Alterations in Structure and Function of Transfer Ribonucleic Acid on Chemical Methylation†

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ABSTRACT: Methylation of mixed yeast tRNA or purified yeast $tRNA^{Phe}$ with dimethyl sulfate gave 7-methylguanine as the major product with smaller amounts of 1-methyladenine and 3-methylcytosine. Methylation of mixed tRNA caused no change in absorbance at 260 nm measured at 25°. However, there was a decrease in hyperchromicity on heating suggesting a partial loss of secondary or tertiary structure. The effect of alkali, salt, and ethylene glycol on the absorbance was consistent with this interpretation. The circular dichroism spectra of mixed or $tRNA^{Phe}$ showed a shift of the positive dichroic band to longer wavelengths and a decrease in amplitude on methylation, confirming loss of structure. The loss of structure was also confirmed by the large decrease in fluorescence of $tRNA^{Phe}$ on methylation. The intrinsic viscosity was not changed on methylation. Methylated tRNA depurinated at a much slower rate than methylated DNA, with an activation energy of 29 kcal/mol, the same as for DNA. Methylated $tRNA^{Phe}$ accepted phenylalanine one-fourth as well as unmethylated $tRNA^{Phe}$, but was still specific for phenylalanine. The methylated Phe-$tRNA^{Phe}$ was one-third as effective as unmethylated Phe-$tRNA^{Phe}$ as a substrate for the synthesis of polyphenylalanine directed by poly(U).

Methylated bases are found in DNA as well as in ribosomal and tRNA. The highest concentration of methylated bases occurs in tRNA (Hall, 1965) and since the minor bases are not randomly distributed (Philipps, 1969), they probably play a structural and functional role. The function of the normally occurring methyl groups in tRNA, with regard to amino acid acceptance and transfer, has been reviewed by Starr and Sells (1969).

In elucidating the effect of methylated bases on the structure and function of tRNA, a useful approach is to study the changes in properties of tRNA on overmethylation with alkylating agents. Hypermethylation of *Escherichia coli* tRNA by dimethyl sulfate decreased the ability of the tRNA to accept amino acids (Pillinger *et al.*, 1969) and to transfer the charged amino acids (Hay *et al.*, 1970). Methylation of tRNA led to a decrease in hyperchromicity on heating (Kriek and Emmelot, 1963; Bollack *et al.*, 1965; Pochon and Michelson, 1967; Pillinger *et al.*, 1969) and was interpreted to suggest that methylation leads to a loss of secondary structure. There have been no detailed studies of the effect of methylation of tRNA on its secondary or tertiary structure. The present investigation was undertaken in order to elucidate the changes in structure of tRNA brought about by chemical methylation and to correlate these changes with those in amino acid acceptance and transfer.

Experimental Section

Materials. Mixed yeast tRNA (stripped) was purchased from General Biochemicals. The protein content was less than 2% (the limit of detection) and was not further decreased by treatment with phenol. No rRNA was detectable by Sephadex chromatography. Purified yeast phenylalanine tRNA was obtained from Boehringer-Mannheim. Dimethyl sulfate (reagent grade) was obtained from Eastman Organic Chemical Co. and [^{14}C]dimethyl sulfate from New England Nuclear Corp. Methylated purines and pyrimidines were purchased from Cyclo Chemical Corp., and ethylene glycol from Fischer Scientific Co.

Alkylation Procedure. The buffer used for the methylation reaction contained 0.01 M cacodylic acid–0.07 M sodium cacodylate–0.001 M Na_2EDTA– and 0.12 M $NaClO_4$ (pH 7.0). It was essential to use this particular buffer since it was shown previously (Uhlenhopp and Krasna, 1969, 1971) that dimethyl sulfate alkylates phosphate, citrate, and chloride ions but is inert to cacodylate and perchlorate. In a typical experiment, 10 mg of tRNA in 2 ml of buffer was treated with dimethyl sulfate (4–40 μl) at room temperature and allowed to react to completion (~4 hr). The pH decreased to 5.5 and no attempt was made to maintain the pH at 7.0 (Uhlenhopp and Krasna, 1969). For higher concentrations of dimethyl sulfate (above 50 mM), it was necessary to periodically add sodium cacodylate to keep the pH above 5.5. At the end of the reaction, the entire mixture was dialyzed against buffer three times at 4°. For the unmethylated tRNA control, dimethyl sulfate, at the same concentration used for alkylation, was added to buffer and allowed to hydrolyze to completion (~4 hr). tRNA was added to this solution (pH 5.5) and dialyzed against buffer after 4 hr. This control tRNA was indistinguishable with respect to absorbance, viscosity, T_m, and circular dichroism (CD) spectra, from the original tRNA.

The concentration of tRNA was determined from the absorbance at 260 nm using the published extinction coefficients (Willick and Kay, 1971). The number of methyl groups introduced into the tRNA was determined by measuring the radioactivity in the tRNA after methylation by [^{14}C]dimethyl sulfate.

Base Analysis. Radioactive methylated tRNA was hydrolyzed with 70% perchloric acid at 100° for 1 hr. The bases were separated by descending paper chromatography in a solvent containing 2-propanol–HCl–H_2O (85:20:20 v/v) (Bollack *et al.*, 1965). The positions of the radioactive spots were determined with a radiochromatogram scanner, cut out, and counted in a liquid scintillation counter. The radioactive areas were identified by comparing their R_F values with those

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of known methylated purines and pyrimidines. The bases were also identified by high-voltage paper electrophoresis at pH 3.9 as previously described (Uhlenhopp and Krasna, 1971). These procedures gave the concentrations of the methylated bases to ±5%.

The Y base was isolated from $tRNA^{Phe}$ as described (Fink *et al.*, 1971; Blobstein *et al.*, 1973).

Hydrolysis of tRNA by snake venom phosphodiesterase was carried out essentially as described by Hall (1964) except that three times as much enzyme was used. tRNA (2.5 mg) and 1 mg of enzyme were dissolved in 1.5 ml of 0.2 M Tris (pH 8.7) containing 0.01 M $MgCl_2$ and incubated at 37° for 24 hr. Under these conditions, hydrolysis was complete, or nearly so, for both methylated and unmethylated tRNA as judged by the large increase in absorbance at 260 nm.

To get some idea whether the methylation was random, as would be expected, or highly selective, $tRNA^{Phe}$ was methylated with radioactive dimethyl sulfate and the methylated $tRNA^{Phe}$ (7.5 methyl groups) digested with pancreatic RNase. The nucleotides were separated on the basis of charge by chromatography on DEAE-cellulose in the presence of 7 M urea (RajBhandary *et al.*, 1967) and the radioactivity was determined in each fraction. Though there was an overlap of radioactivity in the different fractions, the distribution of ^{14}C among the fractions was approximately that predicted from the known sequence assuming random introduction of methyl groups (*i.e.*, 30% of G residues, 4.5% of A residues, and 4.5% of C residues methylated).

To determine the extent of methylation of the G residue in the 2′-*O*-MeGAA anticodon of $tRNA^{Phe}$, radioactive methylated $tRNA^{Phe}$ (7.5 methyl groups) was hydrolyzed with alkali and the alkali-stable dinucleotides (2′-*O*-MeGA and 2′-*O*-MeCU) isolated by DEAE-cellulose chromatography as described by Hall (1971). This fraction contained the same ^{14}C activity per G residue as the nucleotide fraction, suggesting that the G residue in the anticodon is methylated to the same extent (30%) as the other G residues.

Physicochemical Measurements. Viscosity measurements were made with a Couette-type viscometer. Absorbance and melting profiles were measured at 260 nm in a Gilford spectrophotometer (Uhlenhopp and Krasna, 1971). CD spectra were determined in 1-cm cells at 25° on a Jasco J-40 spectropolarimeter. Uncorrected excitation and emission spectra were measured in a Hitachi-Perkin-Elmer MPF-2A fluorescence spectrophotometer.

Depurination Measurements. To determine the rate of depurination, tRNA was methylated with [^{14}C]dimethyl sulfate under conditions which introduced 7.5 mol of methyl groups/mole of tRNA. The tRNA was dissolved (4.7 mg/ml in cacodylate buffer (pH 7.0) (see above)) and one sample was incubated at 37° and another at 70°. From each reaction mixture, 10-μl aliquots were removed at appropriate time intervals and spotted on sheets of Whatman 3MM filter paper. Electrophoresis was run at 3000 V in 0.05 M formate buffer (pH 3.2) for 140 min and the dried sheets scanned in a radiochromatogram scanner. The radioactive areas were cut out and counted in a liquid scintillation counter. The main radioactive area corresponded to 7-methylguanine. There were traces of radioactivity at the 1-methyladenine position.

Amino Acid Acceptance and Transfer. The enzymes and reagents for these assays were supplied by Dr. L. Skogerson of this department. The system used is derived from *Saccharomyces cervisiae* (Skogerson *et al.*, 1973) and was carried out with normal and methylated yeast $tRNA^{Phe}$ (7.5 methyl groups/mole of tRNA). The charging of $tRNA^{Phe}$ was carried out at 30° in a reaction mixture containing 0.04 M Tris (pH 8.0), 0.013 M $MgCl_2$, 0.006 M NH_4Cl, 0.02 M dithiothreitol, 0.003 M ATP, 2.6×10^{-6} M [^3H]phenylalanine (7.8 Ci/mmol), 5.2×10^{-7} M $tRNA^{Phe}$, and the soluble synthetase enzymes. When the 19 other unlabeled amino acids were added, their final concentrations were 2×10^{-4} M. The limiting reactant in this assay is tRNA (Skogerson *et al.*, 1973). Samples were removed at 1-min intervals, precipitated with cold 10% Cl_3CCOOH, filtered onto glass fiber discs, washed, dried, and counted in a liquid scintillation counter.

To study the transfer of [^3H]phenylalanine from the charged tRNA, a preparative incubation similar to that just described was carried out for 20 min at 30°. The mixture was treated twice with phenol to remove protein and the aqueous phase chromatographed on Sephadex G-25 to isolate the Phe-$tRNA^{Phe}$. The fractions containing the $tRNA^{Phe}$ were combined, lyophilized, and dissolved in water.

The transfer of [^3H]phenylalanine from Phe-$tRNA^{Phe}$ was studied in a system synthesizing polyphenylalanine directed by poly(U). The soluble enzymes and ribosomes were derived from *S. cerivisiae* (Skogerson *et al.*, 1973); unmethylated and methylated Phe-$tRNA^{Phe}$ (7.5 methyl groups/mole of tRNA) were used as substrates. The reaction was carried out at 30° in a mixture containing 0.06 M Tris (pH 7.0), 0.012 M $MgCl_2$, 0.05 M NH_4Cl, 0.02 M dithiothreitol, 2×10^{-4} M GTP, 0.16 mg/ml of poly(U) (mol wt >100,000), soluble enzymes, ribosomes, and the charged tRNA containing 1.7×10^5 cpm total. The ribosomes are the limiting factor in this assay. Samples were removed at 1-min intervals, precipitated with cold 10% Cl_3CCOOH, heated at 90° for 10 min to hydrolyze charged tRNA, cooled, filtered onto glass fiber discs, washed, dried, and counted in a liquid scintillation counter. The control (unmethylated) Phe-$tRNA^{Phe}$ transferred 100% of its phenylalanine to polyphenylalanine showing that there was negligible nuclease contamination in the enzyme preparations.

Results and Discussion

Since there are many different species of tRNA, one must consider whether the results found with any particular pure tRNA species are characteristic of all tRNA species or are unique to the tRNA studied. The ideal approach would be to examine a large number of pure tRNA species. This is a very extensive undertaking and in this study the effect of methylation was studied on mixed yeast tRNA and purified yeast $tRNA^{Phe}$. The former substrate gives results which are characteristic of the class of molecules while the $tRNA^{Phe}$ can ascertain the effect of methylation on a particular pure species. In addition, the use of $tRNA^{Phe}$, which contains the fluorescent Y base, offers an additional probe of secondary structure. Phenylalanine tRNA is also convenient for studying amino acid acceptance and transfer.

Products of tRNA Methylation. The major product of methylation of tRNA with dimethyl sulfate was 7-methylguanine (80%). The other two methylated bases were 1-methyladenine (10%) and 3-methylcytosine (10%). These are the products that Bollack *et al.* (1965) reported for the methylation of tRNA in aqueous or dimethylformamide solution. Methylation of TMV RNA was reported (Singer and Fraenkel-Conrat, 1969) to yield 7-methyladenine. High-voltage paper electrophoresis unequivocably demonstrated that 7-methyladenine was not formed on methylation of tRNA with dimethyl sulfate.

Table I shows the dependence of the extent of methylation of tRNA on the concentration of dimethyl sulfate. There was

TABLE I: Extent of Methylation of tRNA as a Function of Dimethyl Sulfate Concentration.[a]

Dimethyl Sulfate Concn (mM)	No. of Methyl Groups (moles of CH_3/mole of tRNA)	% Change in ϵ[b]
25	4.3	±0.9
50	7.5	±0.5
100	14.7	±0.6
200	16.1	±0.6

[a] Methylation was carried out as described in the Experimental Section with [^{14}C]dimethyl sulfate. The number of methyl groups introduced was determined from the radioactivity of the methylated tRNA, assuming an average molecular weight of 25,000 for tRNA. [b] Measured at 260 nm.

a linear dependence until 15 methyl groups were introduced; further increase in dimethyl sulfate concentration did not lead to much further increase in the number of methyl groups. Analysis of the nucleotides formed on digestion with pancreatic RNase suggested that methylation was random and not highly selective (see Experimental Section).

Effect of Methylation on Ultraviolet Absorbance. Methylation of mixed yeast tRNA with 4–16 methyl groups/tRNA molecule caused no change in extinction coefficient (relative to the orcinol value) at 260 nm measured at 25° (Table I). Kriek and Emmelot (1963) reported that methylation of RNA with diazomethane in ether resulted in an increase in molar extinction coefficient. Diazomethane, besides forming 7-methylguanine, caused esterification of the secondary phosphate groups leading to the breakdown of the RNA within 24 hr. This degradation may have led to the increase in extinction. In the present case, no dialyzable fragments were produced, no depurination took place (see below), and there was no change in electrophoretic mobility suggesting that there was no rapid degradation of tRNA. Under these conditions there was no change in extinction coefficient at 260 nm measured at 25° and the absorption spectra from 220 to 320 nm was the same for methylated and unmethylated tRNA.

At first glance the failure to observe hyperchromicity at 25° on methylation would suggest that there is no loss of secondary or tertiary structure. This is not the case as evidenced by the absorption–temperature curve (Figure 1). The left panel compares the hyperchromicity on heating normal and methylated yeast tRNA. The melting curves are broad which is to be expected for the mixed tRNA. Whereas the unmethylated tRNA had 24% hyperchromicity, the methylated species (7.5 methyl groups) had only 16% hyperchromicity.

The increase in absorbance with temperature is indicative of the existence of secondary and tertiary structure in a molecule, and methylated tRNA still has considerable structure. However, the hyperchromicity decreased on methylation and is indicative of the partial loss of structure. The decrease in hyperchromicity on methylation has been previously reported (Kriek and Emmelot, 1963; Bollack *et al.*, 1965; Pochon and Michelson, 1967; Pillinger *et al.*, 1969).

The decrease in hyperchromicity from 24 to 16% on methylation would lead one to expect an 8% hyperchromicity at 25° on methylation, if there is loss of structure. The failure to observe this could be due to a lower extinction coefficient for the molecule on conversion of guanine residues to 7-methylguanine. Hendler *et al.* (1970) have reported that the extinction coefficient of 7-methylguanylic acid is smaller than for guanylic acid. To test this suggestion, equal quantities of unmethylated and methylated mixed tRNA were hydrolyzed with 0.3 N NaOH and adjusted to pH 7.0, and the absorbance of the combined nucleotides was measured at 258–260 nm. The hydrolyzed methylated tRNA had 8% less absorbance than the hydrolysate from unmethylated tRNA. Alkali causes the opening of the imidazole ring of 7-methylguanylic acid yielding a product with a different absorption spectrum than that of 7-methylguanylic acid, with an isosbestic point at 258 nm (Hendler *et al.*, 1970). Since the absorbance of the hydrolysate was measured at 258–260 nm, it is an accurate measure of the extinction coefficient of the methylated nucleotide. Identical results were obtained on hydrolysis with snake venom phosphodiesterase.

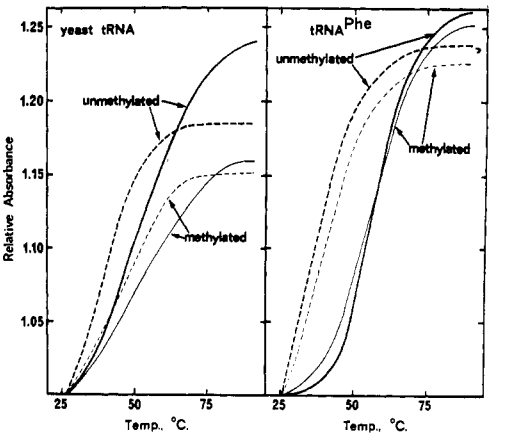

FIGURE 1: Absorbance–temperature curve at 260 nm for tRNA and methylated tRNA. The methylated tRNA contained 7.5 methyl groups/mole of tRNA. The left panel is the data for mixed yeast tRNA and the right panel for yeast tRNA^{Phe}. The solid lines are the data in cacodylate buffer containing 0.2 M Na^+ and the broken lines in cacodylate buffer containing 0.01 M Na^+. The relative absorbance is the ratio of the absorbance at the indicated temperature to that at 25°.

Since the extinction coefficient for the constituent nucleotides is 8% less for methylated tRNA, the finding of no change in absorbance of the intact molecule at 25° on methylation actually represents an 8% hyperchromicity which is indicative of loss of structure. If this 8% hyperchromicity is added to the 16% found on heating it is equal to the 24% found on heating unmethylated tRNA.

The right panel in Figure 1 gives the absorption–temperature profile for unmethylated and methylated yeast tRNA^{Phe}. The melting curves are sharper than for the mixed tRNA and there is very little difference in hyperchromicity. On methylation of tRNA^{Phe} there was a 7% decrease in extinction coefficient at 25°. This was completely due to a decreased extinction coefficient for the constituent nucleotides as determined by hydrolysis with alkali or snake venom phosphodiesterase. For tRNA^{Phe}, therefore, there is no hyperchromicity on methylation which could mean that there is no loss in structure or that the loss in structure is not manifested by increased absorbance at 260 nm. The latter is the correct explanation as shown below. The 8% hyperchromicity found on methylating mixed tRNA represents an average value for the different species of tRNA, some having higher hyperchromicity and some (like tRNA^{Phe}) having lower values.

Figure 1 shows the effect of sodium ions on the absorbance–temperature curve for tRNA and methylated tRNA. In all cases the T_m is decreased as the sodium concentration is lowered from 0.2 to 0.01 M where the negatively charged phos-

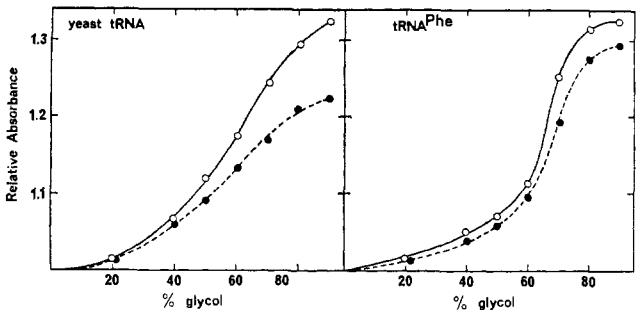

FIGURE 2: Effect of ethylene glycol on absorbance of tRNA and methylated tRNA (7.5 methyl groups) at 25°. The absorbance was measured at 260 nm in solvents containing increasing quantities of ethylene glycol. All solutions contained the same quantity of cacodylate buffer and were 0.02 M in Na^+. The relative absorbance is the ratio of absorbance in ethylene glycol–buffer to that in buffer alone. The left panel represents the data for mixed yeast tRNA while the right panel is the data for yeast $tRNA^{Phe}$.

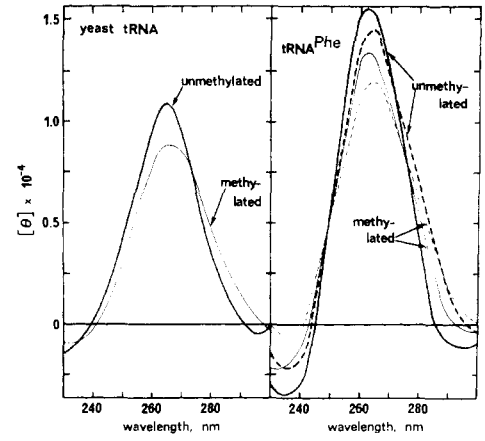

FIGURE 3: CD spectra of tRNA and methylated tRNA (7.5 methyl groups) at 25°. All samples were measured at the same concentration. The solid lines are in cacodylate buffer containing 0.2 M Na^+ and the broken lines in buffer containing 0.01 M Na^+. In all cases, spectra were also run on the constituent nucleotides obtained by hydrolysis for 24 hr with 0.3 N NaOH and readjustment of the pH to 7.0. The ellipticities of the nucleotides were very small and are subtracted from the spectra of the tRNA. The left panel is the data for mixed yeast tRNA and the right panel for yeast $tRNA^{Phe}$.

phate groups repel each other leading to a loss of secondary structure. These findings are the same as those reported by Goldstein *et al.* (1972) for *E. coli* mixed tRNA and formylmethionine tRNA. For unmethylated mixed yeast tRNA, the hyperchromicity on melting decreased from 24 to 18% on lowering the salt concentration (Figure 1). At 25°, the absorbance in 0.01 M salt was 6% higher than in 0.2 M salt, which is exactly the difference in hyperchromicity on melting. For methylated tRNA, however, there was no difference in absorbance at 25° and, as shown in Figure 1, very little difference in hyperchromicity on melting. The unmethylated tRNA at low salt had more hyperchromicity than methylated tRNA at high salt. These results suggest that the loss in structure due to methylation is more extensive than that caused by decreasing the sodium concentration from 0.2 to 0.01 M.

The right panel in Figure 1 shows the effect of low salt on unmethylated and methylated yeast $tRNA^{Phe}$. The T_m is decreased at low salt but there was only a small decrease (~3%) in hyperchromicity. There was no difference in absorbance at 25° as the sodium concentration was lowered from 0.2 to 0.01 M. The small difference in hyperchromicity for unmethylated $tRNA^{Phe}$ between high and low salt shows that the loss in structure (see below) for this species of tRNA is not manifested in absorbance changes at 260 nm. This is consistent with the finding that the hyperchromicity of $tRNA^{Phe}$ is not changed on methylation under conditions which caused significant changes in mixed yeast tRNA.

In order to decide whether the loss in structure of tRNA on methylation is due primarily to its effect on hydrogen bonds electrostatic interactions, or stacking interactions, the effect of ethylene glycol was studied. It has been shown (Fasman *et al.*, 1965; Green and Mahler, 1970) that ethylene glycol disrupts hydrophobic interactions while increasing contributions due to hydrogen bonding. Secondary structure in tRNA is destroyed by ethylene glycol and this suggests that hydrophobic forces are mainly responsible for maintenance of the native configuration. The effect of ethylene glycol on the absorbance of unmethylated and methylated mixed yeast tRNA is shown in the left panel of Figure 2. The curve resembles a melting curve and shows that methylated tRNA, though still maintaining some secondary structure, has less structure than unmethylated tRNA. The midpoint for tRNA was 58% glycol and for methylated tRNA 52%, suggesting that methylation weakens the overall stability of helical regions.

When the absorbance–temperature profile for both tRNA and methylated tRNA was studied in 50% ethylene glycol, the T_m and hyperchromicity were, as expected, much lower than in aqueous solution. The hyperchromicity for methylated tRNA in 50% ethylene glycol was considerably less than for tRNA in the same solvent.

The right panel in Figure 2 shows the effect of ethylene glycol on unmethylated and methylated yeast $tRNA^{Phe}$. Here there is only a small difference due to methylation and this result is consistent with those described above concerning the effect of methylation of $tRNA^{Phe}$ on the absorbance at 260 nm, *i.e.*, the effect is much smaller than that observed with mixed tRNA.

Changes in CD Spectra on Methylation. The absorbance results presented suggest that methylation of tRNA causes a loss in structure similar to that observed on treating tRNA with heat, low salt, or ethylene glycol. However, absorbance changes alone are not sufficient to conclusively establish loss of secondary or tertiary structure as evidenced by the results for $tRNA^{Phe}$. Conformational changes in tRNA can also be observed in the ORD and CD spectra (Fasman *et al.*, 1965).

The CD spectra of tRNA and methylated tRNA are shown in Figure 3. For all curves, the CD spectra were also measured on the constituent nucleotides obtained by hydrolysis of the samples with 0.3 N NaOH and readjustment of the pH to 7.0. The ellipticities of the nucleotides were very small and were subtracted from the spectra of the intact tRNA. The spectra obtained for yeast mixed tRNA is similar to that reported by Fasman *et al.* (1965), Green and Mahler (1970), and Goldstein *et al.* (1972). The spectra for $tRNA^{Phe}$ is the same as that described by Beardsley *et al.* (1970) and similar to that of other purified tRNA species at these wavelengths (Willick and Kay, 1971).

On methylation of tRNA, there is a slight shift of the positive dichroic band to longer wavelengths and a decrease in amplitude. In the ORD spectra (not shown) the Cotton effect is decreased in amplitude with a concomitant shift of the peak and crossover point to longer wavelength. These changes are precisely those observed (Fasman *et al.*, 1965; Green and Mahler, 1970) on heating tRNA or treating with ethylene glycol and confirm that methylation of tRNA causes conformational changes in the molecule similar to those brought about by denaturing agents. Ramstein *et al.* (1971) reported

similar changes in the CD spectra on methylation of DNA with dimethyl sulfate and, though they did not interpret the changes, it is probable that they were also due to a loss of secondary structure.

The right panel in Figure 3 gives the CD spectra for unmethylated and methylated $tRNA^{Phe}$ at 0.2 and 0.01 M Na^+. The effect of low salt is the same as methylation, *i.e.*, shift of the positive band to longer wavelength and decrease in amplitude (Reeves *et al.*, 1970; Goldstein *et al.*, 1972). In the present case, methylation causes a greater conformational change than decreasing the salt concentration. The methylated $tRNA^{Phe}$, though having less secondary structure than the unmethylated $tRNA^{Phe}$, still possesses some structure which is lost on lowering the salt concentration. It should be noted that with $tRNA^{Phe}$ the loss in structure was not evident from absorbance changes at 260 nm but was apparent from the changes in CD spectra.

Changes in Fluorescence on Methylation. An advantage to the use of $tRNA^{Phe}$ in these studies is that it fluoresces due to the Y base and the intensity of fluorescence is indicative of the degree of secondary structure (Beardsley *et al.*, 1970). Figure 4 shows the fluorescence spectra for unmethylated and methylated $tRNA^{Phe}$ in the region where fluorescence is due to the Y base. It is clear from both the emission and excitation spectra that methylation of $tRNA^{Phe}$ appreciably decreases the fluorescence intensity. The fluorescence maximum is shifted to short wavelengths due to the fluorescence of 7-methylguanine (see below). The decrease in fluorescence intensity on methylation is probably due to loss of secondary structure since it has been shown (Beardsley *et al.*, 1970) that the fluorescence intensity of $tRNA^{Phe}$ decreased with increasing temperature as the molecule is denatured.

It is conceivable that the decrease in fluorescence on methylation could be due to methylation of the Y base itself causing a change in the chromophore and that the changes observed may not be due to loss of secondary structure. To establish that the Y base was not changed on methylation, it was isolated from unmethylated and methylated $tRNA^{Phe}$. The fluorescence spectra and intensity of the Y base from both sources were identical. In addition, the Y base isolated from $tRNA^{Phe}$ methylated with radioactive dimethyl sulfate had no radioactivity. Thus it is certain that the decreased fluorescence on methylation is due to loss of structure.

Figure 4 also shows the decreased fluorescence of $tRNA^{Phe}$ in 0.01 M salt compared with 0.2 M salt for both the unmethylated and methylated molecules. This decrease in fluorescence is in accord with the reported effects of salt on the secondary structure of tRNA. As has been noted with respect to the CD spectra, methylation of $tRNA^{Phe}$ causes a greater loss in structure than lowering the sodium concentration to 0.01 M. The methylated $tRNA^{Phe}$ still possess some structure which can be further disrupted by decreasing the salt concentration.

In analyzing the fluorescence spectra of methylated $tRNA^{Phe}$ the fluorescence due to 7-methylguanine must be considered. Leng *et al.* (1968) have shown that 7-methylguanine in nucleosides, nucleotides, and polynucleotides exhibits fluorescence with excitation maxima at 290–330 nm, and emission maxima at 380–390 nm. DNA which had been chemically methylated exhibited emission maxima at 390–400 nm while unmethylated DNA had no fluorescence. In the spectra presented in Figure 4, the wavelengths used primarily measure the fluorescence of the Y base and not 7-methylguanine. In fact, if the latter were contributing significantly, the intensity of fluorescence should be greater for methylated tRNA, not smaller. There undoubtedly is some contribution from 7-methylguanine and the true fluorescence of the Y base in methylated $tRNA^{Phe}$ is probably even less than shown in Figure 4. When the excitation spectra for $tRNA^{Phe}$ was measured at 370 nm (emission maximum for 7-methylguanine in mixed tRNA) instead of 420 nm as in Figure 4, the methylated species had the same fluorescence intensity as the unmethylated species below 300 nm (excitation maximum for 7-methylguanine in mixed tRNA) and considerably less fluorescence above 300 nm. The excitation spectra measured with emission at 480 nm (where 7-methylguanine does not fluoresce) showed very little fluorescence for methylated $tRNA^{Phe}$ compared to the unmethylated species.

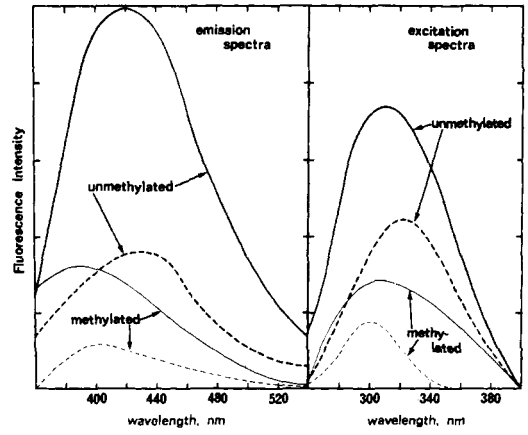

FIGURE 4: Fluorescence spectra (uncorrected) of unmethylated and methylated (7.5 methyl groups) $tRNA^{Phe}$. Both samples were measured at the same concentration in cacocylate buffer containing 0.2 M Na^+ (—) and 0.01 M Na^+ (- - - -). The emission spectra were run with excitation at 310 nm, and the excitation spectra were run with emission at 420 nm. The intensity scales for emission and excitation are not the same and should not be compared.

Viscosity Changes on Methylation. The intrinsic viscosities for tRNA and methylated tRNA were the same within experimental error. In 0.2 M Na^+, the intrinsic viscosities were 0.065 dl/g for tRNA and 0.062 dl/g for methylated tRNA. In 0.01 M Na^+ the values were 0.153 dl/g for tRNA and 0.165 dl/g for methylated tRNA. The higher viscosity of tRNA in low salt is expected (Goldstein *et al.*, 1972) since the molecule is more asymmetric and would have a greater hydrodynamic volume than the helix form. Though methylation causes a loss in structure of tRNA as seen by optical parameters, apparently the change in conformation is not sufficient to bring about changes in hydrodynamic properties.

In agreement with previous reports (Millar and Steiner, 1966; Henley *et al.*, 1966), the intrinsic viscosity of tRNA increased with increasing temperature indicating a loss of structure from a helix form to a random coil form of greater hydrodynamic volume.

Depurination of Methylated tRNA. Methylated DNA undergoes depurination of the methylated bases followed by introduction of single-strand breaks at apurinic sites (Lawley, 1966; Strauss and Hill, 1970; Uhlenhopp and Krasna, 1971). The depurination of methylated tRNA has not previously been studied and Figure 5 presents data for the release of 7-methylguanine from methylated tRNA at pH 7.0. Though most of the radioactivity was in 7-methylguanine, there was some radioactivity in 1-methyladenine. Methylated tRNA depurinated with a first-order rate constant at 70° of 3.1×10^{-4} min^{-1}, and at 37° of 3.6×10^{-6} min^{-1}. The first-order rate constants for depurination of methylated DNA at pH 7.0 were 8.2×10^{-3} min^{-1} at 70° and 8.5×10^{-5} min^{-1} at 37°

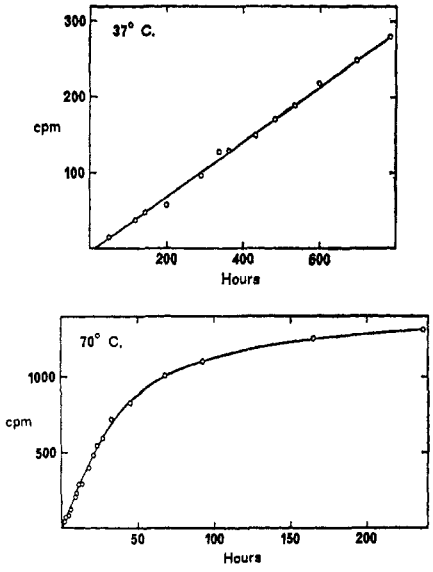

FIGURE 5: Depurination of 7-methylguanine from methylated mixed yeast tRNA. tRNA containing 7.5 methyl groups/mole of tRNA was incubated in cacodylate buffer (pH 7.0) at 37 and 70° as described in the Experimental Section. Samples were removed at intervals and the radioactivity in 7-methylguanine determined as described.

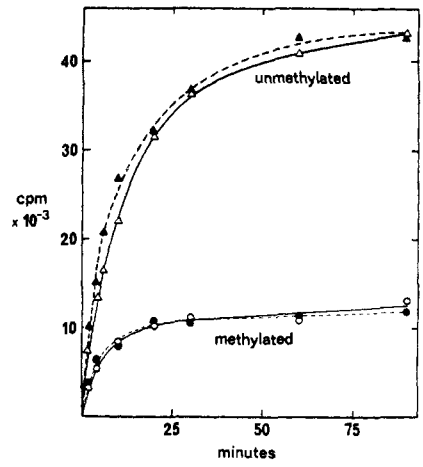

FIGURE 6: Amino acid acceptance by unmethylated and methylated (7.5 methyl groups) $tRNA^{Phe}$. The conditions for charging with [^{3}H]phenylalanine are given in the Experimental Section. The solid lines are for charging with phenylalanine alone and the dashed lines for charging with phenylalanine in the presence of an excess of the nineteen other amino acids.

(Uhlenhopp and Krasna, 1971). It is clear that DNA depurinates more rapidly than RNA which is to be expected from the greater lability of the purine–deoxyribose glycosidic bond compared to the purine–ribose glycosidic bond.

From these rates, the activation energy for depurination of 7-methylguanine is 29 kcal/mol for tRNA and 30 kcal/mol for DNA. It is interesting that the activation energies for RNA and DNA are the same even though the inherent rate of depurination is 25 times greater for DNA. Electrophoresis revealed that after prolonged incubation, tRNA was undergoing degradation in addition to depurination. The rate of depurination was extremely slow and probably played no role during the time required to make the physicochemical measurements described above.

Amino Acid Acceptance by Methylated $tRNA^{Phe}$. Having available methylated $tRNA^{Phe}$ characterized with respect to secondary structure, it was of interest to determine whether methylation of the tRNA affected its ability to accept phenylalanine in the presence of ATP and the synthetase enzyme. Figure 6 compares the rate and extent of charging of unmethylated and methylated $tRNA^{Phe}$. The methylated tRNA accepted phenylalanine at 38% the rate of the unmethylated molecule. The final degree of charging was only 28% that of unmethylated $tRNA^{Phe}$.

To determine whether methylation of $tRNA^{Phe}$ leads to recognition of the wrong amino acid, the same experiment was carried out in the presence of an excess of 19 amino acids in addition to phenylalanine. It is clear from Figure 6 that though methylation decreases the extent of charging with phenylalanine, the fidelity of the reaction is maintained.

The anticodon for $tRNA^{Phe}$ is 2′-*O*′-MeGAA and the G residue is methylated to approximately the same extent (~30%) as the remaining G residues (see Experimental Section). The decreased acceptor activity (loss of 70%) could be due to methylation of the anticodon bases or to the loss of structure. Though it is not possible to decide which effect led to the decreased activity, the degree of inhibition suggests that secondary structure plays a role. This is consistent with the findings of Weil *et al.* (1964) and Pillinger *et al.* (1969) that methylation of tRNA decreases the acceptor activity for amino acids even though the anticodon is not modified.

The present results compare the acceptor activity of normally methylated and hypermethylated $tRNA^{Phe}$. In a comparison of the amino acid acceptance of normal and methyl-deficient tRNA, many studies (Starr, 1963; Peterkofsky, 1964; Peterkofsky *et al.*, 1964; Fleissner, 1967; Stern *et al.*, 1970) have shown no appreciable difference in activity. However, Shugart *et al.* (1968a,b) have reported that methyl-deficient tRNA has decreased acceptor activity which is increased on enzymatic methylation.

Transfer of Phenylalanine by Methylated Phe-$tRNA^{Phe}$ The results just presented suggest that methylated $tRNA^{Phe}$ accepts amino acids only one-fourth as well as unmethylated $tRNA^{Phe}$. Another possible explanation is that the chemical methylation had methylated only 72% of the $tRNA^{Phe}$ molecules leaving 28% of the molecules unmodified. The unmodified molecules could accept amino acids normally while the methylated tRNA is completely inactive. The observed charging with phenylalanine of only 28% could be due to the fact that 28% of the molecules are unmodified. Though it is unlikely that the chemical methylation would leave 28% of the tRNA unmodified, this must be demonstrated.

To establish this point, the unmethylated and methylated Phe-$tRNA^{Phe}$ were used as substrates for the synthesis of polyphenylalanine directed by poly(U) (Figure 7). Both incubations contained the same quantity of charged radioactive phenylalanine. If the charged tRNA in the methylated sample was unmodified, the rate of incorporation should be the same as that of unmethylated tRNA. The results in Figure 7 clearly show that the rate of polyphenylalanine synthesis by methylated Phe-$tRNA^{Phe}$ is only 37% the rate of unmethylated Phe-$tRNA^{Phe}$, showing that the charged tRNA is indeed methylated.

Since the methylated tRNA was only 28% charged, the reaction mixture for polyphenylalanine synthesis contained, in addition to Phe-$tRNA^{Phe}$, three times as much methylated $tRNA^{Phe}$. It is possible that the methylated $tRNA^{Phe}$ inhibited incorporation of phenylalanine from Phe-$tRNA^{Phe}$. To demonstrate that this was not taking place, polyphenylalanine synthesis was studied with unmethylated Phe-$tRNA^{Phe}$ in the presence of three times as much methylated $tRNA^{Phe}$ or un-

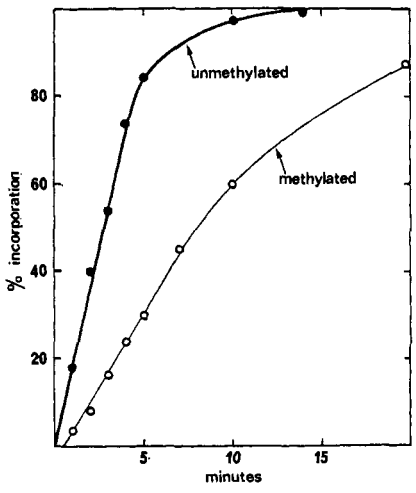

FIGURE 7: Polyphenylalanine synthesis by unmethylated and methylated (7.5 methyl groups) Phe-tRNA^{Phe}. The preparation of charged tRNA and the protocol for polyphenylalanine synthesis are given in the Experimental Section. Both incubations contained the same quantity of charged [^{3}H]phenylalanine.

methylated tRNA^{Phe}. In both cases, the rate of synthesis was exactly the same as with unmethylated Phe-tRNA^{Phe} alone.

The decreased transfer activity of methylated Phe-tRNA^{Phe} is undoubtedly due to modification of the anticodon but since the loss in activity (~70%) is greater than the degree of modification of the anticodon (~30%) the loss in secondary structure is probably also leading to decreased transfer activity.

These results are in essential accord with those of Weil *et al.* (1964) and Hay *et al.* (1970) for tRNA^{Phe}. With respect to polylysine synthesis with tRNA^{Lys}, Weil *et al.* (1964) reported no effect on methylation whereas Hay *et al.* (1970) found significant inhibition on methylation. The latter authors also reported no difference between methylated and unmethylated tRNA^{Phe} with respect to ribosome binding. In a comparison of normal and methyl-deficient tRNA^{Phe} (Stern *et al.*, 1970), there was very little difference in the ability to synthesize polyphenylalanine or natural peptides containing phenylalanine.

The present study has demonstrated that chemical methylation of mixed tRNA or purified tRNA^{Phe} causes a partial loss of secondary and tertiary structure which can be detected by changes in absorbance, hyperchromicity, CD, and fluorescence. The methylated tRNA depurinated very slowly (4% the rate for methylated DNA). Methylated tRNA^{Phe} was less effective than unmethylated tRNA^{Phe} in accepting and transferring phenylalanine.

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