Structural Studies on Transfer Ribonucleic Acid. I. Labeling of Exposed Guanine Sites in Yeast Phenylalanine Transfer Ribonucleic Acid with Kethoxal*

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ABSTRACT: Yeast phenylalanine transfer ribonucleic acid was labeled with tritiated kethoxal in the presence of Mg^{2+} at 37°.

After 30-min reaction time, only two sites were labeled to a significant extent. These were the guanines at positions 20 and 34 of the transfer ribonucleic acid chain. This labeling pattern is consistent with the "folded cloverleaf" model recently proposed by Cramer *et al.* (Cramer, F., Doepner, H., v. d. Haar, F., Schlimme, E., and Seidel, H. (1969), *Proc. Natl. Acad. Sci. U. S. 61*, 1384).

It is generally accepted that involvement of RNA nucleotides in secondary structure decreases their reactivity toward chemical reagents. In a few cases, this principle has been applied in attempts to study the conformation of purified tRNA molecules with known nucleotide sequences. Brostoff and Ingram (1967) showed that although the bases in the anticodon region of yeast $tRNA^{Ala}$ reacted with a water-soluble carbodiimide, those in the Tψ loop region did not react. Nelson *et al.* (1967) showed that certain regions of the $tRNA^{Ala}$ molecule were more susceptible to bromination than others. Cramer *et al.* (1969) have studied the selective N oxidation of adenines in yeast $tRNA^{Phe}$. They have proposed a model for tRNAs in which the dihydrouridine-rich loop, the Tψ loop, and the stem of the cloverleaf are folded together to form a compact structure from which the anticodon loop extends into space.

We have investigated the labeling of tRNA molecules with kethoxal (β-ethoxy-α-ketobutyraldehyde). This reagent reacts specifically with guanine (Staehelin, 1959). Kinetic data which we obtained on the reaction of kethoxal with free guanylic acid as well as with tRNA-bound guanylic acid suggested that involvement in secondary structure protects guanine sites against attack by the reagent (Litt and Hancock, 1967).

Our earlier work with kethoxal was hampered by the lability of the kethoxal–guanine adduct in basic solutions. Recently, Dr. R. Mitchel and Dr. G. M. Tener (personal communication, 1968) have found that the glyoxal–guanine adduct is stabilized by borate and that guanine sites which have reacted with glyoxal are completely resistant to attack by T_1 RNase. In this report, we present evidence that the kethoxal–guanine adduct behaves like the glyoxal–guanine adduct in these respects. We also describe the use of ^3H-labeled kethoxal to label yeast $tRNA^{Phe}$ to a limited extent and hence to identify exposed guanine sites in this molecule.

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Methods

RNA. Unfractionated bakers' yeast tRNA was obtained from Schwarz BioResearch. It was used as the source for preparing $tRNA^{Phe}$ according to the method of Litt (1968).

The purified tRNA accepted phenylalanine to the extent of at least 1800 pmoles/A_{260} unit. Synthetic polynucleotides were obtained from Miles Laboratories.

Kethoxal. Unlabeled kethoxal was a gift from Dr. G. E. Underwood, The Upjohn Co. ^3H-Labeled kethoxal, synthesized by the method of Tiffany *et al.* (1957), was kindly provided by Schwarz BioResearch. [2-^3H]Ethanol of specific activity 5 mCi/mmole was used as the labeled precursor for the kethoxal synthesis. The [^3H]kethoxal was analyzed by thin-layer chromatography (Litt and Hancock, 1967) and Friedemann titration (Friedemann, 1927). The radiochemical purity of the [^3H]kethoxal was estimated as 40%. Most of the radioactive impurities were volatile and could not be recovered after thin-layer chromatography. These impurities probably consisted of [^3H]ethanol which had not been completely removed in the work-up of the product. There was also a nonvolatile impurity which traveled with the solvent front on thin-layer chromatography and which accounted for about 15% of the total radioactivity applied to the origin. This impurity could be removed by extraction of the aqueous solution of kethoxal with benzene. However, as it was found that extracted and nonextracted kethoxal behaved identically when mixed with synthetic polynucleotides and with tRNA, nonextracted kethoxal was used in the experiments described in this paper.

Reaction of Kethoxal with tRNA and with Synthetic Polynucleotides. Reaction mixtures for labeling synthetic polynucleotides or tRNA contained (per milliliter final volume) 1 mg of polynucleotide, 180 μmoles of sodium cacodylate (pH 7.0), 5 μmoles of $MgCl_2$ or EDTA, and 14 μmoles of [^3H]kethoxal. Incubation was at 37°. For kinetic studies, the amount of tritium bound to acid-precipitable material after various times was determined by scintillation counting of trichloroacetic acid precipitates after filtration through a Millipore membrane filter (0.45 μ porosity) (Litt and Hancock, 1967). Kethoxal-labeled $tRNA^{Phe}$ used for structural studies was labeled for 30 min in the presence of Mg^{2+}. It was isolated by ethanol precipitation and gel filtration on Sephadex G-25.

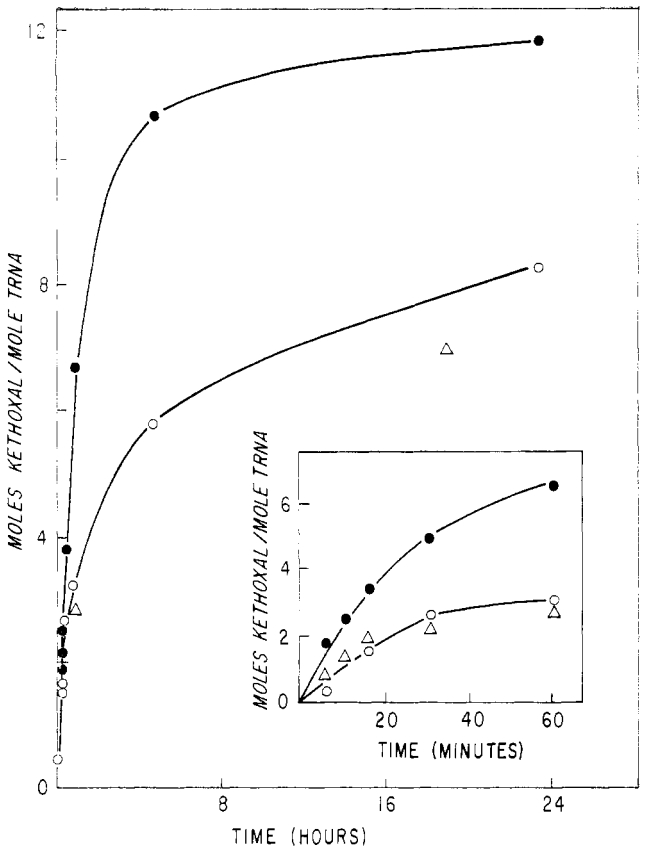

FIGURE 1: Kinetics of the reaction of [^{3}H]kethoxal with tRNA. Closed circles, unfractionated tRNA in the presence of EDTA; open circles, unfractionated tRNA in the presence of Mg^{2+}; triangles, $tRNA^{Phe}$ in the presence of Mg^{2+}. See text for details of reaction conditions. The inset shows the initial phase of the reaction.

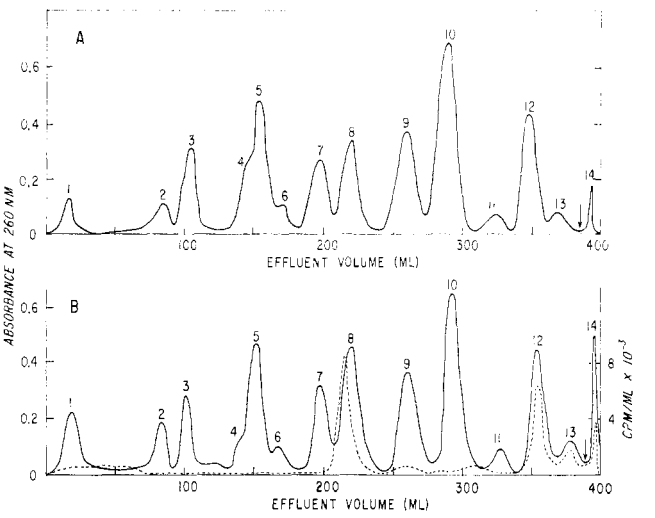

FIGURE 2: Elution profiles of T_1 RNase digests of untreated (A) and kethoxalated (B) $tRNA^{Phe}$. About 50 A_{260} units of the digests were chromatographed on 0.4 × 120 cm columns of DEAE-cellulose. The columns were eluted with linear gradients (total volume 380 ml) from 0 to 0.3 M NaCl in 0.02 M Tris-borate (pH 8.0)–7 M urea. After the gradient had finished (at the point shown by the arrow), the columns were stripped with 0.6 M NaCl in Tris-borate–urea. Fraction volumes were 1.4 ml (2A) or 0.9 ml (B). Flow rates were 7–9 ml/hr. Solid line, A_{260}; dashed line, radioactivity of ^{3}H from the [^{3}H]kethoxal used.

Enzymatic Digestions. T_1 RNase was obtained from Calbiochem. During the course of this work, it was found to contain significant phosphatase contamination. This could be eliminated by extraction of the T_1 RNase into phenol; the enzyme was recovered from the phenol phase by precipitation with acetone (Rushizsky *et al.*, 1963). Digestion mixtures contained (per milliliter final volume) 4 mg of tRNA, 0.25 ml of 0.2 M $Na_2B_4O_7$ adjusted to pH 8.0 with HCl, 800 units of T_1 RNase, and 8 μmoles of EDTA. Digestion was for 2 hr at 37°.

Escherichia coli alkaline phosphatase and pancreatic RNase were purchased from Worthington. Digestions of oligonucleotides with pancreatic RNase or T_1 RNase combined with alkaline phosphatase were performed according to Raj-Bhandary *et al.* (1968a,b).

Chromatography of T_1 RNase Digests on DEAE-cellulose in the Presence of Urea. Fractionation of T_1 RNase digests of $tRNA^{Phe}$ was performed according to RajBhandary *et al.* (1968a,b) with the exception that 0.02 M Tris-borate (pH 8.0) replaced 0.02 M Tris-chloride (pH 7.6) in the eluting solutions. Aliquots of column fractions were counted by liquid scintillation after dissolving them in a toluene–methoxyethanol-based scintillation fluid (Litt and Hancock, 1967). A Packard liquid-scintillation counter equipped with automatic external standardization was used.

Paper and Thin-Layer Chromatography. Paper chromatography was carried out on Whatman No. 1 paper. Thin-layer chromatography was carried out on plastic-backed cellulose layers with fluorescent indicator (MN Polygram Cel 300/UV_{254}, Brinkmann Instruments). The following solvent systems were used: solvent A, 2-propanol–concentrated ammonium hydroxide–water (7:1:2, v/v); solvent B, 2-propanol–water (7:3, v/v) with ammonia in the gas phase (Markham and Smith, 1952); solvent C, 40 g of ammonium sulfate dissolved in 100 ml of 0.1 M sodium phosphate (pH 7.0) (Rushizsky and Sober, 1962a). Ultraviolet spectral data on spots eluted from chromatograms were obtained with a Cary Model 15 spectrophotometer.

Amino Acid Acceptor Assays. These were performed essentially as previously described (Litt, 1968), except that the enzyme preparations and the incubation mixtures used were prepared according to Wimmer *et al.* (1968). Sufficient enzyme was used to produce maximal charging of $tRNA^{Phe}$ in 15 min at 25°.

Results

Reaction of [^{3}H]Kethoxal with Synthetic Polynucleotides, Mixed tRNA, and $tRNA^{Phe}$. After 20 hr at 37°, the tritiated kethoxal preparation did not react to a significant extent with poly A or poly U under conditions which gave rise to labeling of 50% of the guanines in unfractionated tRNA. Under identical conditions, poly C bound [^{3}H]kethoxal to the extent of about 0.02 mole/mole of cytosine. The significance of this slight degree of reaction is not clear; we assume that labeling of C does not contribute significantly to the tritium content of tRNA labeled for 30 min.

The time course of the labeling of mixed tRNA and

TABLE I: Analysis of Pancreatic RNase and Alkaline Phosphatase Digest of Peak 12 (Figure 2A) from Untreated $tRNA^{Phe}$.[a]

Spot	Product[a]	Mole % Found	Mole % Expected
1	5MeC	21	20
2	U	15	20
3	G	19	20
4	Ap2'OMeCpU	24	20
5	2'OMeGpApApYpApψ	20	20

[a] Spots 1, 2, and 3 were identified by their chromatographic properties in solvent A and by their ultraviolet spectra. Spot 4 had an ultraviolet spectrum at pH 1 which closely resembled the spectrum computed for an equimolar mixture of Ap, Cp, and U. Spot 5, which remained at the origin in solvent A and which fluoresced strongly under ultraviolet light, had an ultraviolet spectrum identical with that reported for the hexanucleotide 2'OMeGpApApYpψp (RajBhandary *et al.*, 1968a).

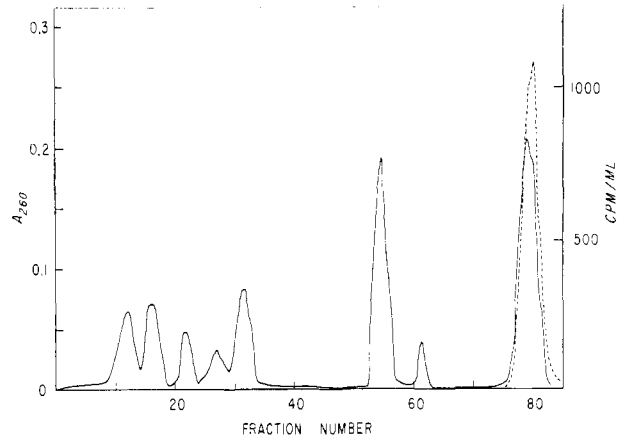

FIGURE 3: Elution profile of pancreatic RNase digest of peak 12 (Figure 2B); 3.5 A_{260} units of a pancreatic RNase digest of peak 12 was chromatographed on a 0.4 × 50 cm column of DEAE-cellulose. Elution was with a linear gradient from 0 to 0.3 M NaCl, 0.02 M Tris-borate (pH 8.0), and 7 M urea. The total volume of the gradient was 180 ml and the fraction size was 1.5 ml. Solid line, A_{260}; dashed line, counts per minute[^{3}H]kethoxal.

$tRNA^{Phe}$ with kethoxal is shown in Figure 1. These data were obtained at a higher pH and a lower kethoxal concentration than our previous kinetic data (Litt and Hancock, 1967). The new data confirm our previous observations that Mg^{2+} decreases the reactivity of tRNA guanines with kethoxal.

Stability of Kethoxal-tRNA in the Presence of Borate. A sample of mixed yeast tRNA was labeled to the extent of 12 moles of kethoxal/mole of tRNA by overnight incubation at 37° in the presence of EDTA. The tRNA was isolated by ethanol precipitation and was taken up in a small volume of water. An aliquot of this solution was adjusted to 0.02 M Tris-Cl (pH 8.0); another aliquot was adjusted to 0.02 M Tris-borate (pH 8.0). After 43 hr at 25°, the Tris-Cl^--containing sample retained 65% of its acid-precipitable radioactivity; the Tris-borate-containing sample retained 100% of its acid-precipitable radioactivity.

These observations are in agreement with the results of Mitchel and Tener (R. Mitchel and G. M. Tener, personal communication, 1968) on the glyoxal–guanine adduct. These authors have suggested that the stabilization of the adduct is probably due to formation of a complex between borate and the vicinal hydroxyl groups of the adduct.

Resistance of Kethoxalated Guanine Sites to T_1 RNase. It is possible to label all of the guanines in tRNA with kethoxal (Litt and Hancock, 1967). When a T_1 digest of such fully kethoxalated mixed tRNA was chromatographed on DEAE-cellulose in 7 M urea, nearly all of the ultraviolet-absorbing material emerged as a broad peak at salt concentrations greater than 0.25 M. Control digests of untreated tRNA gave a series of peaks; most of the ultraviolet-absorbing material was eluted from the column at salt concentrations lower than 0.25 M. This is in agreement with the results of Mitchel and Tener (personal communication, 1968) who found that extensive glyoxal treatment of tRNA restricts cleavage by T_1 RNase to sites adjacent to inosinic acid residues.

Specificity of Labeling of $tRNA^{Phe}$ by Kethoxal. $tRNA^{Phe}$ was labeled with [^{3}H]kethoxal for 30 min in the presence of Mg^{2+}. About 2 moles of kethoxal/mole of $tRNA^{Phe}$ was incorporated, as expected from the kinetic data of Figure 1. Elution profiles of T_1 RNase digests of this material and of untreated $tRNA^{Phe}$ are shown in Figure 2. Two major peaks and two minor peaks of radioactivity are found; these account for about 80% of the radioactivity placed on the column. The remaining 20% of the radioactivity is distributed broadly over the entire elution profile. Essentially similar results were found in four different experiments.

Identification of the Sites of Attack of Kethoxal on $tRNA^{Phe}$. PEAK 12. The oligonucleotide present in peak 12 from T_1 RNase digests of untreated $tRNA^{Phe}$ was hydrolyzed with pancreatic RNase and alkaline phosphatase; the products were separated by paper chromatography in solvent A. The analysis,shown in Table I, indicates that peak 12 is Ap2'OMeCpUp2'OMeGpApApYpApψ p5MeCpUpGp (corresponding to peak 17 of RajBhandary *et al.*, 1968a,b).

In three different experiments with $tRNA^{Phe}$ kethoxalated for 30 min in the presence of Mg^{2+}, the degree of labeling of the dedecanucleotide in peak 12 varied between 0.6 and 0.8 mole of kethoxal per mole of oligonucleotide. When a portion of the kethoxalated dodecanucleotide was digested with pancreatic RNase and chromatographed on DEAE-cellulose, all of the radioactivity was associated with a single peak (Figure 3). This peak, which had twice the specific radioactivity of the original dodecanucleotide, fluoresced strongly with an excitation maximum at 320 nm and an emission at 440 nm indicating that it contained the base "Y" (Yoshikami *et al.*, 1968). Its absorption spectrum at neutral pH had λ_{max} 248 nm; $\epsilon_{250}/\epsilon_{260} = 1.1$; $\epsilon_{280}/\epsilon_{260} = 0.50$. This spectrum closely resembles that published for the hexanucleotide 2'OMeGpApApYpApψp (RajBhandary *et al.*, 1968a).

These results suggest that one of the major sites of kethoxalation is at the 2'OMeGp residue which forms the 5'-terminal end of the anticodon. This site is, of course, normally resistant to T_1 RNase because the 2'-hydroxyl group is methylated. Hence, kethoxation at this site does not affect the size of the

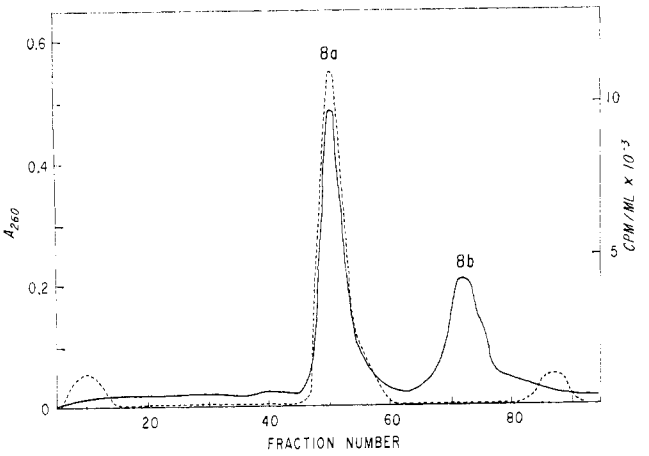

FIGURE 4: Rechromatography of peak 8 (Figure 2B) at pH 4.0; 4.9 A_{260} units of the radioactive portion of peak 8 was chromatographed on a 0.4 × 120 cm column of DEAE-cellulose. The column was eluted with a linear gradient from 0 to 0.2 M NaCl, 0.02 M ammonium formate, and 7 M urea (pH 4.0). The total volume of the gradient was 180 ml and the fraction size was 0.95 ml. Solid line, A_{260}; dashed line, counts per minute [^{3}H]kethoxal.

oligonucleotide produced by T_1 RNase digestion. Although we have no reason to believe that kethoxal reacts with the Y base in $tRNA^{Phe}$, our results do not rule out this possibility.

PEAK 13. Our ultraviolet absorbance peak 13 is probably identical with peak 18 of RajBhandary *et al.* (1968b). These authors suggest that this peak differs from the one immediately preceding it only in the nature of the base Y. Our observation that the specific radioactivity of peak 13 is approximately the same as that of peak 12 is in agreement with this.

PEAK 8. The first major radioactive peak in the elution profile of Figure 2B was more difficult to identify. This peak, which eluted with the front portion of the ultraviolet-absorbing peak 8, was desalted on Bio-Gel P-2 (Uziel and Cohn, 1965) and was rechromatographed on DEAE-cellulose in 7 M urea at pH 4.0 (Rushizky *et al.*, 1964) (Figure 4). Peak 8a from this pH 4.0 column was desalted by absorption to and elution from a small DEAE-cellulose column at pH 8.6 (Rushizsky and Sober, 1962b), a process which removed all of the kethoxal. After lyophilization to remove ammonium bicarbonate, peak 8a was digested with T_1 RNase and alkaline phosphatase. The digest was chromatographed on thin-layer cellulose in solvent B. The products were identified as guanosine, ApG, and a band which remained at the origin and whose ultraviolet spectrum at pH 7 was identical with that of CpCpApGp. (We had previously shown CpCpApGp to be a normal constituent of our peak 8 from digests of $tRNA^{Phe}$.) The molar ratio of guanosine to ApG was 0.75.

These results suggest that the radioactivity found in the leading part of peak 8 was associated with the trinucleotide G*pApGp.[1] This oligonucleotide would have survived the first T_1 RNase digestion because of the kethoxal bound to the 5′-terminal guanine. Furthermore, it might be expected to chromatograph like a tetranucleotide because borate complexing by the kethoxal–guanine adduct would give it an extra negative charge. After removal of kethoxal, it would become susceptible to T_1 RNase attack to yield the observed products.

The assignment of the structure G*pApGp to the radioactive oligonucleotide in peak 8 of Figure 2B is supported by the changes produced by kethoxalation in the relative abundance of certain peaks in the elution profiles of T_1 RNase digests. These data, which are presented in Table II, show the ratios of the areas under certain peaks to the area under peak 7. Peak 7, which comprised a relatively constant proportion of the total A_{260} units recovered in all our experiments, consisted of pGp and CpApCpCpA in equimolar amounts (M. Litt, unpublished experiments, 1968). The data in Table II show that kethoxalation caused a consistent decrease in peak 3 (Gp) and peaks 4 and 5 (CpGp, ApGp, and UpGp) with a concomitant increase in peak 8 (normally containing CpCpApGp and TpψpCpGp). Furthermore, thin-layer chromatography of peaks 4 and 5 in solvent C showed that the dinucleotides in the kethoxalated digest had a decreased proportion of ApGp as compared with the control (Table III).

Our evidence is also compatible with the assignment of the structure ApG*pGp to the radioactive trinucleotide in peak 8 of Figure 2B. However, this structure is ruled out because the sequence ApGpGp, from which this labeled trinucleotide would have arisen, does not occur in $tRNA^{Phe}$ (RajBhandary and Chang, 1968).

TABLE II: Relative Areas under Peaks in Elution Profiles of T_1 RNase Digests of Untreated and Kethoxalated $tRNA^{Phe}$.

Peak	Area Relative to Peak 7	
	Untreated	Kethoxalated
3	1.17, 0.95, 0.97,[a] 0.97[a]	0.77, 0.73, 0.73[a]
4 + 5[b]	2.38, 2.42, 2.24,[a] 2.35[a]	1.68, 1.90, 1.81[a]
8	1.31, 1.31, 1.41,[a] 1.48[a]	1.48, 1.66, 2.04[a]

[a] These peaks were derived from digests made with T_1 RNase which had been extracted into phenol to remove phosphatase (see text). [b] Peaks 4 and 5 were frequently poorly resolved; they were pooled together before further analysis.

TABLE III: Analysis of Peaks 4 and 5 from Untreated and Kethoxalated $tRNA^{Phe}$.

Sample	Molar Ratios ApGp:UpGp:CpGp
Untreated	2.6:1.0:1.0[a]
Kethoxalated	2.1:1.0:1.0[a,b]

[a] In solvent C, CpGp and UpGp chromatographed as a single band, well ahead of ApGp. The ultraviolet spectrum at acid and neutral pH of this fast-moving band agreed closely with the spectrum calculated for an equimolar mixture of UpGp and CpGp, using the data of Aoyagi and Inoue (1968). [b] This analysis was confirmed by analysis of an alkaline hydrolysate of a portion of peaks 4 and 5.

[1] Abbreviation used is: G*p, the kethoxal adduct of guanylic acid.

PEAK 14. This peak was obtained when the column was stripped with 0.6 M NaCl (see legend to Figure 2). In the elution profile of a T_1 digest of kethoxalated $tRNA^{Phe}$ (Figure 2B), peak 14 contained 5% of the radioactivity and 2.7% of the A_{260} units applied to the column. In a separate experiment, a T_1 RNase digest of kethoxalated $tRNA^{Phe}$ was chromatographed on DEAE-cellulose under conditions similar to those shown in the legend of Figure 2B, except that the NaCl concentration in the limit buffer was increased to 0.6 M and the total volume of the gradient was increased to 630 ml. Under these conditions, no distinct peaks of absorbance or radioactivity were observed to elute after peak 13.

Discussion

Our results can be discussed in terms of the known nucleotide sequence for $tRNA^{Phe}$. This sequence, arranged in a cloverleaf type of secondary structure, is shown in Figure 5 (reproduced from RajBhandary and Chang, 1968). In the presence of Mg^{2+} at 37°, initial attack on $tRNA^{Phe}$ by kethoxal occurs primarily at two specific sites. One of these sites is the guanine at position 34. Attack at the other site results in the appearance of a kethoxalated trinucleotide G*pApGp and in a decrease in the amount of Gp and ApGp in T_1 RNase digests. These results can be explained only if this site is the guanine at position 20 of the chain.

In the cloverleaf model, seven guanines are located in loops. We find that only two of these are readily attacked by kethoxal. We propose that the guanines located at positions 15, 18, 19, 45, and 57 interact with other bases and help to maintain the tertiary structure of $tRNA^{Phe}$.

In the folded cloverleaf model recently proposed by Cramer *et al.* (1969) the only two guanines which are not base paired are those at positions 20 and 34. Hence, our results provide strong support for this model.

In preliminary experiments, we have found that $tRNA^{Phe}$, labeled with kethoxal in the presence of Mg^{2+} for 30 min, loses about 50% of its phenylalanine acceptor activity. We are presently attempting to separate active and inactive molecules of $tRNA^{Phe}$ after partial kethoxalation by chromatography on benzoylated DEAE-cellulose. If successful, this will allow us to characterize the structural changes which have occurred in both active and inactive species and hence to provide clues about the nature of the enzyme recognition site.

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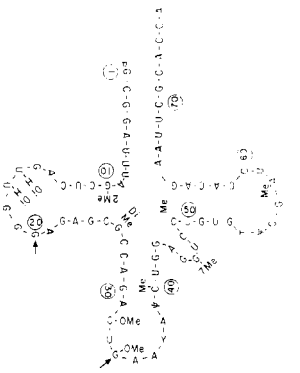

FIGURE 5: Cloverleaf model for yeast $tRNA^{Phe}$ (RajBhandary and Chang, 1968). Arrows indicate major sites of reaction with kethoxal.

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