Modifications of Ribonucleic Acid by Chemical Carcinogens. Modification of *Escherichia coli* Formylmethionine Transfer Ribonucleic Acid with *N*-Acetoxy-2-acetylaminofluorene†

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ABSTRACT: *Escherichia coli* formylmethionine transfer RNA has been modified by reaction with *N*-acetoxy-9-[^{14}C]acetylaminofluorene, a drug which is known to cause the covalent attachment of the acetylaminofluorene group to the 8 position of guanosine residues. After separation of radioactive from nonradioactive molecules, the modified tRNA was cleaved by T_1 or pancreatic ribonuclease. The localization of the modified guanosine residues in the resulting oligonucleotides was determined after DEAE-Sephadex chromatography. The primary target of *N*-2-acetylaminofluorene modification is the guanosine residue in the dihydrouridine loop at position 20. The methionine acceptor and transformylase activities of the modified tRNA decreased to 40%, and had a K_m threefold higher than that of the unmodified molecules. The A-U-G codon recognition in a ribosomal binding assay was not affected by the attachment of acetylaminofluorene to this tRNA.

Although the primary structure of tRNA and its function in protein synthesis are now reasonably well understood, the relationship between its structure and biological functions remains to be elucidated. Chemical modification of tRNA can provide useful information, not only about the specific structural requirements for multiple functions of tRNA, but also about the three-dimensional structure of tRNA molecules (see reviews: von der Haar *et al.*, 1971; Chambers, 1971).

Miller and coworkers (1966) have established that 2-acetylaminofluorene, a potent carcinogen which is covalently bound to liver tRNA when administered *in vivo* (Henshaw and Hiatt, 1963; Agarwal and Weinstein, 1970), requires metabolic activation as a prerequisite for complexing with nucleic acids. The final metabolite for this interaction is an ester of *N*-hydroxy-AAF.[1] With a synthetically prepared ester, *N*-acetoxy-AAF, it is possible to directly bind AAF to the 8 position of G residues in nucleic acids at neutral pH *in vitro*, thereby producing the same type of modification as that which occurs *in vivo* (Miller *et al.*, 1966; Kriek *et al.*, 1967). In preceding papers in this series we have described that this modification causes major changes in the conformational properties of oligonucleotides which include rotation of the guanine base about the glycosidic linkage and the intramolecular stacking of fluorene with an adjacent base (Nelson *et al.*, 1971). These results provided essential chemical background for the use of *N*-acetoxy-AAF for the modification of a specific tRNA whose primary structure is known. Previously, a similar approach was used in this laboratory for the *in vitro* modification of unfractionated tRNA (Fink *et al.*, 1970). These results did indicate that AAF modification can affect the functional properties of tRNA but a precise interpretation was not possible since we were dealing with a complex mixture of tRNAs.

The present report describes the chemical modification of *Escherichia coli* tRNA^{fMet} with *N*-acetoxy-AAF, localization of the modified guanosine residues in the primary structure of the tRNA molecule, and the effects of this modification on aminoacylation and codon recognition. A preliminary report of some of our results has appeared elsewhere (Weinstein *et al.*, 1971).

Experimental Procedure

Materials. *N*-Acetoxy-9-[^{14}C]AAF was prepared by the method of Miller *et al.* (1961) from *N*-2-hydroxy-9-[^{14}C]AAF, which was purchased from ICN. The specific activity was 12.2 mCi/mmole. A stock solution of drug in ethanol was prepared just prior to use. Highly purified preparations of *E. coli* tRNA^{fMet} were kindly supplied by Dr. S. Nishimura, National Cancer Center Research Institute, Tokyo, Japan, and the Oak Ridge National Laboratory. These accepted approximately 1300 pmoles of methionine/A_{260} unit. A-U-G was a product of Miles Laboratories. BD-Cellulose (20–50 mesh) was purchased from Schwarz BioResearch, Inc. DEAE-Sephadex A-25 (capacity 3.5 ± 0.5 mequiv/g and particle size 40–120 μ) was obtained from Pharmacia Fine Chemicals. The anion-exchange resin AG 1-X8 (200–400 mesh, Cl^- form), Bio-Gel P-2 (50–100 mesh), and DEAE-cellulose (Cellex-D), capacity 0.6 mequiv/g, were obtained from Bio-Rad Laboratories. Ribonuclease T_1 and Hepes were purchased from Calbiochem, ribonuclease A and alkaline phosphatase (*E. coli*) from Worthington Biochemical Corp., and ribonuclease T_2 from Sankyo Co. Inc., Tokyo, Japan.

Methods. SPECTRAL METHOD. Spectrophotometric measurements were made on a Zeiss spectrophotometer Model PMQ II.

REACTION OF tRNA^{fMet} WITH *N*-ACETOXY-AAF. *N*-Acetoxy-9-[^{14}C]AAF (1.30 mM) was incubated in 2 ml with 25 A_{260} units of *E. coli* B tRNA^{fMet} in 0.025 M Tris-HCl buffer (pH

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[1] Abbreviations used are: AAF, 2-acetylaminofluorene; tRNA^{fMet}, formylmethionine transfer ribonucleic acid; tRNA$_{AAF}^{fMet}$, formylmethionine tRNA modified with AAF; BD-cellulose, benzoylated diethylaminoethylcellulose; DEAE, diethylaminoethyl-; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

7.2) and 30% ethanol at 37°. After 1.5 or 5.5 hr of incubation, the mixture was chilled to 4° and extracted twice with an equal volume of ether. The aqueous phase was adjusted to 0.1 M sodium acetate, and the tRNA was precipitated by adding 2 volumes of ethanol at −20° and was dissolved in 1 ml of water.

To determine the rate of modification, aliquots were taken after various incubation times, added to 0.5 ml of cold 5% trichloroacetic acid with crude tRNA as a carrier, and filtered on Whatman glass fiber filters GF/C (24-mm diameter). The filters were washed with ethanol three times, dried, and assayed for radioactivity in a Nuclear Chicago Mark II liquid scintillation system in 5 ml of toluene scintillation solution.

SEPARATION OF MODIFIED FROM UNMODIFIED tRNA. After the AAF modification, $tRNA^{fMet}$ was separated from $tRNA_{AAF}^{fMet}$ by chromatography on a BD-cellulose column (Gillam *et al.*, 1967). The column was first washed with 0.01 M sodium acetate (pH 5.0)–0.01 M $MgCl_2$. After applying the sample, the column was eluted with 120 ml of the starting buffer containing 1.0 M NaCl, followed by 100 ml of 30% ethanol in 1.5 M NaCl and buffer.

Fractions were measured for absorbancy at 260 nm, and the radioactivity of the aliquots was counted in 10 ml of Bray's solution. The radioactive $tRNA_{AAF}^{fMet}$ was found in the ethanol region of the eluate, and unmodified $tRNA^{fMet}$ appeared in the 1.0 M NaCl eluate. These two peaks were pooled separately, dialyzed against water overnight at 4°, lyophilized, dissolved in water, and precipitated with ethanol as described above. The first peak, which had not been modified by AAF, was used as a control in some of the subsequent experiments.

ENZYMATIC DIGESTION OF MODIFIED tRNA. All incubations were carried out at 37° for 16 hr. For T_1 RNase hydrolysis the incubation mixture contained, in 0.4 ml: 10 A_{260} units of tRNA–0.02 M Tris buffer (pH 7.1)–200 units of T_2 RNase. The incubation mixture for pancreatic RNase splitting contained, in 0.4 ml: 10 A_{260} units of tRNA–0.02 M Tris buffer (pH 7.9)–5 μg of RNase A (70 units/mg).

SEPARATION AND IDENTIFICATION OF [^{14}C]AAF-MODIFIED FRAGMENTS OF $tRNA^{fMet}$ AFTER T_1 RNase OR PANCREATIC RNase HYDROLYSIS. Chromatography on a DEAE-Sephadex A25-column (0.6 × 125 cm) was essentially as described by Seno *et al.* (1969) using a linear gradient of 400 ml each of 0.14 and 0.7 M NaCl in 0.02 M Tris-HCl buffer (pH 7.9)–7 M urea. Each fraction (3.2 ml) was measured for absorbancy at 260 nm, and 0.1-ml aliquots were assayed for radioactivity in 10 ml of Bray's solution. The major radioactive fractions were pooled, diluted five times with water, and applied to a BD-cellulose column to remove urea and to separate [^{14}C]-AAF-modified oligonucleotides from unmodified ones. BD-Cellulose was previously equilibrated with 0.01 M ammonium bicarbonate, and, after applying the diluted sample, the column was eluted stepwise with 0.01 and 1.3 M ammonium bicarbonate, and finally with 30% ethanol in 1.3 M ammonium bicarbonate. The radioactive fractions, which were eluted in the last step, were pooled, evaporated, and further purified on a DEAE-cellulose column (0.5 × 100 cm), which was equilibrated previously with 7 M urea in 0.02 M ammonium formate (pH 4). Chromatography was carried out with a linear gradient of 200 ml each of 0 and 0.3 M NaCl in 0.02 M ammonium formate–7 M urea. Absorbancy at 260 nm and radioactivity in 0.1-ml aliquots of each fraction placed in 10 ml of Bray's solution were measured. The radioactive peaks were pooled, diluted with two or three volumes of water, and then desalted on a DEAE-cellulose column (0.5 × 5 cm), which was washed with 0.005 M triethylamine bicarbonate buffer (pH 8.1) and eluted with a 2 M solution of the same buffer. After evaporation, samples were hydrolyzed to nucleotides with RNase T_2 as described previously (Grunberger and Weinstein, 1971). The resulting nucleotides were separated on a Bio-Rad AG 1-X8 column (0.4 × 20 cm). After applying the sample, chromatography was carried out with a convex gradient of NaCl, using 60 ml of 0.0035 M HCl in the mixing chamber and 0.5 M NaCl in the reservoir (Shima *et al.*, 1970). The elution profiles of the nucleotides were recorded with a Beckman Recording quartz spectrophotometer (Model DUR) and a Gilford multiple-sample absorbance recorder (Model 2000) at 260 nm using a full-scale deflection of 0.15. Identification of each nucleotide was provided by their elution position and the amount was determined by their A_{260} absorbancy area with a planimeter. These values were compared with those of authentic nucleotides. After detection of nucleotides the [^{14}C]AAF-modified Gp was eluted with 1 M NaCl in 30% ethanol. The amount of [^{14}C]AAF-modified guanosine 3′-monophosphate was calculated from the total radioactivity and specific activity of *N*-acetoxy-[^{14}C]AAF. The amount of dihydrouridine monophosphate was determined quantitatively in an aliquot of the sample by observing the loss of absorption at 234 nm in 0.02 N NaOH (Batt *et al.*, 1954).

ASSAY OF METHIONINE ACCEPTANCE ACTIVITY OF $tRNA_{AAF}^{fMet}$. *E. coli* aminoacyl tRNA synthetase was prepared from the 105,000*g* supernatant fraction by chromatography on a DEAE-cellulose column. The material which eluted with 0.25 M NaCl in 10 mM Tris-HCl (pH 7.5) was used as the enzyme fraction. The assay system contained, in a total volume of 0.25 ml: 0.1 M Hepes (pH 8.0)–0.01 M magnesium acetate–0.01 M KCl–4 mM ATP–0.2 μCi of [^{14}C]methionine, the amount of tRNA indicated in the figures and about 0.05 mg of crude aminoacyl-tRNA synthetase. The reaction mixture was incubated at 37° for 10 min, precipitated with 3 ml of 10% cold trichloroacetic acid, filtered on membrane filters, and washed with 3 ml of 5% cold trichloroacetic acid three times, and the radioactivity was measured in 5 ml of toluene scintillation solution.

RIBOSOMAL BINDING ASSAY was that of Nirenberg and Leder (1964) and the incubation mixture (0.05 ml) contained: 10 mM Tris-HCl (pH 7.2), 10 mM ammonium acetate, 20 mM magnesium acetate, 1.7 A_{260} units of *E. coli* ribosomes, 89 pmoles of [^{14}C]Met-$tRNA^{fMet}$ (sp act. 650 pmoles/A_{260}) or [^{14}C]Met-$tRNA_{AAF}^{fMet}$ (sp act. 351 pmoles/A_{260}), and 0.15 A_{260} units of A-U-G. Incubation was carried out at 24° for 20 min. The reaction mixture was diluted with cold 0.1 M Tris-HCl (pH 7.2), 0.01 M $MgCl_2$, and 0.1 M ammonium acetate and filtered onto Millipore membranes (type HA), and the membranes were washed three times with 5 ml of cold buffer. Radioactivity was measured in a liquid scintillation spectrometer with 5 ml of a toluene scintillation mixture.

Results

Modification of G Residues in $tRNA^{fMet}$ with N-Acetoxy-AAF. The time course of $tRNA^{fMet}$ modification obtained with increasing concentrations of *N*-acetoxy-[^{14}C]AAF is shown in Figure 1. After about 4 hr of incubation maximum binding of AAF was reached at all 3 concentrations of carcinogen used in the reaction mixture. The increased binding of AAF obtained with higher concentrations might be the result of either modification of more G residues in the same tRNA molecule or of a single G residue in a larger number of tRNA molecules. In order to decide between these two

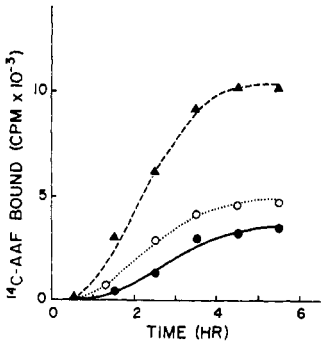

FIGURE 1: Kinetics of the reaction of tRNA^{fMet} with increasing concentrations of *N*-acetoxy-[^{14}C]AAF. Incubation mixture (0.25 ml) contained 2.8 A_{260} units of *E. coli* tRNA^{fMet} in 0.025 M Tris-HCl (pH 7.2), containing 33% ethanol and the indicated amount of *N*-acetoxy-[^{14}C]AAF. Incubation was carried out at 37° and 0.025-ml aliquots were precipitated with 10% trichloroacetic acid at indicated time intervals and processed as described in Methods. The concentrations of *N*-acetoxy-AAF were: (●—●) 0.66 mM; (○- - -○) 1.30 mM; and (▲- - -▲) 2.60 mM.

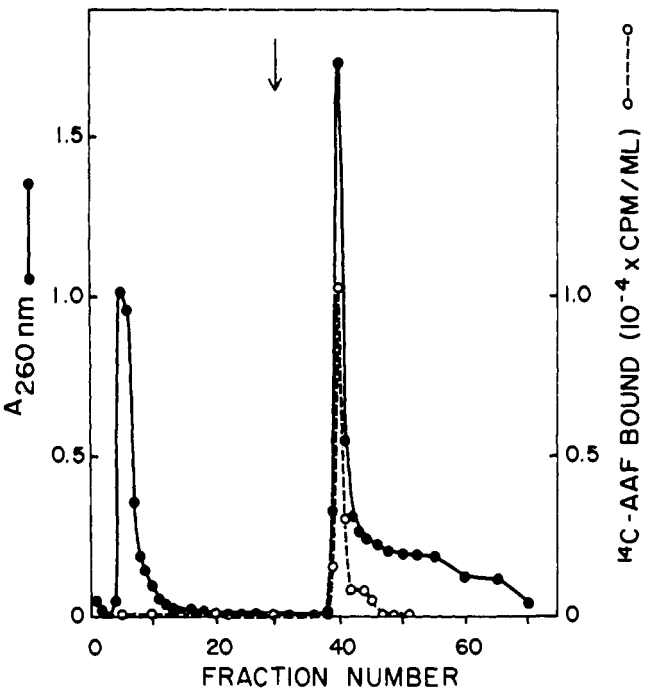

FIGURE 2: Separation of tRNA^{fMet} modified with [^{14}C]AAF from unmodified tRNA^{fMet} on a BD-cellulose column. The reaction mixture contained, in a total volume of 2 ml: 39.5 A_{260} units of tRNA^{fMet}–1.63 mM *N*-acetoxy-[^{14}C]AAF–0.3 M Tris-HCl (pH 7.2)–33% ethanol. Incubation was carried out for 3.5 hr at 37°, the mixture was extracted twice with ether, and the tRNA in the aqueous phase was precipitated with 2 volumes of ethanol at −20° and finally dissolved in 1 ml of water. The sample was applied to a BD-cellulose column (0.3 × 20 cm) and eluted as described in Methods.

possibilities it was necessary to separate the modified from the unmodified molecules. Due to the hydrophobic character of the modified tRNA, BD-cellulose column chromatography proved to be extremely useful for this purpose. As shown in Figure 2, the unmodified tRNA molecules were eluted with just 1 M NaCl, but the radioactive-modified material remained bound to the column and was eluted with 30% ethanol and 1.5 M NaCl. By employing this procedure it was possible to demonstrate that increasing concentrations of *N*-acetoxy-[^{14}C]AAF in the reaction mixture led to an increase in the fraction of tRNA molecules modified by AAF (Figure 3). After 4 hr of incubation in the presence of 3 mM *N*-acetoxy-[^{14}C]AAF almost 85% of the molecules were modified. Measurements of the specific activity of the modified fraction indicated that the moles of AAF residues per mole of tRNA^{fMet} remained almost constant. As the concentration of drug was increased in the reaction mixture this value remained at 1–1.5 moles of AAF residues per mole of tRNA^{fMet}. These results suggested that AAF was binding specifically to one or at most a few G residues in tRNA^{fMet}.

Localization of Modified Guanosine Residues in tRNA$_{AAF}^{fMet}$. The tRNA^{fMet} contains 25 guanosine residues and it was of interest to determine which G residue is the major site of modification. A sample of tRNA$_{AAF}^{fMet}$ containing approximately 1 mole of [^{14}C]AAF per mole of tRNA, purified as described above, was digested with ribonuclease T_1, and the oligonucleotides were separated on a DEAE-Sephadex A-25 column with 7 M urea and a NaCl gradient (Figure 4). One major radioactive peak was detected (in the region of tube 43) and this was followed by smaller radioactive peaks in the region of larger oligonucleotides. The amount of oligonucleotides between fractions 100–110 is lower than that in the nonmodified tRNA (Seno *et al.*, 1969). This is probably the result of the action of excess T_1 ribonuclease used for the splitting of the modified molecules which could cause some nonspecific cleavage in A-rich sequences as observed previously by Zachau *et al.* (1966). An authentic sample of AAF-modified Gp eluted at the same position as the major radioactive peak and the latter also cochromatographed with an authentic sample of AAF-GMP on paper chromatography. Due to the specificity of ribonuclease T_1 splitting we could conclude that in the structure of the tRNA the modified guanosine residue must have another G residue adjacent to it on the 5′ side. The primary structure of tRNA^{fMet} indicates that there were 9 G residues as possible candidates (Figure 8).

To obtain further localization of the modified G a separate sample of radioactive tRNA$_{AAF}^{fMet}$ was digested with pancreatic RNase and the oligonucleotides were separated on a DEAE-Sephadex A-25 column in 7 M urea (Figure 5). A major radioactive peak and a smaller peak were found in the region of tubes 76–89. These eluted considerably later than the previously described AAF-modified Gp present in the T_1 digest. This region was pooled and the radioactive oligonucleotides were further separated from unmodified oligo-

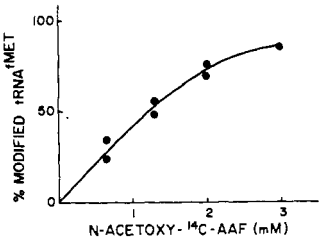

FIGURE 3: Percentage of tRNA^{fMet} which was modified by AAF as a function of increasing concentrations of *N*-acetoxy-AAF. The reaction mixture, the processing of the samples, and the BD-cellulose column chromatography were essentially as described in the legend to Figure 2. The concentration of *N*-acetoxy-[^{14}C]AAF used in the reaction mixture is given in the ordinate. The "% modified tRNA^{fMet}" = (the amount of tRNA eluted in the ethanol region × 100) divided by the total amount of tRNA eluted from the column.

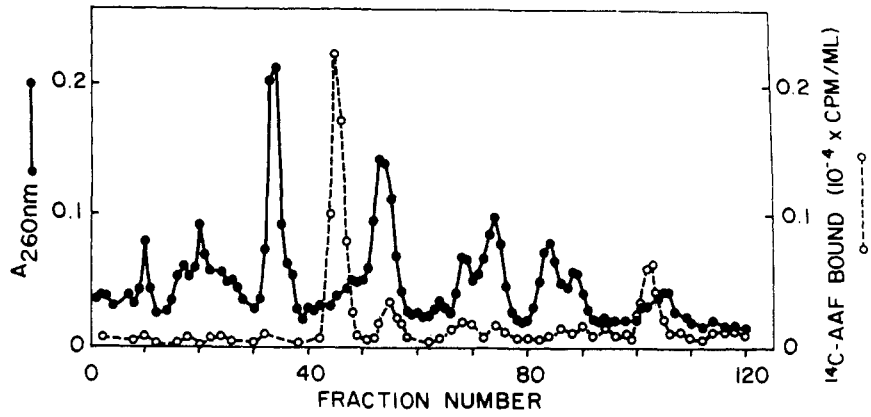

FIGURE 4: DEAE-Sephadex A-25 column chromatography of a RNase T_1 hydrolysate of $tRNA_{AAF}^{fMet}$. 10 A_{260} units of modified $tRNA^{fMet}$ were hydrolyzed with RNase T_1 and chromatographed on a DEAE-Sephadex A-25 column as described in Methods.

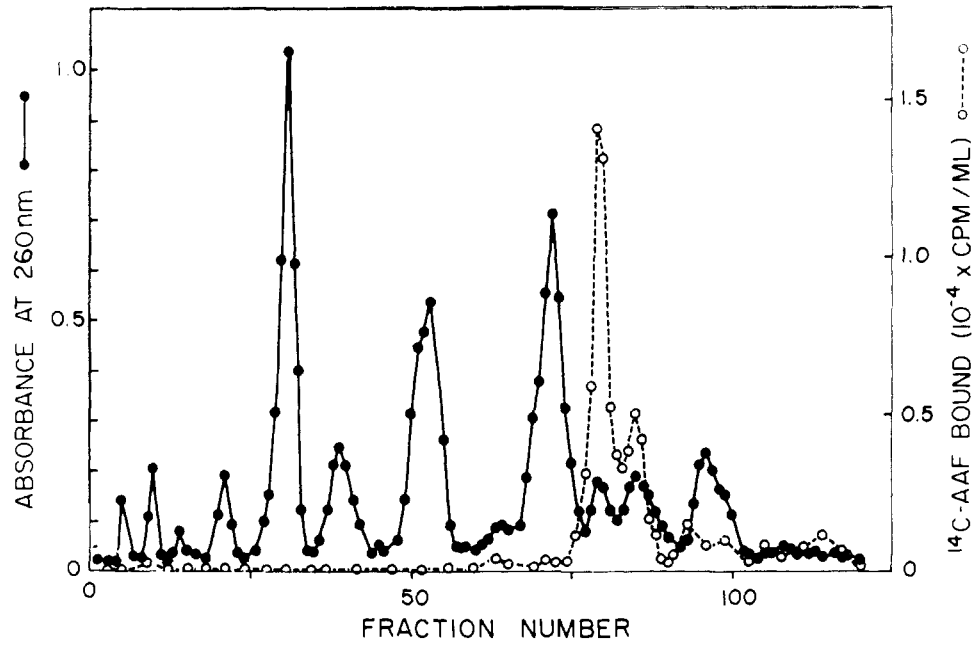

FIGURE 5: DEAE-Sephadex A-25 column chromatography of a RNase A hydrolysate of $tRNA_{AAF}^{fMet}$. 30 A_{260} units of [^{14}C]AAF-modified $tRNA^{fMet}$ were hydrolyzed with RNase A and chromatographed on a DEAE-Sephadex A-25 column as described in Methods.

nucleotides present in the same region by chromatography on a BD-cellulose column.

As shown in Figure 6, the unmodified oligonucleotides were eluted from the column with 1.3 M ammonium bicarbonate and the oligonucleotides containing [^{14}C]AAF were next eluted with 30% ethanol. By using this column there was no necessity for desalting the fractions because the urea carried over from the previous column eluted prior to the ethanol region and the ammonium bicarbonate present in the ethanol region could be easily evaporated. The radioactive oligonucleotides were further chromatographed on a DEAE-cellulose column using a NaCl gradient in 7 M urea at an acidic pH, as shown in Figure 7. By this method the smaller peak from the radioactive region of the original DEAE-Sephadex column (Figure 5) was clearly separated from the major radioactive peak.

The base composition of the major radioactive peak was determined by hydrolysis to mononucleotides with RNase T_2 and an aliquot of this material was separated on a Bio-Rad AG 1-X8 column using a convex gradient of NaCl (Shima *et al.*, 1970). The effluent was continuously monitored for 260 nm and only Gp was detected. When the column was subsequently washed with 30% ethanol and 1 M NaCl a radioactive peak corresponding to AAF-modified Gp was detected. An aliquot of the hydrolysate also revealed the presence of dihydrouridine (which does not absorb at 260 nm) since there was a sharp loss of absorption at 234 nm after alkalization.

The primary structure of $tRNA^{fMet}$ as established by Dube *et al.* (1968) is given in Figure 8. Taken together, the results of the T_1 and RNase A hydrolysis experiments indicate that the major site of AAF modification must be the G residue on the 5′ side of the dihydrouridine (position 20). This is compatible with the detection of AAF-Gp in the T_1 hydrolysate and the base composition analysis of the major radioactive oligonucleotide obtained from an RNase A digest indicating the presence of Gp, AAF-Gp, and D. The A_{260} absorbance of the minor peak obtained from the RNase A

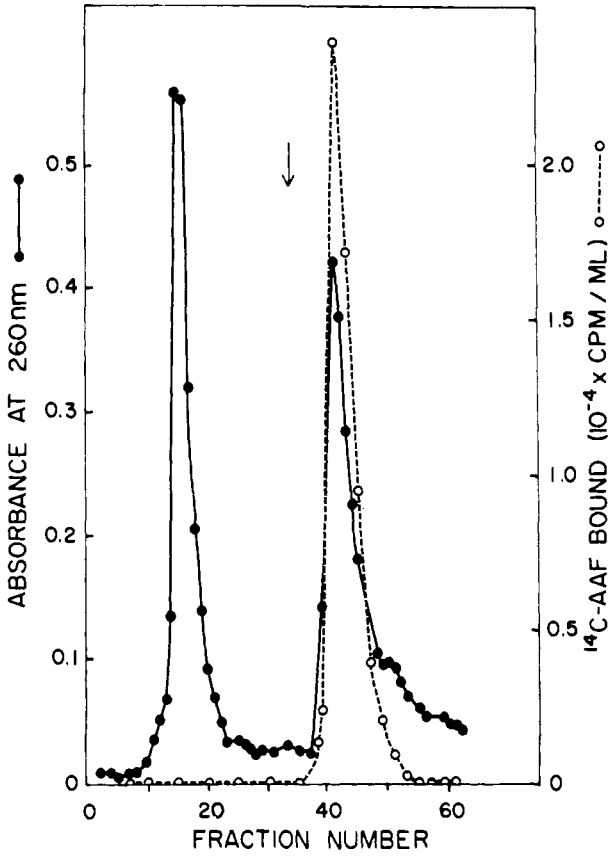

FIGURE 6: Separation of the major radioactive region from Figure 5 by BD-cellulose column chromatography. Fractions 76–89 from Figure 5 were pooled, diluted 5 times with water, and applied to a BD-cellulose column. The column was washed with 0.01 M ammonium bicarbonate until fraction 10, then eluted with 1.3 M ammonium bicarbonate until fraction 33, followed by 1.3 M ammonium bicarbonate in 30% ethanol. Fractions of 3 ml were collected at a flow rate of 15 ml/hr. Fractions 35–55 were pooled and evaporated. The recovery of radioactivity was 88.9%.

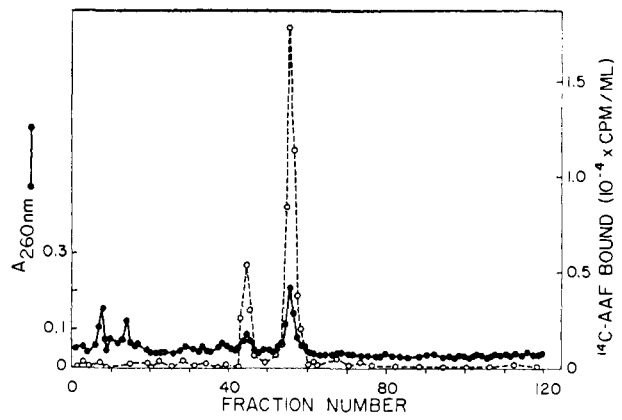

FIGURE 7: Further purification of [^{14}C]AAF-modified oligonucleotides obtained from the BD-cellulose column (Figure 6). A DEAE-cellulose column (0.5 × 120) was equilibrated with 7 M urea in 0.02 M ammonium formate (pH 4). After applying the sample, the column was washed with the same buffer until fraction 6 and then eluted with a linear gradient of 200 ml each of 0 and 0.3 M NaCl in the same buffer. Fractions (3 ml) were collected and assayed for radioactivity and absorbancy at 260 nm.

digest (tubes 40–45) was insufficient to determine its base composition and therefore the minor site of modification cannot be assigned with certainty.

Methionine Acceptance Activity of Modified $tRNA^{fMet}$ The acceptance activity for methionine of $tRNA^{fMet}$ after modification with AAF was decreased by about 60% when compared to that of equivalent amounts of unmodified molecules and this was true over a sevenfold range of tRNA concentrations (Figure 9) and a fourfold range of enzyme concentration. Figure 10 shows Lineweaver–Burk plots of the aminoacylation of the modified and unmodified $tRNA^{fMet}$. From the diagram it is obvious that the K_m for the modified tRNA was more than threefold higher than that observed for the control. No change in V_{max} was observed. Separate studies (not shown here) indicate that the fraction of AAF-modified $tRNA^{fMet}$ which accepted methionine could also be fully formylated by the transformylase enzyme.

To determine a possible change in specificity of synthetase recognition for $tRNA_{AAF}^{fMet}$ we tested the acceptance activity for 18 ^{14}C-labeled amino acids, in the absence of methionine, using a crude preparation of *E. coli* aminoacyl tRNA synthetase. Neither the unmodified nor the modified tRNAs accepted any amino acid under these experimental conditions. Attempts to restore the original acceptance activity by heating the modified samples to 75° in 0.01 M $MgCl_2$ for 5 min and then cooling (Lindahl *et al.*, 1966) had no effect on acceptor activity.

Codon-Dependent Binding of Modified $tRNA^{fMet}$ to Ribosomes. Since the major site of G modification with AAF was in the dihydrouridine loop of $tRNA^{fMet}$, it was of interest to determine if this modification affects the ability of this tRNA to recognize its specific codon A-U-G. As shown in Table I, A-U-G stimulated the binding of both the unmodified and the modified tRNA to ribosomes. Since the modified tRNA accepted only 53% as much methionine as the control sample, the binding of [^{14}C] Met-$tRNA_{AAF}^{fMet}$, expressed as pmoles of [^{14}C] methionine, was approximately 50% of that obtained with the unmodified tRNA. This corresponds with the decreased specific acceptance activity of the modified molecules. Since it was shown previously that the codon–anticodon interaction in the ribosomal binding assay takes place with aminoacylated as well as with deacylated tRNAs (Levin and Nirenberg, 1968; Levin, 1970), we also expressed the binding of tRNA to ribosomes correcting for the difference in specific activities of the modified and unmodified

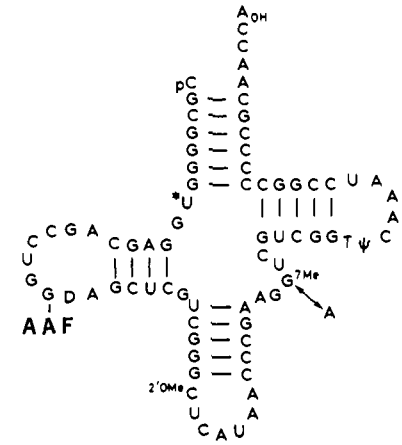

FIGURE 8: Primary structure of $tRNA^{fMet}$ indicating the presence of AAF on the G_{20} residue. The primary structure of $tRNA^{fMet}$ is taken from Dube *et al.* (1968).

TABLE I: Binding of $tRNA^{fMet}$ and $tRNA_{AAF}^{fMet}$ to Ribosomes Stimulated by A-U-G.[a]

Codon (0.15 A_{260})	$tRNA^{fMet}$ Added (89.6 pmoles)	[^{14}C]Met-$tRNA^{fMet}$ Bound pmoles	Δ pmoles	Total $tRNA^{fMet}$[b] Bound (Δpmoles)	$tRNA^{fMet}$[c] Bound (%)
	Unmodified	2.91			
A-U-G	Unmodified	13.94	11.03	28.22	31.60
	Modified	2.81			
A-U-G	Modified	8.71	5.90	27.56	30.70

[a] Reaction mixtures and processing of the samples are described in Methods. The specific activity of [^{14}C]Met-$tRNA^{fMet}$ was 650 pmoles/A_{260} and of $tRNA_{AAF}^{fMet}$ 351 pmoles/A_{260}. [b] The total $tRNA^{fMet}$ bound is a derived value which includes the binding of uncharged $tRNA^{fMet}$ (see text). [c] % = (total $tRNA^{fMet}$ bound × 100)/$tRNA^{fMet}$ added.

tRNAs (Table I). With this correction, the binding of modified $tRNA^{fMet}$ to ribosomes seemed to be the same as that of the unmodified molecules. Assuming that this correction is valid, we can conclude that AAF modification of G at position 20 in the dihydrouridine loop of $tRNA^{fMet}$ does not markedly affect the codon recognition.

Discussion

Several models, based on data obtained from various types of modifications and physical measurements, have been proposed for the secondary and tertiary structure of tRNA molecules. For a review of this subject see Cramer (1971). Although these models differ from each other in certain aspects most of them place the dihydrouridine loop at the outer surface of the molecule with certain nucleotides in this loop protected from chemical modification by base pairing. Litt (1969, 1971) has found that kethoxal, which modifies only G residues in tRNA, is bound preferentially to G in the dihydrouridine loop of $tRNA^{Phe}$ at position 20.

The present studies indicate that in the reaction of $tRNA^{fMet}$ with *N*-acetoxy-AAF the major target is also the G residue at position 20. This is in accord with our proposal on the conformation of AAF-modified G which predicts that single-stranded regions of RNA are more accessible to AAF than are double-stranded ones (Fink *et al.*, 1970; Grunberger *et al.*, 1970). The cloverleaf model of tRNA predicts that 18 of the 25 G residues in $tRNA^{fMet}$ are in base-paired regions. Five of the remaining G residues are presumably buried within the three-dimensional structure, and the remaining two (present in the sequence G-G-D) of the dihydrouridine loop would be exposed to react with AAF. It is interesting to note that in Levitt's model (1969) of $tRNA^{fMet}$ G residue 20 is in the syn conformation, since our conformational studies predict that this would also markedly enhance the interaction of AAF with this particular G.

Modification of $tRNA^{fMet}$ to the extent of approximately 1.3 moles of AAF/mole of tRNA caused about 60% decrease in methionine acceptor activity. There are several possible explanations why we did not see an "all or none" effect. (1) In addition to the major AAF modification at G-20, it appears that the drug was bound to a lesser extent to one or more other G residues in the molecule (see Figures 4 and 7). Perhaps only those molecules which had two modified G residues were not recognized by the aminoacyl-tRNA synthetase. The quantitative aspects of the data make this explanation unlikely but do not exclude it. (2) The AAF-modified tRNA molecules may exist in two different conformations, perhaps related to a hydrophobic interaction between AAF on G-20 with neighboring bases; one of these conformations may be completely inactive and the other normally active in methionine acceptor activity. (3) A third possibility is that all of the AAF-modified $tRNA^{fMet}$ has the same structure and conformation but that the 60% decrease in acceptor activity is due to steric hindrance by the bulky AAF residue preventing appropriate orientation of the tRNA during its binding to the aminoacyl-tRNA synthetase. In support of

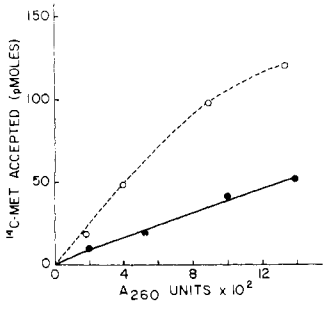

FIGURE 9: Methionine acceptance activity of $tRNA^{fMet}$ and $tRNA_{AAF}^{fMet}$. The $tRNA^{fMet}$ was obtained after reaction with *N*-acetoxy-AAF and isolation from the salt region of a BD-cellulose column and $tRNA_{AAF}^{fMet}$ was from the ethanol region of the same column. Incubation was for 10 min at 37°. Processing of samples as described in Methods. (●—●) $tRNA_{AAF}^{fMet}$; (○- -○) $tRNA^{fMet}$.

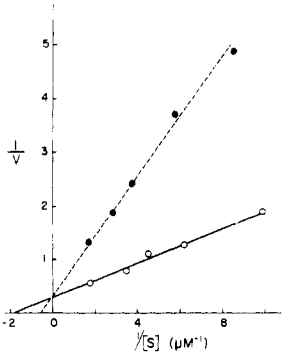

FIGURE 10: Lineweaver–Burk plots of aminoacylation of $tRNA^{fMet}$ and $tRNA_{AAF}^{fMet}$. Experimental conditions as described in legend to Figure 7 except that incubation was carried out for 1 min at 37°. The *S* value was calculated with an assumption that 1 A_{260} unit of tRNA was equal to 1.66 mμmoles of tRNA. The units of $1/v$ are arbitrary and equal for both tRNAs. (●- -●) $tRNA_{AAF}^{fMet}$; (○—○) $tRNA^{fMet}$.

this interpretation is the higher K_m value found with the modified $tRNA^{fMet}$. A fraction of the tRNA molecules may be bound to the enzyme in a form which prevents aminoacylation. Additional explanations also exist but the present data do not permit a precise interpretation for this finding. It is of interest that other investigators have also described partial inhibition of amino acid acceptor activity following chemical modifications with other agents and tRNAs (Litt, 1969, 1971).

Despite the above complexities in interpreting the decreased acceptance activity and increase in K_m observed with $tR\text{-}NA_{AAF}^{fMet}$ it is tempting to speculate that the G-20 region of the dihydrouridine loop is normally involved in the recognition between $tRNA^{fMet}$ and methionyl-tRNA synthetase. On the other hand, several approaches to the problem of tRNA-synthetase recognition suggest that the synthetase recognizes the tertiary structure of the $tRNA^{fMet}$ molecule rather than specific regions in the primary sequence (Seno *et al.*, 1970; Schulman, 1971; Siddiqui and Offengand, 1970, 1971).

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