° for long periods of time (Ishida and Sueoka, 1966; Henley *et al.*, 1966), it was possible to examine whether the difference in CD spectra between these two forms corresponds to some combination of double- or single-strand basis spectra.

The denatured conformers of $tRNA_3^{Leu}$ and $tRNA^{Trp}$ can be prepared by adding $MgCl_2$ and buffer at 25° to the single-strand RNA described previously. This material shows no biological activity unless it is subsequently heated to 50° (for $tRNA^{Trp}$) or 60° (for $tRNA_3^{Leu}$) for 10 min (Ishida and Sueoka, 1967; Henley *et al.*, 1966). As long as the temperature is kept below 40° there will be no interconversion and both forms will be stable in the same solvent. Since denatured tRNA can be converted to native tRNA by simply heating and cooling, it is possible to obtain CD spectra of the two forms with the same sample and without altering the cell position. Since changes of sample and cell position are sources of measurement error, the difference in CD between native and denatured tRNA can be obtained with greater accuracy than other measurements.

The native → denatured transitions of the two tRNAs are markedly different. The denaturation of $tRNA^{Leu}$ results in an increase in absorption and a decrease in the magnitude of the CD maximum accompanied by a red shift of 2 mμ. For $tRNA^{Trp}$, denaturation is also accompanied by an increase in absorption, and a slight red shift of the CD maximum. However, in this case the magnitude of the CD increases upon denaturation. The different behavior of the two cases is

TABLE III: Comparison of CD of Experimental Native tRNA at 40° and Single-Strand Plus Base-Pairing Interactions at 40°.

		λ_{min} (nm)	$[\theta]_{min}$ ($\times 10^{-4}$)	λ_{max} (nm)	$[\theta]_{max}$ ($\times 10^{-4}$)	λ_{calcd} (nm)	λ_{max} (nm)	$[\theta]_{max}$ ($\times 10^{-4}$)	Fit
fMet (*E. coli*)	Exp	297	−0.12	267	2.19	244	230	−0.37	0.244
	Calcd	298	−0.05	268	1.77	242	222	−0.33	
Leu (yeast)	Exp			263	2.57	242	223	−0.16	0.216
	Calcd			266	2.14	243	221	+0.11	
Phe (*E. coli*)	Exp			263	2.26	239	226	−0.08	0.315
	Calcd	300	−0.02	268	1.60	238	222	−0.20	
Phe (wheat)	Exp	296	−0.15	264	2.07	244	226	−0.40	0.139
	Calcd	300	−0.04	265	2.05	244	222	−0.23	
Phe (yeast)	Exp	295	−0.19	263	2.15	246	226	−0.35	0.276
	Calcd			265	1.69	244	218	−0.11	
Tryp (*E. coli*)	Exp	299	−0.02	265	1.95	243	226	−0.41	0.177
	Calcd			268	1.83	240	219	−0.29	
Tyr (*E. coli*)	Exp			264	2.20	244	224	−0.22	0.209
	Calcd			268	2.21	239	221	−0.01	
Tyr (yeast)	Exp	299	−0.04	265	1.83	242	236	−0.19	0.103
	Calcd	298	−0.02	267	1.80	239	221	−0.02	
Val (*E. coli*)	Exp	298	−0.06	267	2.16	243	225	−0.46	0.217
	Calcd			268	2.15	240	222	+0.03	

illustrated in Figure 7, showing the difference spectra between the native and denaturated tRNA.

Comparison of the difference spectra for $tRNA^{Leu}$ with average double-strand spectra corresponding to different numbers of base pairs indicates that the difference between native and denatured tRNA corresponds to the loss of four base-pairing interactions or five base pairs (Figure 7). The somewhat anomalous increase in the CD upon denaturation for $tRNA^{Trp}$ leads to a difference spectrum with the crossover shifted so far to the blue that it will not fit well with any combination of double-strand polymers. This suggests that the structural change upon denaturation of $tRNA^{Trp}$ is different from that observed in $tRNA^{Leu}$.

Discussion

We have shown that the CD spectra of nine different tRNAs are distinguishable and that each spectrum is nearly equal to the spectrum of the tRNA under conditions where it is single stranded plus a sum of the CD contributions of the formation of double-strand interactions given by the cloverleaf model. Thus, the contribution of tertiary structure to the CD spectrum of tRNA is quite small. The fact that each tRNA has a different CD spectrum is due to the nucleotide sequence and not to major differences in structure among these molecules.

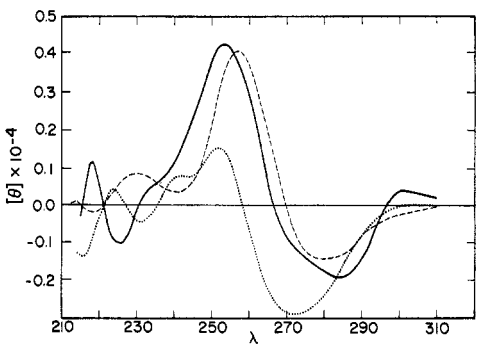

FIGURE 7: Difference CD spectrum of native minus denatured yeast $tRNA_3^{Leu}$ (——) and *E. coli* $tRNA^{Trp}$ (· · · · ·) compared with the difference CD of an average double strand of five base pairs (60% GC) minus its single strands (-----). Denaturation of yeast $tRNA_3^{Leu}$ is consistent with a loss of five base pairs; denaturation of *E. coli* $tRNA^{Trp}$ is not consistent with the loss of any number of base pairs.

In general, the calculated spectra have a somewhat lower magnitude of the major band than the experimental spectra, thereby suggesting that the native molecule has three or four more base pairs than in the cloverleaf model. However, since agreement between calculated and experimental spectra is much better for some tRNAs such as yeast $tRNA^{Tyr}$ (fit = 0.11) than for others such as *E. coli* $tRNA^{Phe}$ (fit = 0.33), all observed disagreements may simply be due to the fact that the library of spectra corresponding to the formation of the ten double-stranded pairing interactions is far from complete. If one or more of the assumptions in eq 4 is incorrect, the poor fit of a calculated spectrum may just reflect a large proportion of that interaction in the tRNA. It is clear that a library of the CD spectral contribution of all ten double-strand interactions should allow the calculation to be more accurate.

The calculation of CD spectra of single-stranded RNA and of the single-stranded portions of native tRNA using single-stranded basis spectra obtained from dimers was not very successful. Although a complete set of basis spectra was not available due to the many modified bases in tRNA, it is unlikely that this is the cause for the poor fit. A more likely explanation is that the dinucleoside monophosphates are not good models for the corresponding interactions in longer RNA molecules. Two adjacent residues probably can take up a larger variety of conformations in a dimer than as part of a longer sequence. This difference will be reflected in the CD spectra. Perhaps single-stranded basis spectra obtained from the interior positions of longer oligomers would be more accurate. However, it is also unlikely that the residues in the looped regions of RNA have a conformation (and hence a

CD spectrum) similar to their single-strand conformation. It has been shown that unlike single-strand RNA, the single-stranded regions in tRNA probably have a very well-defined structure (Uhlenbeck, 1972; Uhlenbeck *et al.*, 1970). Furthermore, work on model loops suggests that the CD spectra of the residues in the loop are different than the same residues as single strands (O. Uhlenbeck, P. Borer, B. Dengler and I. Tinoco, Jr., in preparation). These results suggest that it will be difficult to predict the CD contribution of single-strand portions of native RNA.

One means of improving the general method of fitting experimental spectra with basis spectra is to increase the amount of data by measuring spectra over a wider wavelength range. For example, using tRNA solutions of $A_{260} = 30$ in 0.5-mm path length cells, CD spectra have been obtained down to 200 mμ (Willick and Kay, 1971). However, it is likely that the tRNA is aggregated at this concentration (Millar and Steiner, 1966). The 4tU CD present in many *E. coli* tRNAs at about 335 mμ may also be studied using concentrated solutions in a 1-cm path length cell (Watanabe and Imahori, 1971; Scott and Schofield, 1969).

A number of previous studies of the CD of tRNA involved an analysis of the tRNA in the presence and absence of magnesium at 25° (Reeves *et al.*, 1970; Willick and Kay, 1971). We have shown that even in very low ionic strength, tRNA at 25° in the absence of magnesium still has considerable secondary and possibly tertiary structure. This somewhat surprising result emphasizes that in very low Mg^{2+} concentrations, the absorbance–temperature profile should be examined to determine what point on the structure → single-strand transition one is at before making structural conclusions about tRNA.

The temperature–absorbance curves in Figure 4 indicate that in the absence of magnesium tRNA has a somewhat greater absorption limit than it does in the presence of 1 mM Mg^{2+}. This suggests some interaction between the magnesium and the tRNA even at quite high temperatures. Apparently the Mg^{2+} somewhat alters the geometry of the bases or the overall structure of the tRNA, even at 90°. It would be interesting to compare the upper limit for the melting of single-strand and double-strand oligomers in the presence and absence of Mg^{2+} to see if their structure is also sensitive to Mg^{2+} at high temperatures, and to see if any of the optical properties of monomers and oligomers change in the presence of magnesium.

Conclusion

Our CD study of nine species of tRNA suggests the following.

(1) The difference in the CD of various species of tRNA may be explained by the different primary and secondary structure of these tRNAs.

(2) tRNA in the absence of magnesium and salt is single stranded at 40°, but not at 25°.

(3) The difference between native and denatured $tRNA^{Leu}$ may be represented by the loss of five base pairs, but the difference for $tRNA^{Trp}$ does not correspond to a loss of base pairs.

(4) The CD of dimers is only a qualitative model for calculating the CD of polymers.

(5) The CD of native tRNAs at 40° may be represented by a sum of the single-strand tRNA spectrum and an appropriate sum of spectra of base-pairing interactions.

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Localization of Polyadenylic Acid Sequences in Messenger Ribonucleic Acid of Mammalian Cells†

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ABSTRACT: The location of the poly(A) sequences in the mRNA of cultured L and HeLa cells was determined by submitting the mRNA, or poly(A) fragments excised from the mRNA, to hydrolysis with a highly purified exoribonuclease specific for 3′-OH termini. Analysis of the reaction products by a variety of means indicated that most and possibly all of the poly(A) sequences are at the 3′-OH termini of the mRNAs.

Polyadenylic acid is present in the large heterogeneous nuclear RNA (HnRNA)[1] and the relatively smaller mRNA of mammalian cells, as well as in vaccinia virus mRNA and adenovirus specific nuclear and polyribosomal RNA (Hajivassiliov and Brawerman, 1966; Edmonds and Caramela, 1969; Kates, 1970; Lim and Canellakis, 1970; Darnell *et al.*, 1971a,b; Edmonds *et al.*, 1971; Lee *et al.*, 1971; Burr and Lingrel, 1971; Philipson *et al.*, 1971). The poly(A) region is approximately 200 nucleotides long and is covalently linked to the RNA. Recent experiments by Darnell *et al.* (1971b) and Philipson *et al.* (1971) indicate that the poly(A) is added posttranscriptionally to the nuclear RNA, suggesting that the poly(A) is located near a terminus of the nuclear RNA molecules.

The following question arises. Are the poly(A) sequences in mRNA located at a terminus, and if so, at which one? Kates (1970) has presented evidence derived from chemical end-group analysis indicating that the poly(A) region is located at the 3′-OH end of *in vitro* synthesized vaccinia virus RNA. Burr and Lingrel (1971) have shown by base sequence analysis that oligonucleotides derived from the 3′-OH terminus of rabbit hemoglobin mRNA consist of five to six adenine nucleotides. These oligonucleotides were produced by digestion with pancreatic ribonuclease under low ionic strength conditions in which poly(A) is partially hydrolyzed (Beers, 1960), and thus might be derivatives of a larger poly(A) sequence described by Lim and Canellakis (1970). In the present study we have investigated the location of the large poly(A) sequences in the mRNA of cultured mammalian cells using a highly purified nuclear exoribonuclease specific for 3′-OH termini.

The nuclear exoribonuclease is a processive enzyme (RNA molecules are hydrolyzed to completion before the enzyme dissociates from them) which attacks the 3′-OH end of an RNA molecule and produces only 5′-mononucleotides (Lazarus and Sporn, 1967; Lazarus *et al.*, 1968). An RNA molecule with a phosphate in the 3′-terminal position cannot be degraded by the enzyme (Sporn *et al.*, 1968). The enzyme appears to prefer substrates with relatively little helix content, and has been observed to hydrolyze poly(A) and rapidly labeled (presumably mRNA-like) RNA at roughly comparable rates (Lazarus and Sporn, 1967). If the poly(A) region of mRNA is located at or near the 3′-OH end of the molecule, then incubation of mRNA with the exoribonuclease, under conditions approaching enzyme excess, should result in the disappearance of poly(A) at a rate much faster than that of total RNA. Moreover, poly(A) fragments excised from mRNA with pancreatic ribonuclease should be susceptible to exoribonuclease digestion only if they have come from the 3′-OH end. The results of such experiments with mRNA from mouse L cells and HeLa cells[2] provide strong evidence that

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[1] Abbreviation used is: HnRNA, heterogeneous nuclear RNA.

[2] L cell and HeLa cell mRNA contained poly(A) regions of essentially the same size as judged by polyacrylamide gel analyses of poly(A) fragments excised from the mRNA with T1 and pancreatic ribonuclease. However, in order to produce poly(A) fragments of maximum size, the ribonuclease digestion had to be carried out at a higher salt concentration (0.27 N NaCl) for L cell mRNA than that which is suitable for HeLa mRNA (0.1 M NaCl); digestion of the L cell mRNA at 0.1 M NaCl resulted in considerable degradation of the poly(A). Whether this difference in ribonuclease susceptibility is due to some subtle differences in the structures of the two kinds of mRNA is presently not clear.