Effects of the Ionic Environment on Modification of Yeast Tyrosine Transfer Ribonucleic Acid with *N*-Acetoxy-2-acetylaminofluorene†

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ABSTRACT: The effects of Mg^{2+} and K^+ ions on modification of purified yeast tyrosine transfer ribonucleic acid with *N*-acetoxy-2-acetylaminofluorene, a potent carcinogen which specifically binds to the 8 position of guanosine residues, were studied. In the presence of 3 mM Mg^{2+} and 0.1 M K^+ when the native form of tRNA is preserved, only 1.08 mol of the drug was bound/mol of tRNA. After pancreatic ribonuclease digestion of the modified tRNA, DEAE-cellulose chromatography of the resulting oligonucleotides, and Aminex A-6 separation of the component nucleosides, it was found that the major targets of the modification were two residues in the dihydrouridine loop, 2′-*O*-methylguanosine at position 18 and guanosine at position 19. In addition, a minor target was found in a dinucleotide probably derived from the anticodon region. When the modification was performed in the absence of Mg^{2+} and K^+ and in the presence of 3 mM EDTA, the binding of the drug increased to 1.6 mol/mol of tRNA. DEAE-cellulose chromatography of a pancreatic ribonuclease hydrolysate revealed, in addition to the previously described modifications, a modified tetranucleotide. The latter was presumably due to modification of a guanosine at position 15, on the 5′ side of the dihydrouridine loop. The amino acid acceptor activity of tyrosine tRNA modified in the presence of Mg^{2+} and K^+ was decreased by 18%; whereas the tyrosine acceptor activity of tRNA modified in the absence of Mg^{2+} and K^+ and in the presence of EDTA was decreased to 50% of that of the unmodified tRNA.

There is considerable evidence that tRNA possesses a highly organized three-dimensional structure which depends on the presence of divalent and/or univalent cations (Fresco *et al.*, 1966). Multiphase melting curves of individual purified tRNAs and other physicochemical measurements indicate that several "metastable" conformations can exist between tRNA in the "native" and "denatured" forms (Cole *et al.*, 1972; Cole and Crothers, 1972). Magnesium ions seem to be the most important factor in stabilization of the secondary and tertiary structures (Goldstein *et al.*, 1972), though they can be substituted to a considerable extent by other divalent cations, particularly Mn^{2+} and Ca^{2+}, and to a lesser extent Zn^{2+} and Co^{2+} (Fresco *et al.*, 1966). The univalent cations K^+ and Na^+ can also stabilize the structure of tRNA; however, only at much higher concentrations than magnesium ions (Fresco *et al.*, 1966; Cole *et al.*, 1972; Cole and Crothers, 1972). A partial renaturation of tRNA can also be achieved by polyamines (Tissières, 1959; Mahler and Mehrotra, 1963).

Chemical modification of tRNA appears to be a useful approach for providing information on the three-dimensional structure of tRNA and on certain structure–function relationships. *N*-Acetoxy-2-acetylaminofluorene,[1] a derivative of a potent carcinogen *N*-2-acetylaminofluorene, is a particularly useful reagent for chemical modification, since it binds preferentially to the C-8 position of guanosine residues located in single-stranded regions of nucleic acids (Miller *et al.*, 1966; Kriek *et al.*, 1967; Fujimura *et al.*, 1972; Levine *et al.*, 1974).

In the preceding paper of this series (Fujimura *et al.*, 1972), we described modification of purified *E. coli* $tRNA^{fMet}$ by reaction with AcN(OAc)Fln, and determined that the primary target in this tRNA was a guanosine residue at position 20 in the dihydrouridine loop.

In the present study we describe the effects of the ionic environment on AcN(OAc)Fln modification of purified yeast $tRNA^{Tyr}$. The extent and specific sites of base modification have been determined in a high and in a low ionic strength environment. In addition, the effects of drug modification on aminoacylation and codon recognition of this tRNA are presented.

Experimental Section

Materials. *N*-Acetoxy-9-[^{14}C]-2-acetylaminofluorene was prepared by the method of Miller *et al.* (1966) from *N*-2-hydroxy-9-[^{14}C]acetylaminofluorene, which was purchased from the International Chemical and Nuclear Co. The specific activity was 1.264 Ci/mol. A 20 mM stock solution of this drug in ethanol was prepared immediately prior to use. Purified yeast $tRNA^{Tyr}$ was prepared according to Gillam *et al.* (1967) from total tRNA purchased from the Plenum Scientific Research Inc. The steps included BD-cellulose chromatography of total tRNA to isolate the $tRNA^{Tyr}$ rich fraction. This material was then charged with tyrosine and run on a second BD-cellulose column where the charged tRNA now eluted in the ethanol region. The final material accepted approximately 1100 pmol of tyrosine per A_{260} unit. The triribonucleoside diphosphate U-A-U was prepared by enzymatic (ribonuclease A) synthesis from uridine 2′,3′-cyclic phosphate and A-U as described by Srinivasan *et al.* (1971).

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[1] Abbreviations used are: AcNH-Fln, *N*-2-acetylaminofluorene; AcN(OAc)Fln, *N*-acetoxy-2-acetylaminofluorene; $tRNA^{Tyr}$, tRNA specific for tyrosine; tyrosyl-$tRNA^{Tyr}$, the same tRNA acylated with tyrosine; BD-cellulose, benzoylated diethylaminoethyl-cellulose; G* denotes guanosine residues modified with *N*-2-acetylaminofluorene. Standard abbreviations were used according to IUPAC-IUB Commission on Biochemical Nomenclature (CBN) (1970), *Biochemistry* 9, 4022.

BD-cellulose (20–50 mesh) and [^{14}C]tyrosine (specific activity 50 Ci/mol) were purchased from Schwarz/Mann Biochemical. Prior to use for isolation of modified oligonucleotides, the BD-cellulose was washed sequentially with acetone–ethanol (1:1), 95% ethanol, 30% ethanol in 1.3 M NaCl, and, finally, with 0.02 M NH_4HCO_3. DEAE-cellulose (Cellex-D) (capacity 0.6 mequiv/g) was obtained from Bio-Rad Laboratories. Ribonuclease A and alkaline phosphatase (*Escherichia coli*) were purchased from Worthington Biochemical Corp., and snake venom phosphodiesterase (*Crotalus adamanteus*) from Sigma Chemical Co. Aminex-6 was purchased from Bio-Rad Laboratories, Rockville Center, N. Y.

Methods. Spectral Methods. Spectrophotometric measurements were made either on a Beckman spectrophotometer, Model DB-G, or a Zeiss spectrophotometer, Model PMQII. Continuous monitoring of eluates from the DEAE-cellulose and Aminex A-6 columns and dihydrouridine determinations were made on a Gilford multiple sample recording spectrophotometer, Model 2000.

Reaction of Yeast tRNA^{Tyr} with AcN(OAc)Fln. A 6-ml reaction system contained: 140 A_{260} units of tRNA, 10 mM sodium cacodylate (pH 7.2), 2 mM AcN(OAc)-9-[^{14}C]Fln, and 30% ethanol. As indicated in the individual experiments, the system also contained 3 mM EDTA, 3 mM $MgCl_2$, or 0.1 M KCl. The reaction mixture was incubated for 4 hr at 20° in the dark and then extracted five times with an equal volume of ether. The aqueous phase was adjusted to 0.2 M with potassium acetate (pH 5.0) and precipitated with 2.5 volumes of ethanol and stored at −20°. After centrifugation, the precipitate was dissolved in 2 ml of water.

To determine the rate of modification, aliquots were taken after various incubation times, coprecipitated with carrier tRNA, filtered through glass fiber filters, and washed five times with absolute ethanol and five times with ether. The filters were dried, and radioactivity was measured in a Nuclear-Chicago Mark II liquid scintillation spectrometer in 5 ml of a toluene scintillation solution.

Separation of Modified tRNA from the Unmodified Fraction. Modified tRNA was separated from the unmodified fraction by chromatography on a BD-cellulose column (2 × 5 cm). The column was first equilibrated with 0.01 M sodium acetate (pH 5.0). After the sample (140 A_{260} units) was applied, the column was washed with 50 ml of the same buffer, followed by 50 ml of 1.3 M NaCl in 0.01 M sodium acetate (pH 5.0). This eluted the unmodified fraction of tRNA, whereas the drug-modified fraction remained bound to the column and was then eluted with 1.3 M NaCl and 30% ethanol in 0.01 M sodium acetate (pH 5.0). Fractions (1.5 ml) were monitored for absorbancy at 260 nm, and the radioactivity of 50-μl aliquots was assayed in 5 ml of Bray's solution (Bray, 1960). The unmodified tRNA^{Tyr} from the "salt region" and the modified tRNA^{Tyr} from the "ethanol region" were pooled separately, dialyzed against water overnight at 4°, and lyophilized. The unmodified material obtained from the "salt region" was used as a control in subsequent assays.

RNase A Digestion of Modified tRNA. The mixture (final volume 0.2 ml) contained: 0.02 M Tris-HCl (pH 7.5), 50 A_{260} units of tRNA, and 100 μg of RNase A. Incubation was carried out for 30 min at 37°.

Separation of Oligonucleotides by DEAE-Cellulose Chromatography. DEAE-cellulose column chromatography of the RNase A digests of tRNA^{Tyr} was performed essentially as described by Madison *et al.* (1967). A column (0.45 × 50 cm) was equilibrated with 0.02 M Tris-HCl (pH 7.0) and 7 M urea. The sample was brought to 7 M with solid urea before applying it to the column. Oligonucleotides were eluted using a linear 200-ml gradient of 0–0.3 M NaCl in 0.02 M Tris-HCl (pH 7.0) and 7 M urea. Fractions (2 ml) were collected and monitored for uv absorbance at 260 nm. Radioactivity was assayed in 50-μl aliquots in 5 ml of Bray's solution.

Isolation of Modified Oligonucleotides on a BD-Cellulose Column. The major radioactive peaks obtained from the above column were pooled, diluted fivefold with water, and applied to a BD-cellulose column (1 × 3 cm) to remove the urea and NaCl and also to separate the modified oligonucleotides from overlapping unmodified ones. The BD-cellulose column was preequilibrated with 0.01 M ammonium bicarbonate and, after the sample was applied, the column was eluted with 50 ml of 0.01 M ammonium bicarbonate, followed by 50 ml of 1.3 M ammonium bicarbonate, and, finally, with 30% ethanol in 1.3 M ammonium bicarbonate. The eluate was monitored with an LKB recording spectrophotometer for uv absorbancy and the fractions were assayed for radioactivity. The first eluates contained some nonradioactive oligonucleotides, whereas the ethanol eluate contained the purified radioactive oligonucleotides. The latter material was pooled, evaporated, suspended in distilled water, and reevaporated to remove all of the ammonium bicarbonate.

Purification of Modified Oligonucleotides by Thin Layer Chromatography (Tlc). Cellulose plates (Eastman Kodak Co. 6065) were previously washed with water and with isobutyric acid–0.5 M NH_4OH (5:3, v/v) and dried, and the samples from the BD-cellulose column were applied. The plates were then developed in the same isobutyric acid–ammonia system.

Enzymatic Digestion of Modified Oligonucleotides. The uv absorbing and radioactive spots purified on the tlc plates were removed by scraping, eluted with water, evaporated to dryness on a flash evaporator, and dissolved in 100 μl of 0.2 M ammonium bicarbonate; 10 μg of *E. coli* alkaline phosphatase and 10 μg of snake venom phosphodiesterase were added to each sample and the mixture was incubated for 16 hr at 37°.

Aminex A-6 Column Chromatography. Chromatographic separation of nucleosides was essentially as described by Uziel and coworkers (1968). The Aminex A-6 column (0.45 × 70 cm) was previously washed with 0.4 M ammonium formate (pH 4.7). The above enzymatic digests were applied directly to the column and were eluted with the same buffer. The elution profiles of nucleosides were continuously recorded with a Gilford multiple sample recorder (Model 2000) at 260 nm using a full scale deflection of 0.15 A_{260}. The identification of nucleosides was provided by their elution position and by their uv spectra. The amount of a nucleoside was determined by measuring its A_{260} absorbancy area with a planimeter and then comparing this to the area obtained with known amounts of authentic samples.

Dihydrouridine Assay. Dihydrouridine was determined both qualitatively and quantitatively on an aliquot of the sample by observing the loss of absorbancy at 235 nm in 0.02 M NaOH according to Batt and coworkers (1954). The method was applied directly to samples obtained from the Aminex A-6 column.

Assay of Tyrosine Acceptor Activity. Rat liver aminoacyl-tRNA synthetase was prepared from the 105,000*g* supernatant fraction by protamine sulfate precipitation, ammonium sulphate precipitation (70% of saturation at 20°), and dialysis against 50% glycerol, 0.01 M Tris-HCl (pH 7.5), 0.06 M KCl, 0.01 M Mg^{2+}, and 0.05 M mercaptoethanol, as previously described by Nishimura and Weinstein (1969). The assay system contained in a total volume of 100 μl: 0.01 M potassium cacodylate (pH 7.5), 0.01 M KCl, 0.016 M $MgCl_2$, 2 mM ATP, 0.2 μCi of

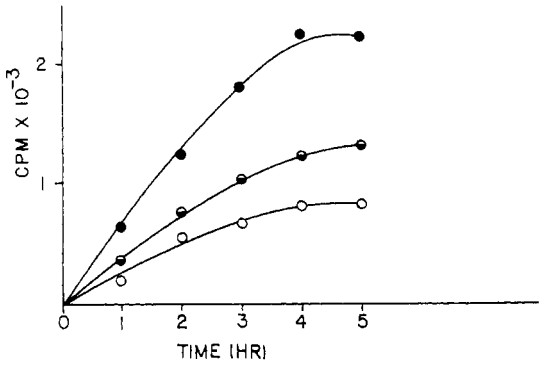

FIGURE 1: Kinetics of the reaction of yeast $tRNA^{Tyr}$ with AcNH-(AcOH)Fln under different ionic conditions. Incubation mixture (0.3 ml) contained 5 units of yeast $tRNA^{Tyr}$ in 10 mM sodium cacodylate (pH 7.2), 2 mM [^{14}C]AcN(OAc)Fln in 33% ethanol, and indicated amounts of $MgCl_2$, KCl, or EDTA. Incubation was carried out at 37°, and 50-μl aliquots were precipitated with 10% trichloroacetic acid at indicated time intervals and processed as described in Methods: (○) 3 mM $MgCl_2$–0.1 M KCl; (◒) 3 mM $MgCl_2$ (●) 3 mM EDTA.

[^{14}C]tyrosine, 0.05 A_{260} of tRNA, and about 0.05 mg of crude aminoacyl-tRNA synthetase. The reaction mixture was incubated at 37° for 15 min, precipitated with 3 ml of 5% trichloroacetic acid, filtered through Millipore membrane filters (type HA), and washed three times with 3 ml of 5% trichloroacetic acid. Radioactivity was measured in 5 ml of toluene scintillation solution.

Ribosomal Binding Assay. The ribosomal binding of [^{14}C]-tyrosyl-tRNA stimulated by the triplet U-A-U was carried out according to Nirenberg and Leder (1964). The incubation mixture (final vol 50 μl) contained: 0.01 M Tris-HCl (pH 7.2), 0.01 M ammonium acetate, 0.02 M $MgCl_2$, 1.7 A_{260} units of *E. coli* ribosomes, approximately 125 pmol of [^{14}C]tyrosyl-$tRNA^{Tyr}$, and 0.15 A_{260} unit of U-A-U. Incubation was carried out at 24° for 20 min. The reaction mixture was diluted with 3 ml of cold 0.1 M Tris-HCl (pH 7.2) containing 0.01 M Mg^{2+} and 0.1 M ammonium acetate, filtered on a Millipore membrane (type HA), washed three times with 5 ml of cold buffer, and assayed for radioactivity.

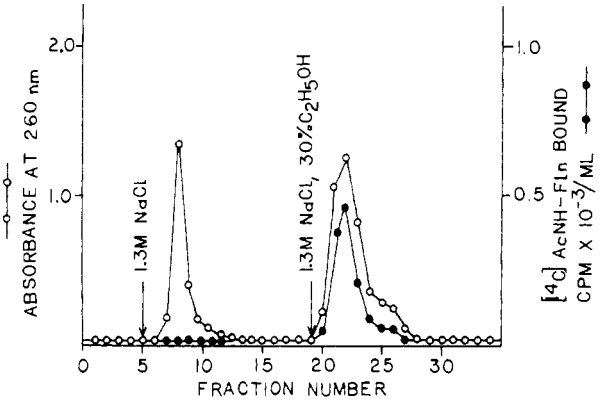

FIGURE 2: Separation of yeast $tRNA^{Tyr}$ modified with [^{14}C]AcN-(AcOH)Fln from unmodified $tRNA^{Tyr}$ on a BD-cellulose column. The reaction mixture contained in a total volume of 6 ml: 140 A_{260} units of $tRNA^{Tyr}$, 2.0 mM [^{14}C]AcN(OAc)Fln, 10 mM sodium cacodylate (pH 7.2), and 33% ethanol. Incubation was carried out for 4 hr at 37° in the dark; samples were extracted five times with ether, and the tRNA in the aqueous phase was precipitated with 2.5 vol of ethanol at −20° and finally dissolved in 2 ml of water. The sample was applied to a BD-cellulose column (2 × 5 cm) and eluted as described in Methods.

TABLE I: Effects of Ionic Conditions on Extent of AcNH-Fln Modifications and Tyrosine Acceptor Activity of Yeast $tRNA^{Tyr}$.

Modified in	Moles of AcNH-Fln Bound/Mol of tRNA	Tyrosine Acceptor Activity Expressed as % of Control[a]
3 mM $MgCl_2$–0.1 M KCl	1.08	81
3 mM $MgCl_2$	1.20	Not done
3 mM EDTA	1.63	50

[a] The data were obtained on AcNH-Fln-modified tRNA which was previously separated from the unmodified fraction on a BD-cellulose column. Reaction conditions and processing of the samples are described in Methods. A per cent inhibition is related to the acceptor activity of an equivalent amount of unmodified $tRNA^{Tyr}$ taken from the 1.3 M region of a BD-cellulose column.

Results

Effects of Salts on Extent of Modification of Yeast $tRNA^{Tyr}$ with N-Acetoxy-2-acetylaminofluorene. The time course of $tRNA^{Tyr}$ modification in the presence of either 3 mM EDTA, 3 mM $MgCl_2$, or 3 mM $MgCl_2$ and 0.1 M KCl is shown in Figure 1. In all cases maximum binding was reached after about 4 hr of incubation. To calculate the exact number of radioactive acetylaminofluorene moieties bound per molecule of tRNA, it was necessary to separate the modified tRNA molecules from those that might have escaped modification. This was achieved by fractionating the tRNA obtained from the reaction mixture on a BD-cellulose column (Figure 2). As demonstrated previously (Fink *et al.*, 1970; Fujimura *et al.*, 1972) with other tRNAs, the unmodified tRNA molecules were eluted with 1.3 M NaCl whereas the modified tRNA molecules, due to the hydrophobic character of the carcinogen, were eluted only with a mixture of 30% ethanol and 1.3 M NaCl. In all three reaction conditions about 50% of the $tRNA^{Tyr}$ molecules were modified. The different ionic conditions did, however, influence the specific activity of the drug-modified fraction. Table I indicates that in the presence of 3 mM $MgCl_2$ and 0.1 M KCl only 1.08 AcNH-Fln residues were bound per molecule of tRNA. When 0.1 M KCl was omitted from the reaction mixture the ratio of drug to tRNA increased to 1.20. When both 3 mM $MgCl_2$ and 0.1 M KCl were omitted and 3 mM EDTA was added to the reaction mixture the modification ratio increased to 1.63. Under these experimental conditions not necessarily all of the stabilizing cations present in the "interior" of the tRNA molecule were removed (Goldstein *et al.*, 1972).

RNase A Hydrolysis and Location of Modified Guanosine Residues in $tRNA^{Tyr}$. Preparations of yeast $tRNA^{Tyr}$ reacted with AcN(OAc)Fln in the presence of $MgCl_2$ and KCl or in the absence of $MgCl_2$ and KCl and, in the presence of EDTA, were digested with pancreatic ribonuclease, and the oligonucleotides were separated on a DEAE-cellulose column with 7 M urea and a NaCl gradient (Figures 3 and 4). Eight uv absorbing peaks were reproducibly obtained, and the profile was similar to that previously described by Madison *et al.* (1967). The fact that only two or three radioactive peaks were obtained (see below) indicates that the drug specifically modifies only a few nucleoside residues in yeast $tRNA^{Tyr}$ and does not attack randomly.

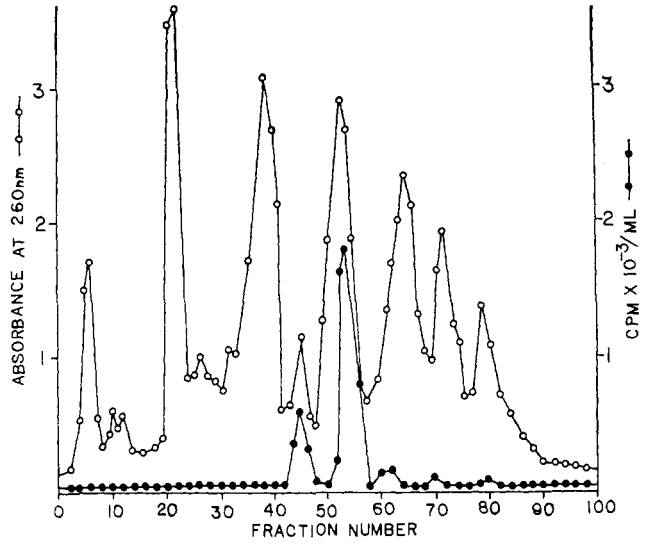

FIGURE 3: DEAE-cellulose column chromatography of a RNase A hydrolysate of modified yeast tRNA^{Tyr}. Fifty A_{260} units of tRNA^{Tyr} modified with [^{14}C]AcNH-Fln in 3 mM $MgCl_2$ and 0.1 M KCl were hydrolyzed with RNase A and chromatographed in a DEAE-cellulose column as described in Methods.

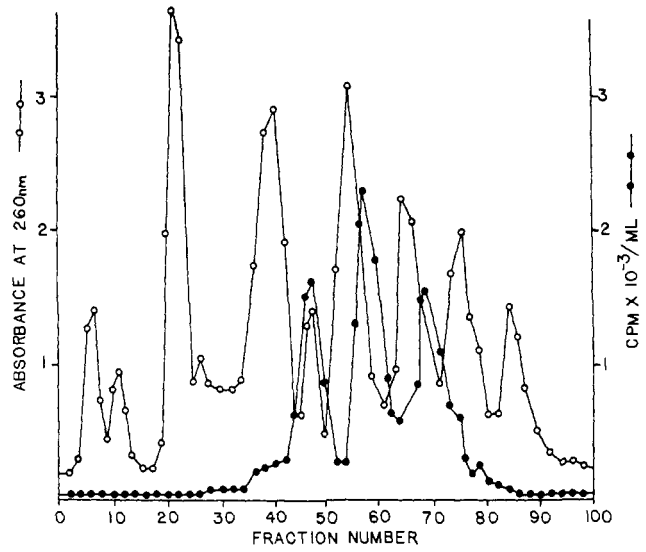

FIGURE 4: DEAE-cellulose column chromatography of a RNase A hydrolysate of modified yeast tRNA^{Tyr}. Fifty A_{260} units of tRNA^{Tyr} modified with [^{14}C]AcNH-Fln in 3 mM EDTA were hydrolyzed with RNase A and chromatographed on a DEAE-cellulose column as described in Methods.

In the hydrolysate of tRNA modified in the presence of 3 mM $MgCl_2$ and 0.1 M KCl, only two significant radioactive peaks were observed and these occurred in a ratio to each other of approximately 1 : 4 (Figure 3). The smaller peak was in the region of dinucleotides (fractions 42–48, Figure 3), and the major one in the region of trinucleotides (fractions 52–58, Figure 3). The oligonucleotides containing bound drug actually elute somewhat later from the DEAE-cellulose column than the corresponding unmodified materials. This is consistent with previous evidence that oligonucleotide containing AcNH-Fln have a higher affinity for DEAE-Sephadex (Fujimura *et al.*, 1972). In the hydrolysate of tRNA modified in the presence of EDTA, three major radioactive peaks were obtained; one in the region of dinucleotides (fractions 44–52, Figure 4); one in the region of trinucleotides (fractions 56–62, Figure 4), and the third in the region of tetranucleotides (fractions 67–76, Figure 4) were pooled and separated from unmodified oligonucleotides on BD-cellulose columns as described in Methods.

The major radioactive peak obtained from tRNA modified in the presence of $MgCl_2$ and KCl (Figure 3) was collected and further purified on a BD-cellulose column and by thin-layer chromatography (see Methods). Three uv spots were detected on tlc but only one of these (R_F 0.38) was both fluorescent and radioactive. The two other spots were non-radioactive. The base composition of the radioactive spot was obtained by complete digestion with snake venom phosphodiesterase and *E. coli* alkaline phosphatase and nucleoside analysis on the Aminex A-6 column. The results of the chromatographic separation are shown in Figure 5. Guanosine and 2′-*O*-methylguanosine (2′-MeGuo) in a ratio of approximately 2:1 were detected. Evidence for the presence of dihydrouridine in the same oligonucleotide was provided by a sharp loss of absorption at 235 nm after alkalinization of either an aliquot of the digested oligonucleotide or of the fractions which were eluted in the uridine region of the Aminex A-6 column (Figure 5). None of the radioactivity applied to the column appeared in the eluate. This is consistent with separate studies indicating that AcNH-Fln modified guanosine residues are bound extremely tightly to Aminex A-6.

The primary structure of yeast tRNA^{Tyr}, established by Madison and Kung (1967), is shown in Figure 6. Since the base composition of the major AcNH-Fln modified oligonucleotide indicated the presence of Guo, 2′-MeGuo, and H_2Urd, we concluded that this modification occurred in the dihydrouridine loop in the region 18–20 (Figure 6). Since both free Guo and 2′-MeGuo were obtained in the hydrolysate of the purified AcNH-Fln modified triplet, it appears that the drug had modified in certain molecules of tRNA Guo at position 18 whereas in other molecules 2′-MeGuo modification occurred at position 19. In separate studies we were able to demonstrate that AcN(OAc)-Fln reacts with 2′-MeGuo nucleoside as well as with Guo.

The amount of material present in the minor peak located in the dinucleotide region (fractions 42–47, Figure 3) and in the tetranucleotide (fractions 67–76, Figure 4) was in-

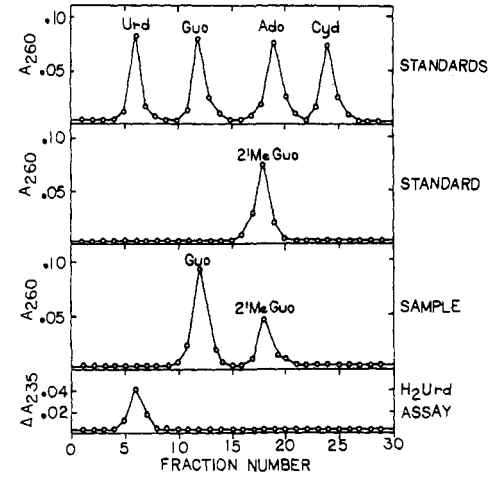

FIGURE 5: Aminex A-6 column chromatography of nucleosides. Purified radioactive oligonucleotide was digested with snake venom phosphodiesterase and *E. coli* alkaline phosphatase, and applied on an Aminex A-6 column (0.45 × 70 cm). The elution profile was compared with that obtained with authentic nucleosides. Dihydrouridine was assayed in fractions obtained from the column as described in Methods.

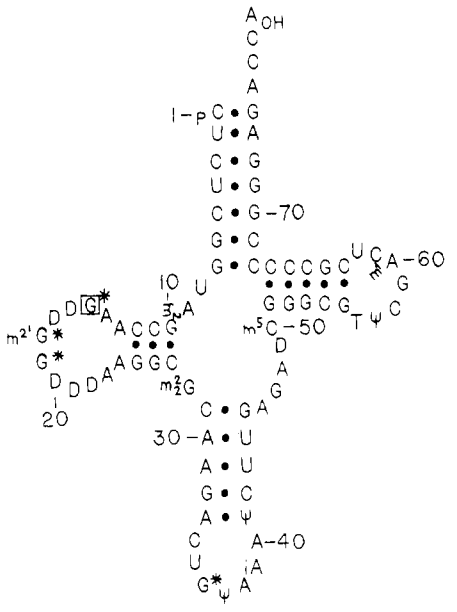

FIGURE 6: Primary structure of yeast $tRNA^{Tyr}$ with probable sites of AcNH-Fln modification. The primary structure of $tRNA^{Tyr}$ is taken from Madison *et al.* (1967): (*) apparent sites of AcNH-Fln modification in the native form; (□*) additional site of modification in the denatured form.

TABLE II: UAU Stimulated Binding of Tyrosyl-tRNA to Ribosomes.[a]

Type of tRNA Added	[^{14}C]Tyrosyl-$tRNA^{Tyr}$ Bound[b]		Total $tRNA^{Tyr}$ Bound[c]	
	Δ pmol	%	Δ pmol	%
Control	5.22	100	19.43	100
AcNH-Fln-modified	3.05	58	15.99	82

[a] Reaction mixtures contained 125 pmol (as bound tyrosine) of either control or AcNH-Fln-modified [^{14}C]tyrosyl-tRNA. Their specific activities were 430 and 350 pmol of [^{14}C]tyrosine/A_{260} of tRNA, respectively. The AcNH-Fln-modified tRNA had been reacted with the drug in the presence of $MgCl_2$ and KCl and is the same sample as that in line 1, Table I. Assays and the processing of the samples were as described in Methods. [b] [^{14}C]tyrosyl-tRNA bound is the amount of tyrosyl-tRNA bound in the presence of UAU minus the amount bound in the absence of UAU. [c] Total $tRNA^{Tyr}$ bound is a derived value which corrects for the differences in specific activities of modified and unmodified $tRNAs^{Tyr}$, assuming that both the aminoacylated as well as the nonacylated tRNAs bind to ribosomes.

sufficient to determine their base composition. It is likely, however, that the dinucleotide was derived from the G-ψ sequence (positions 36 and 37; Figure 6), and the tetranucleotide from the A-A-G-D (positions 13–16, Figure 6); see Discussion.

Tyrosine Acceptor Activity of Modified Yeast $tRNA^{Tyr}$. The tyrosine acceptor activity of yeast $tRNA^{Tyr}$ which had been reacted with AcNH(OAc)Fln in a medium of either high or low ionic strength is given in Table I. In these studies the modified molecules were first separated from the unmodified fraction by the BD-cellulose procedure and their acceptor activities were then compared. It can be seen that the tRNA modified with drug in the presence of $MgCl_2$ and KCl retained 81% of the acceptor activity of an equivalent amount of unmodified tRNA. The tRNA modified in the absence of $MgCl_2$ and KCl and in the presence of EDTA retained only 50% of the acceptor activity of the control material, presumably reflecting the greater extent of modification (Table I).

It is possible that the 19% decrease in the case of tRNA modified in the presence of $MgCl_2$ and KCl relates to that fraction of molecules which were modified in the anticodon. This explanation is consistent with the quantitative relations of the two peaks seen in Figure 3, although other explanations cannot be excluded.

Codon Dependent Binding of Modified tRNA to Ribosomes. In Table II is shown U-A-U-stimulated binding of [^{14}C]-tyrosyl-tRNA to ribosomes. Results obtained with control tRNA are compared to those obtained with tRNA previously modified with the drug in the presence of $MgCl_2$ and KCl. The binding to ribosomes of the [^{14}C]tyrosyl-tRNA modified with AcNH-Fln was only 58% of that obtained with the unmodified tRNA. In part, this could reflect the fact that the modified sample was less fully charged with [^{14}C]tyrosine than the control sample, since there is evidence that interaction with codon and ribosomes takes place with nonacylated tRNA as well as aminoacylated tRNA (Levin and Nirenberg, 1968; Levin, 1970). A correction for the difference in specific activities of the two tRNAs was therefore made to calculate the total amount (both acylated and nonacylated) of $tRNA^{Tyr}$ bound to ribosomes. Based on this assumption, the binding of AcNH-Fln modified tRNA was only 18% less than that obtained with the control tRNA (Table II). This decrease corresponds approximately to our estimate of the fraction of tRNA molecules which were modified in the dinucleotide present in the anticodon of this tRNA (see above and Discussion).

Discussion

Based on data obtained from chemical modification studies and physical measurements, several models have been proposed for the secondary and tertiary structures of tRNA (for a review, see Cramer, 1971). Recently the tertiary structure of yeast phenylalanine tRNA was determined by X-ray crystallography studies (Kim *et al.*, 1973). It is of interest, therefore, to analyze our results in terms of previous proposals for the structure of tRNA. *N*-2-Acetylaminofluorene is a particularly interesting probe for analyzing the secondary and tertiary structures of tRNA in solution because of its preferential interaction with G residues in single-stranded regions of nucleic acids (Fujimura *et al.*, 1972; Levine *et al.*, 1974).

According to the cloverleaf model of yeast $tRNA^{Tyr}$ (Figure 6), 16 out of 23 guanosine residues are in double-stranded regions. Several of the proposed tRNA models suggest an additional base pair between G-15 and the C residue (C-58) in the sequence T-Ψ-C (Cramer *et al.*, 1968, 1969). This would diminish the probability of interaction of AcN-(OH)-Fln with either G-15 or G-59. Previous studies indicated that residues in the dihydrouridine loop are readily attacked by a variety of chemical agents (Fujimura *et al.*, 1972; Cramer *et al.*, 1968; Igo-Kemenes and Zachau, 1969; Litt, 1969; Chang *et al.*, 1972). The data obtained in the present study indicate that residues 18 and 19 in the dihydrouridine loop of yeast $tRNA^{Tyr}$ are very susceptible to AcNH-Fln modification, suggesting that in this respect the tertiary structure of $tRNA^{Tyr}$ is similar to that of previously studied tRNAs.

The anticodon regions of tRNAs should also be relatively exposed since they must interact with codons in mRNA. Kethoxal modification of a guanosine residue in the anticodon loop of yeast tRNA^{Phe} has been described (Litt, 1969; Litt and Hancock, 1967). Other residues in the anticodon region of tRNAs are susceptible to chemical modification by borohydride (Igo-Kemenes and Zachau, 1969) and monoperphthalic acid (Cramer *et al.*, 1968).

In the RNase A digest of AcNH-Fln modified yeast tRNA^{Tyr} we observed, in addition to the major modification described above, lesser amounts of an AcNH-Fln containing dinucleotide. We have been unable to obtain sufficient amounts of this material for direct analysis of its base composition. There are, however, only three dinucleotides which have the general structure G-Y in an RNase A digest of yeast tRNA^{Tyr}: (1) m_2^2G-C (positions 28 and 29); (2) G-T (positions 55 and 56), and (3) G-Ψ (positions 36 and 37). Modification of M_2^2G-C is unlikely since it is sterically buried and thus far no chemical modifications of this region have been reported. G-55 is probably base paired with C-63 (see above) and, therefore, would not be readily susceptible to modification. We conclude, therefore, that the AcNH-Fln containing dinucleotide probably represents modification of G-36 which is present in the anticodon of tRNA^{Tyr}. The decreased activity of AcNH-Fln modified [^{14}C]tyrosyl-tRNA in a codon stimulated ribosomal binding assay is consistent with this interpretation, and it is known that AcNH-Fln modification of G residues in oligo- or polynucleotides impairs their base-pairing capacity (Grunberger *et al.*, 1970; Grunberger and Weinstein, 1971; Levine *et al.*, 1974). On the other hand, we have not excluded the possibility that the decrease of the acceptor activity as well as that of the ribosomal binding could be due to an overall conformational change in the modified tRNA.

Exposure of tRNA^{Tyr} to AcN(OAc)Fln in a low rather than a high ionic strength environment resulted in at least one additional site of drug modification. After RNase A digestion this modification was recovered in a tetranucleotide. Only two tetranucleotides containing G are present in a RNase A digest of yeast tRNA^{Tyr} (Madison *et al.*, 1967, and Figure 6), A-A-G-D (positions 13–16) and G-G-G-C (positions 51–55). AcNH-Fln modification of the former is most likely to occur because: (1) the latter sequence is in a double-stranded region, and (2) in a low ionic environment, partial unfolding of the tRNA molecule would be associated with a loss of the G-15 to C-58 base pair, thus rendering G-15 susceptible to chemical modification. Streeck and Zachau (1972) found that with denatured yeast tRNA^{Phe} T_2 RNase produced a split in the dihydrouridine loop at G-15 which did not occur with native tRNA^{Phe}, suggesting that denaturation does selectively unfold this region of the tRNA molecule.

Although additional studies are required to more firmly establish each of the sites of AcNH-Fln modification in tRNA^{Tyr}, it is apparent that the modification is quite specific and sensitive to the conformation of the tRNA molecule. This drug should prove to be a valuable tool for further exploring the various conformations of a single species of tRNA as a function of the solvent and ionic environment. From the biological point of view, it may also be of interest that the present results suggest that the extent and specific sites of nucleic acid modification by this carcinogen are a function of the ionic environment.

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