$$C_j = K_l C_1{}^j = \frac{1}{D}\begin{vmatrix} 1 & C & 1 & 1 \\ 1 & \Phi_{(w)} & m & n \\ 1 & \Phi_{(N)} & \frac{1}{m} & \frac{1}{n} \\ \eta_1 & \tau & \eta_m & \eta_n \end{vmatrix}$$

$$C_m = K_m C_1{}^m = \frac{1}{D}\begin{vmatrix} 1 & 1 & C & 1 \\ 1 & l & \Phi_{(w)} & n \\ 1 & \frac{1}{l} & \Phi_{(N)} & \frac{1}{n} \\ \eta_1 & \eta_l & \tau & \eta_n \end{vmatrix}$$

$$C_n = K_n C_1{}^n = C - C_1 - C_l - C_m$$

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Phenylalanine Transfer Ribonucleic Acid from *Escherichia coli* B. Isolation and Characterization of Oligonucleotides from Ribonuclease T_1 and Ribonuclease A Hydrolysates*

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ABSTRACT: Phenylalanine transfer ribonucleic acid of *Escherichia coli* B, exhaustively hydrolyzed by either ribonuclease T_1 or ribonuclease A, yields a total of 34 oligonucleotides. These have been isolated and characterized, and account for all the bases present in the molecule, including 4-thiouracil, which is apparently in an unstable state. The oligonucleotide from each enzyme hydrolysate that contains the single 7-methylguanosine residue is routinely isolated in only 60–70% yield.

E. coli B phenylalanine transfer ribonucleic acid has an unique anticodon region, A-ψ-U-G-G-A-A-A-ψ, that includes three possible anticodons in an overlapping configuration. The unexpected anticodon triplet GGA is in the most favored position stereochemically. The full potential of the remaining overlaps may be realized if the protein-synthesizing complex undergoes a conformational shift so as to utilize AAA for the UUU codon and GAA for the UUC codon. On the other hand, as in yeast phenylalanine transfer ribonucleic acid, only the GAA sequence is needed to fulfill the anticodon requirements.

The large number of known tRNA sequences has permitted a number of structural comparisons (Holley *et al.*, 1965; Goodman *et al.*, 1968; Dube *et al.*, 1968; Bayev *et al.*, 1967; RajBhandary and Chang, 1968; Zachau *et al.*, 1966; Madison and Kung, 1967; Takemura *et al.*, 1968; Madison, 1968). By judicious choice of deleted regions it is possible to align all the known tRNA sequences so that there are a large number of sequence positions where the same base occurs (Jukes, 1966; Madison, 1968). Attempts to correlate these structural features unequivocally with the variety of biological roles attributed to tRNA have been hampered by the absence of independent measures of function. The location of the amino acid acceptor site has been readily proven because of the relative ease of isolation of covalently linked aminoacyl ester. However, the enzyme recognition site and the topological interactions of the tRNA with ribosomes and mRNA are

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still not well understood, because we are as yet unable to identify the linkages that characterize these complexes.

The first system to yield RNA-dependent incorporation of amino acid into a polypeptide *in vitro* (Nirenberg and Matthaei, 1961) utilized *Escherichia coli* B $tRNA^{Phe}$. Because of the general interest in the biological properties of this tRNA, we began the study of its chemistry to contribute to the understanding of the relation of structure to function (Uziel and Gassen, 1968). We were aided by the availability of purified preparations (Kelmers, 1966).

This report discusses the isolation and characterization of the oligonucleotides obtained by exhaustive enzymatic hydrolysis with either pancreatic RNase or Takadiastase RNase T_1, and presents a partial sequence of $tRNA^{Phe}$, including the anticodon region. This sequence has the unexpected property of having three potential anticodons all overlapping and within the anticodon loop.

Materials

Only two of the $tRNA^{Phe}$ preparations available to us (13-105 and 13-104) are discussed in detail in this paper, although data from the others are used to augment our results.[1]

All ion-exchange materials were purchased from various commercial sources. Sephadex A-50, G-25, SE-C-50, and A-25 were obtained from Pharmacia Corp.; DE-32 was purchased from Reeve Angel; and P-2 is a product of Bio-Rad Laboratories. RNase T_1 was purchased from Sankyo Corp., Tokyo, or Cal-Biochem. Alkaline phosphatase and venom diesterase were obtained from Worthington Biochemical Corp. (BAP-C and SVD, respectively). Unless specified otherwise, all reactions were performed in 0.4-ml capacity polyethylene tubes with caps. The glassware and end pieces used for column chromatography were either purchased from Fisher Porter Co. or constructed in our work shops. All nucleoside analyses were carried out according to Uziel *et al.* (1968).

Methods

tRNA was stored frozen in the original chromatographic effluent (Kelmers, 1966) and was recovered by alcohol precipitation and Millipore filtration or by gel filtration and flash evaporation. The samples were taken to dryness when necessary under a stream of N_2 (O_2 free) at 48°. Final drying was done in an evacuated desiccator over paraffin strips or NaOH pellets.

Oligonucleotides were desalted by standardized procedures (Table I). The final traces of NH_4HCO_3 from the anion-exchange concentration step may be removed by evaporation from 50% ethanol. Stock solutions of the oligonucleotide were stored frozen (−40°) in H_2O. Ammonium acetate was removed by lyophilization. The last traces of ammonium acetate were removed in the final concentration step by rotary evaporation *in vacuo*. The removal of water to concentrate oligonucleotide samples for analysis was done with a stream of N_2 gas (O_2 free) blown over the surface of the sample set in a 48° water bath.

The large number of routine analyses described in the following paragraphs are, for convenience, listed alphabetically and are referred to by their respective letters throughout the text.

A. Nucleoside formation by alkaline hydrolysis followed by alkaline phosphatase treatment was performed by either of the following procedures.

A-1. The salt-free oligonucleotide sample (*ca.* 1 A_{260} unit) was concentrated to 20 μl, and 10 μl of 1 N NaOH was added. Hydrolysis was completed after 40 min at 80° (Uziel *et al.*, 1968). Under these conditions, even $(Ap)_{>50}$[2] was completely hydrolyzed. There were occasional losses of the amino group of cytidine of up to 20%, probably because of spurious metal ion catalysis.

A-2. Alternatively, the alkaline hydrolysis was done at 37° for 18 hr.

The sample (A-1 or A-2) was partially neutralized with 9 μl of 1 M acetic acid; 15 μl of 0.2 M ammonium acetate (pH 8.8) and 5 μl of alkaline phosphatase (Worthington BAP-C) were added. This enzyme solution was capable of an initial rate of hydrolysis of 4 μmoles of *p*-nitrophenyl phosphate/min per ml in 1 M Tris-Cl^- (pH 8, 23°). The oligonucleotide solution was incubated for 3 hr at 37°, and the total sample was analyzed by cation-exchange chromatography (*K*) according to Uziel *et al.* (1968).

B. Hydrolysis to nucleosides by combined alkaline phosphatase and venom diesterase treatment is a chemically milder technique than procedure *A*. Mg^{2+} is necessary to obtain quantitative hydrolysis because of the relatively high concentration of P_i formed during phosphatase action (*ca.* 10^{-3}–10^{-4} M). The salt-free sample, in about 30 μl of H_2O (*ca.* 1 A_{260} unit), was diluted with 20 μl of 0.2 M NH_4OAc (pH 8.8) and 5 μl of 0.3 M $Mg(OAc)_2$. To this was added 10 μl of a stock enzyme mixture capable of an initial rate of hydrolysis of the synthetic nitrophenyl phosphate ester substrates of 0.75 (diesterase) and 5 μmoles/min per ml (phosphatase) at 23°. The oligonucleotide solution was incubated at 45° for 4 hr (Uziel *et al.*, 1968).

C. The release of pyrimidine nucleosides from sequences -N-Y-Y'p and identification of -N-Yp was achieved by sequential hydrolysis of oligonucleotides with pancreatic RNase A and alkaline phosphatase. The sample oligonucleotide from an RNase T_1 hydrolysate (*ca.* 1 A_{260} unit) was dried in the reaction tube and dissolved in 20 μl of 0.3 M NH_4OAc (pH 7.2) containing 0.2 mg of RNase A/ml. After incubation at 37° for 3 hr, the sample was adjusted to between pH 8.5 and 9.0 with 12 μl of 0.1 N NaOH. The sample was then treated with alkaline phosphatase as described in *O*.

[1] All preparations were prepared at the Oak Ridge National Laboratory under a program supported by the U. S. Atomic Energy Commission and the National Institute of General Medical Sciences. Preparation 13-104 is the same as the product distributed by the NIGMS (Dury, 1967).

[2] Abbreviations are S (4-thiouridine) and H (5,6-dihydrouridine). Sp and Hp refer to the 3′ nucleotides. In $(Ap)_{>50}$, the subscripts refer to chain length.

TABLE I: Desalting Procedures.

Compound	Medium	Sample: Salt Concn (M)[b]	Sample: pH	Sample: Maximum Size[a] (Bed Volumes or ml of A-25)	Sample: Maximum Flow Rate (ml/min per cm^2)
Gp or pG	P-2[c]		8 ± 0.5	10%	4
Cp, Ap, Up	P-2		8 ± 0.5	20%	4
Dinucleotides	P-2[d]		8 ± 0.5	30%	4
	A-25[e]	<0.08	9 ± 0.1	200 units[f]/ml	6
Trinucleotides	P-2		8 ± 0.5	30%	4
	A-25	<0.13	9 ± 0.1	400 units[f]/ml	6
Tetranucleotides	A-25	<0.2	9 ± 0.1	100 units[f]/ml	6
	A-25	<0.1	9 ± 0.1	400 units[f]/ml	6
	G-25[g]		8	25%	10
	P-2[d]		8 ± 0.5	30%	4
Pentanucleotides	G-25[d]		8	30%	10
	A-25	<0.25	9 ± 0.1	270 units[f]/ml	6
	P-2		9 ± 0.1	30%	4
Hexanucleotides and larger	G-25[d]		8	30%	10
	P-2			30%	4
	A-25	<0.29	9 ± 0.1	250 units[f]/ml	6

[a] The limitations on sample size for gel filtration depend upon the difference between the K_D values for the nucleotide and the contaminating compounds to be removed (Uziel, 1967). For ion-exchange desalting, the volume of sample is generally less important. Three variables control this desalting process. (1) The salt concentration of the sample must be low enough to permit adsorption, but the total amount of salt must also not be enought to elute the oligonucleotide before the sample addition is complete. (2) The size of the oligonucleotide determines the permissible levels of salt. If the amount of salt exceeds the capacity of the ion-exchange column, the amount of exchanger must be increased to permit adsorption of the nucleotides. (3) The ion-exchange capacity is determined by the net charge of the oligonucleotide and the availability of exchange sites. Oligonucleotides larger than tetranucleotides are excluded from the A-25 matrix; thus the capacity is reduced. Comparing 2 and 3, the size factor is generally the stronger effect. [b] The capacity to bind oligonucleotides can be increased further by lowering the salt concentration. [c] The P-2 was 100–200 mesh, and distilled water was the eluent (Uziel, 1967). [d] The preferred method when alternatives are listed. [e] With A-25, after the sample was added, the column was washed with two to three bed volumes of H_2O, then with about five bed volumes of 0.05 M $(NH_4)_2CO_3$ (until no more Cl^- is eluted), and finally with H_2O to remove the $(NH_4)_2CO_3$. The oligonucleotide was eluted with 1 M NH_4HCO_3. At least four bed volumes are needed at a flow rate of 0.5 ml/min per cm^2. [f] Units = A_{260} units. The units refer to the absorbance at neutral pH of the sample at 260 nm if it is dissolved in a total volume of 1 ml and measured with a light-path length of 1 cm. [g] The eluent for G-25 was distilled water.

D. The release of free guanosine from sequences containing adjacent guanylate residues (-N-G-G) was achieved by sequential hydrolysis of oligonucleotides with RNase T_1 and alkaline phosphatase or by RNase T_1 only; 5–20 nmoles of oligonucleotide was dissolved in 10 μl of H_2O. Ribonuclease T_1 (5 μl) (1 mg/ml) and 5 μl of 0.2 N NH_4OAc were added, and the mixture was incubated at 37° for 6 hr. After the addition of 5 μl of alkaline phosphatase (1 mg/ml) and 5 μl of 1 M NH_4OAc (pH 8.8), it was incubated an additional 3 hr at 37°. The sample was analyzed by either of two ways.

D-1. With the sequential hydrolyses, the sample was applied directly to the analytical column (*K*).

D-2. When only RNase T_1 was used, the nucleotides were separated on thin-layer electrophoresis (*L*) (Gassen, 1968).

E. Terminal nucleoside content was assayed on 4 nmoles of oligonucleotide by treatment as in *A*-1 followed by cation-exchange analysis (*K*) (Uziel *et al.*, 1968).

F. Terminal (5′-hydroxyl) bases were determined as nucleosides after venom diesterase treatment. The sample (*ca.* 1 A_{260} unit) was dissolved in 30 μl of H_2O and diluted with 20 μl of 0.2 M NH_4OAc (pH 6.5). Diesterase (5 μl) (initial rate 1.5 μmoles/min per ml of synthetic substrate at 23°) was added and the solution was incubated for 15 min at 37°. Under these conditions, 3 nmoles of A-U-Gp were completely hydrolyzed to nucleotides plus terminal nucleoside.

G. The identity of the 3′ end of the oligonucleotide isolated from the enzyme hydrolysates was established by enzymatic removal of the terminal phosphate (see *O*) treatment with alkali (see *E*) and nucleoside assay (*K*).

J. All spectra were recorded on a Cary Model 14 with either the normal slide wire or a 0–0.1 slide wire.

K. Nucleoside analysis was performed according to Uziel *et al.* (1968).

L. Thin-layer electrophoresis was carried out according to Gassen (1968).

M. Terminal phosphate obtained by alkaline phos-

TABLE II: Chromatography Gradients.[a]

Gradient No.	Column Dimensions[b] (cm)	Flow (ml/min)	Initial Solvent	Vol. (ml)	Limiting Solvent	Vol. (ml)
7	140 × 0.6	0.66	0.05 M NH_4OAc, pH 8.3	750	0.75 M NH_4OAc, pH 8.3	750
7A	120 × 0.5	0.20	0.05 M NH_4OAc, pH 8.3	380	0.75 M NH_4OAc, pH 8.3	380
7B	120 × 0.5	0.20	0.05 M NH_4OAc, pH 8.3	500	0.75 M NH_4OAc, pH 8.3	500
11	140 × 0.6	0.66	0.10 M NH_4 formate, pH 3.7	750	0.75 M NH_4 formate, pH 3.7	750
11B	120 × 0.5	0.20	0.10 M NH_4 formate, pH 3.7	380	0.75 M NH_4 formate, pH 3.7	380
1	140 × 0.6	0.30	0.02 M Tris Cl^-, 7 M urea, pH 7.8, 0.02 M NH_4OAc	500	0.02 M Tris Cl^-, 7 M urea, pH 7.8, 0.5 M NH_4OAc	500
2	100 × 0.5	0.20	0.02 M Tris Cl^-, 7 M urea, pH 7.8, 0.02 M NaCl	250	0.02 M Tris Cl^-, 7 M urea, pH 7.8, 0.3 M NaCl	250
3	50 × 0.5	0.20	7 M urea adjusted to pH 3.3 with HCl, 0.02 M NaCl	150	7 M urea adjusted to pH 3.3 with HCl, 0.1 M NaCl	150
4	50 × 0.5	0.20	7 M urea adjusted to pH 3.3 with HCl, 0.02 M NaCl	180	7 M urea adjusted to pH 3.3 with HCl, 0.15 M NaCl	180
5	50 × 0.5	0.20	7 M urea adjusted to pH 3.3 with HCl, 0.02 M NaCl	180	7 M urea adjusted to pH 3.3 with HCl, 0.3 M NaCl	180
6	50 × 0.5	0.20	7 M urea adjusted to pH 9.6, 0.05 M NH_4OAc	180	7 M urea adjusted to pH 9.6, 0.5 M NH_4OAc	180

[a] All experiments were performed on Whatman DE-32. [b] The liquid volume above the exchange bed was generally kept at a minimum. The column dimensions are length by inside diameter.

phatase treatment (*O*) was measured after removal of the RNA and protein by filtration through a bed of Dowex 50 (0.2 ml) and charcoal (0.2 ml) at pH 3.

N. Table II lists and describes the various chromatographic systems used for separation and rechromatography of the oligonucleotides.

O. The conditions for phosphate removal with alkaline phosphatase were usually as follows. The sample was dissolved in 30 μl of water (*ca.* 1 A_{260} unit), and the hydrolysis conditions were adjusted with 15 μl of 0.2 M NH_4OAc (pH 8.8) and 5 μl of alkaline phosphatase (1 mg/ml with a specific activity of 2 μmoles/min per mg per ml at pH 8.0 in 1 M Tris at 23°). The solution was incubated at 37° for 3 hr to ensure complete hydrolysis.

P. Periodate oxidation was performed with a modification of published procedures (Neu and Heppel, 1964; Khym and Uziel, 1968). The released base was separated from the remaining oligonucleotides by gel filtration through P-2 (Uziel and Cohn, 1965). The base region was pooled, concentrated by flash evaporation, and analyzed according to Uziel *et al.* (1968). The oligonucleotide was then concentrated to about 0.1 ml and treated with phosphatase (as in *O*). To remove the phosphatase, the solution was diluted to 1 ml and adjusted to pH 4.2 with acetic acid and passed through a 0.2-ml bed of SE-C-50 equilibrated with 0.001 M NH_4OAc (pH 4.2). The phosphatase is absorbed. The bed was washed with this solvent to recover all the oligonucleotide and leave the phosphatase on the column.

R. The following procedures were used to measure dihydrouridine.

R-1. A colorimetric assay based on that of Coulombe and Favreau (1963) for ureido compounds gave a molar extinction coefficient of 3800 at 535 nm. This value is sometimes variable so standards were always run simultaneously for comparison. The sample containing 20 nmoles was made 0.1 N in NaOH in a volume of 0.2 ml. After 1 hr at room temperature, 0.8 ml of the mixed reagents (II) were added. The reaction mixture contained the following reagents: (I) diacetylmonoxime (3 mg/ml) and thiosemicarbazide (0.15 mg/ml in H_2O); (II) 2 ml of reagent I plus 10 ml of 60% phosphoric acid were mixed and used immediately. The tube was closed, mixed vigorously, and heated at 95° for exactly 20 min, then cooled in tap water. The solution was transferred to a 1-ml cuvet for absorbancy measurements.

R-2. Alternatively, the procedure described in Batt *et al.* (1954) was used.

Results

RNase T_1 Oligonucleotides. The nucleotide fragments were initially separated on gradient 7, Table II. Table III and Figure 1, the results of a typical experiment, list and describe the oligonucleotides isolated in significant yield from RNase T_1 treatment of *E. coli* $tRNA^{Phe}$ (13-104). The molar quantity of tRNA is based upon the terminal adenosine content (procedure *E*) which is 1.32 nmoles/A_{260} unit measured in H_2O. Only 40 nmoles of the total 60 nmoles of tRNA used in this experiment accepted phenylalanine and this is the basis for our claim of 65% $tRNA^{Phe}$. A number of contaminating oligonucleotides were found in very low quantity (5–35%). These were not considered possible components of $tRNA^{Phe}$ without further evidence and argument.

The evidence, presented below, supports the oligo-

nucleotide sequences described in Figure 1 and Table III. The numbers below refer to the various chromatographic peaks. The analysis of peak 15 is used as an illustration of the result obtained by our standardized techniques (Figures 2 and 3).

PEAK 1. Free guanylic acid was recovered at a level of 10–12 residues/tRNA chain from all the preparations studied. It was identified by its spectrum, chromatographic position on DE-32 (gradient 7),[3] and formation of guanosine after phosphatase treatment. A value of 11 residues per mole tRNA was obtained from an 85% pure $tRNA^{Phe}$.

PEAK 2. The dinucleotide and trinucleotide positions on gradient 7 were established with known substances prepared in this laboratory. C-Gp is present at a level of 1 mole/tRNA chain present, and was identified by thin layer electrophoresis, spectrum, total composition, chromatographic position (DE-32), and nucleosides formed in analysis *C*.

PEAK 3. U-Gp is found in variable amounts depending upon the preparation (from 0.2 to 0.7 residue per mole of tRNA). Approximately 22 nmoles of U-Gp were recovered from the original 60 nmoles of tRNA (13-104) used for this experiment. The structure was proven by the same criteria as in 2.

PEAK 4. pGp was identified by spectrum, composition, electrophoresis at pH 3.5, and by nucleoside analysis after phosphatase treatment (*O* and *K*). It is present at a level of one per chain.

PEAK 5. H-C-Gp was identified by nucleoside analysis, spectrum, chromatographic position (gradient 7), and lack of an ultraviolet-absorbing nucleoside after venom diesterase treatment. The presence of dihydrouridine was confirmed by the first-order loss of absorbancy at 235 nm in 0.1 N NaOH. Twenty-four nmoles (*R*-2) were found in the material isolated from the H-C-G position after electrophoresis at pH 3.5. This was the only band present except for an expected weak band at the A-Gp position because of contamination by the neighboring A-Gp peak.

PEAK 6. Electrophoresis at pH 3.5 showed one major component, A-Gp, and two minor components, C-C-Gp and H-C-Gp. A-Gp was identified by electrophoresis, chromatographic position, composition, and spectrum. The presence of adenine in an oligonucleotide causes a retardation of the peak beyond the dinucleotide region. Under these chromatographic conditions the effect is equivalent to one additional charge to the oligonucleotide, which explains its position in the midst of the Y_2G region (M. Uziel, unpublished observations). Correcting for the C_2G and H-C-Gp content, there are at least two residues of A-Gp present per mole of $tRNA^{Phe}$. This has been confirmed in the 85% purified preparation.

PEAK 7. This chromatographic peak was divided asymmetrically to reduce the contamination by the overlapping peak 8. The major component is H-A-Gp. This was split between peaks 7 and 8 so that 34 nmoles was in peak 7 and 8 nmoles was recovered from peak 8. Separation of the oligonucleotide was accomplished by electrophoresis at pH 3.5. Dihydrouridylate was identified and quantitated colorimetrically (*R*-1) and spectrophotometrically (*R*-2). At least 42 nmoles of H-A-Gp was present. The sequence location of the dihydrouridine was determined by pancreatic RNase A and phosphatase treatment. No guanosine was released. A small amount of U-U-G was present but was not considered a component of $tRNA^{Phe}$.

PEAK 8. This peak gave 3 bands on electrophoresis. The weak band was a mixture of H-A-Gp and U-A-Gp; both were present at low levels. The two main bands were C-A-Gp and C-A-C-C-A. The sequence was established for C-A-Gp by system *C*. Only cytidine was found as the free nucleoside, fixing its position as terminal; 51 nmoles of C-A-Gp were found.

There was 43 nmoles of C-A-C-C-A. The absence of guanosine suggests that this sequence came from the terminal position. Alkaline hydrolysis yielded free adenosine. Assay *C* released two cytidines and one adenosine, which eliminates the possibility of an ApA linkage. Venom diesterase (*F*) yielded a terminal cytidine. The final placement of bases as C-A-C-C-A was based on sequential degradation with periodate, which will be discussed elsewhere (M. Uziel, in preparation).

PEAK 9. This chromatographic peak contains three tetranucleotides, C-C-C-Gp, U-C-C-Gp, and T-ψ-C-Gp. They were separated by rechromatography on gradient 11A (Figure 4A). Composition established the first as C_3G. Treatment with venom diesterase confirmed cytidine as the terminal. Similarly, composition, relative chromatographic position, and venom diesterase (*F*) confirmed the sequence of the second as U-C-C-Gp. T-ψ-C-Gp was established by composition (*B* and *K*), venom diesterase (*F*), and one periodate cycle (*P*) followed by phosphatase and alkali treatment (*G*).

PEAK 9a. This small peak was a mixture of oligonucleotides with low guanosine content. It was not studied further.

PEAK 10. Peaks 10 and 10a contained the tetranucleotide A-ψ-U-G plus several contaminating oligonucleotides; 40 nmoles of A-ψ-U-Gp were recovered. Assay *C* released uridine and guanosine, and venom diesterase (*F*) released the terminal adenosine. The dinucleotide monophosphate recovered after treatment *C* contained only adenosine and pseudouridine, proving the sequence A-ψ-U-Gp.

PEAK 10a. This is a mixture of oligonucleotides, none of which were present in sufficient quantity to have been derived from $tRNA^{Phe}$.

PEAK 11. This pentanucleotide has been recovered from other experiments in yields corresponding to 95% of theory for 1 mole/$tRNA^{Phe}$. RNase and phosphatase treatment (*C*) gave two cytidines and one uridine, fixing the sequence A-Gp. The third position was established as cytidine by periodate degradation (*P*) through two cycles and by phosphatase plus alkali treatment of the remaining oligonucleotide (*G*). Venom diesterase (*F*) released a terminal cytidine. This sequence is therefore C-U-C-A-G.

PEAK 12. The heptanucleotide U-m^7 G-C-C-U-U-G was eluted earlier than the hexanucleotide A-U-U-C-C-

[3] Gradient numbers in the text are taken from Table II.

TABLE III: Oligonucleotides Isolated from 13-104[a] after RNase T_1 Hydrolysis.

Peak No.	A: Oligonucleotide[b]	A: nmoles Isolated	B: Analyses[c] and Remarks	C: Total Composition of Peaks (nmoles) H	ψ	U	T	S	G	A	C
1	Gp		*B, K; A, K*						655		
2	C-Gp	55	*N; B, K; C, K; J;* U_2A contaminant			17			54	8	57
3	U-Gp	25[d]	*N; B, K*			25			28	3	13
4	pGp	57	*C, K; O, K; J; R-2*			13			57	2	13
5	H-C-Gp	45	*N; A, K; C, K; F, K; J*	24[g]		3			45		45
6	A-Gp[e]	130[f]	*N; G, K; F, K; C, K; J;* C_2G contaminant			8			163	138	47
7	H-A-Gp	34	*B, K; N, B, K; C, K; J; L, B, K;* U_2G contaminant	34		24			62	51	17
8	Total composition[h]		*A, K; B, K*			36			90	176	232
	H-A-Gp	8	*N; L, B, K*								
	C-A-C-C-A	43	*N; E, K; B, K; C, K; F; P, K*								
	C-A-Gp	51	*N; C, K; B, K; F*								
	U-A-Gp	17	*N; L, B, K*								
9	Total composition[h]		*A, K; B, K;*		40	110	(U + T)		174	14	350
	C-C-C-Gp	54	*N, B, K; F*								
	U-C-C-Gp	58	*N, B, K; F; L, B, K*								
	T-ψ-C-Gp	46	*N, B, K; F; C, K; P, G, K*								
9a	Total composition[h]		*B, K;* mixture of oligonucleotide			20			14	20	29
10	Total composition[h]		*B, K*		36	66			50	48	17
	A-ψ-U-Gp	40	*N, B, K; C, K; F*								
10a	Total composition[h]		*B, K*			52			34	23	55
	(C_3AG)	6	*N, B, K*								
	(U_3CG)	9	*N, B, K*								
	(U_2CAG)	1	*N, B, K*								
11	C-U-C-A-Gp	35	*N, B, K; F, K; P^2, G, K*			34			29[a]	28[a]	62
12	U-m^7G-C-C-U-U-Gp	25	*B, K; N; C, K; P^3, G, K; F, K*			111	(ψ + U)[i]		39[j]	20	59
13	A-U-U-C-C-Gp	40	*B, K; N; C, K; P^3, G, K; F, K*			98			44	42	93
14	A-S-C-A-Gp	30	*N, B, K*			7		6[k]	39	60	30
15	A-A-A-ψ-C-C-C-C-Gp	40	*B, K; N; C, K; F, K; C, K, P^2, N, K*		40				42	120	160

[a] This tRNA preparation contains 65% $tRNA^{Phe}$ (40 nmoles of $tRNA^{Phe}$ in a total of 60 nmoles of tRNA). The oligonucleotides were separated on gradient 7 (Figure 1). The table is divided into three sections. Section A incorporates the peak number (Figure 1), the proven sequence of the oligonucleotide(s) in the peak, and the total amount of that particular oligonucleotide recovered from this experiment. Section B lists the various steps used in the determination of each sequence, and section C gives the total composition of each peak (Figure 1). The small discrepancies between the sum of the oligonucleotides found and the total composition are due in part to the low analytical values for A and G when they were in an A-G sequence and to the presence of low levels of a number of oligonucleotides that were not analyzed. [b] Known sequences are indicated with dashes between bases. The amounts are calculated from the original chromatogram peaks that were homogeneous. The mixed peaks were calculated after the first rechromatography. [c] Letters in section B refer to procedures described in the Experimental Section, and superscripts on the *P* analyses refer to the number of periodate cycles. [d] Because of the increased probability of obtaining dinucleotides and trinucleotides from contaminating tRNAs, there is uncertainty in assigning the source of these oligonucleotides when they have been recovered in low yield. The assignment of such oligonucleotides as part of $tRNA^{Phe}$ is based upon a proportionately higher yield from the more purified preparations. [e] The position of the oligonucleotide is proportional to its charge and adenine content. [f] As in footnote *d*, the excess of A and G over two residues is probably due to oligonucleotides from the contaminating tRNA. [g] This value was measured (*R*) after separation of the RNase A hydrolysate by electrophoresis. [h] The total composition of the pooled peak is given in section *C*. [i] With analysis *K* the normal alkaline breakdown products of 7-methylguanosine are found in the uridine region, causing the observed high uridine value. The area under the curve used for calculations spanned the pseudouridine and uridine positions. [j] This includes 5 nmoles of 7-methylguanosine. [k] The recovery of thiouridine from oligonucleotides has been generally poor. Monitoring the DE-32 chromatographic effluent at 310 nm yields calculated values for 4-thiouridine within 10% of the theoretical based upon the guanosine content. It is assumed that the loss of thiouridine gives rise to uridine plus other unknown products.

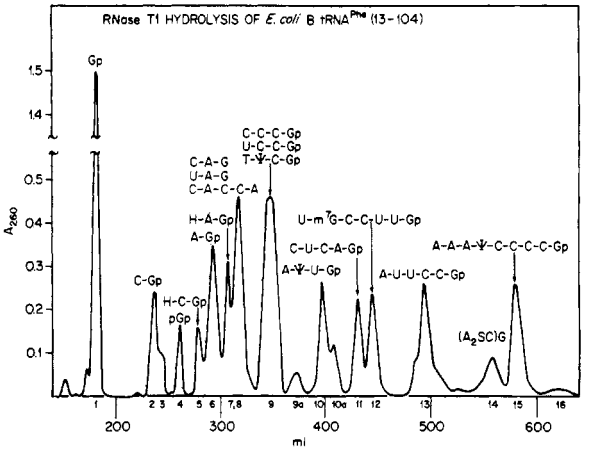

FIGURE 1: A total of 47 absorbance units (*ca.* 60 nmoles of $tRNA^{Phe}$ (13-104, 65% $tRNA^{Phe}$)) was dissolved in 2 ml of 0.02 M Tris (pH 7.3) containing 0.1 mg/ml of RNase T_1. The solution was incubated overnight (16 hr) at 37° in a closed tube in the presence of toluene. The sample was concentrated by flash evaporation to about 0.2 ml and applied to a DEAE column (gradient 7A). The pooled peaks were lyophilized to remove water and salt prior to analysis.

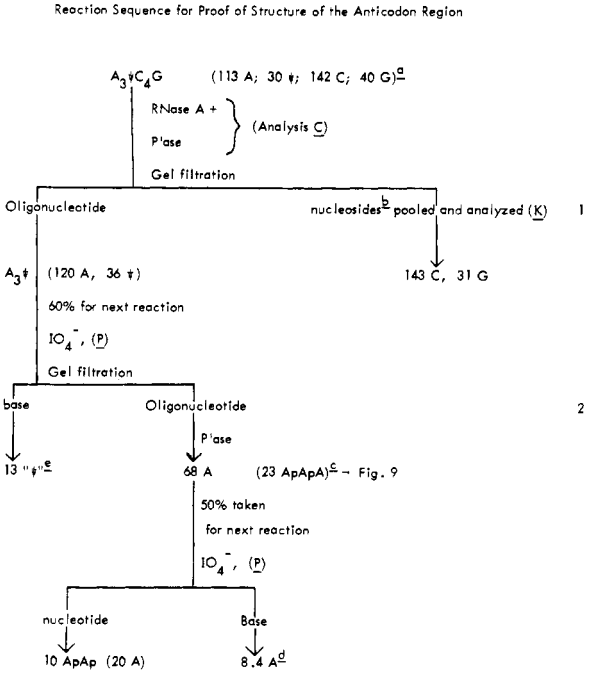

FIGURE 2: Reaction sequence for proving structure of the anticodon region. (a) All analytical values given in nanomoles. See Results, *RNase T_1 Oligonucleotide* section, Peak 15. (b) These values were obtained by integration of the ultraviolet peaks from the gel filtration step, since cytosine and guanine separate on P-2. (c) Figure 3 demonstrates the chromatographic identity of synthetic ApApA and the product of IO_4^- and phosphatase treatment of $A_3\psi$. (d) This value is the sum of released adenine and the adenosine-like fragment present as a result of incomplete periodate overoxidation (Neu and Heppel, 1964). (e) Pseudouridine gives rise to several products on periodate oxidation. We observed only one of the two major products and used a millimolar extinction coefficient of 10 to calculate the number of moles present.

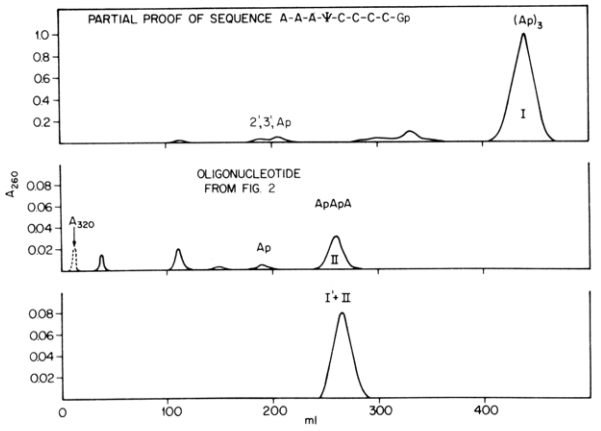

FIGURE 3: Synthetic ApApAp (I) was purified with gradient 7A, then treated with one cycle of *P* and *O* to give I′. The oligonucleotide product of step 1 (Figure 2) was subjected to the same procedure, and then was chromatographed on gradient 7A (II). The chromatographic product II was cochromatographed with I′.

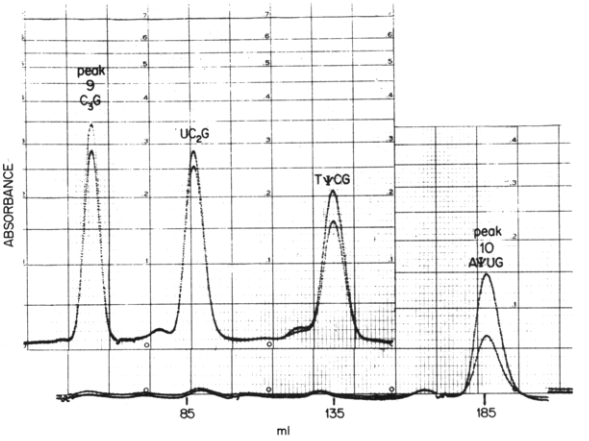

FIGURE 4: Rechromatography of peaks 9 (a) and 10 (b) from the RNase T_1 hydrolysate of 13-104 (Figure 1), using gradient 11A.

G, probably because of the hydrophilic 7-methylguanosine present in the heptanucleotide. This oligonucleotide has never been isolated in greater than 70% yield (based on $tRNA^{Phe}$ content). Its sequence was established by the same series of steps used for the pentanucleotide plus an additional periodate cycle (*P*). 5′-Terminal uridine was established by venom diesterase treatment (*F*). Treatment with RNase A did not release one of the two cytidines; thus the 7-methylguanylic acid is linked to the 5′ end of a cytidine residue. Three sequential degradations with periodate (*P*) established the sequence U-U-G. The oligonucleotide remaining after four periodate cycles was analyzed after hydrolysis with venom diesterase and phosphatase (*B*) and was found to contain one uridine, one cytidine, and the alkaline hydrolysis products of 7-methylguanosine. The latter transformation occurs during the isolation steps (Figure 5).

PEAK 13. The sequence of the hexanucleotide A-U-U-C-C-G was determined by a similar approach. The first three periodate cycles (*P*) released the terminal guanosine and two cytosine residues; RNase A and phosphatase treatment (*C*) released two cytidines and one uridine; and treatment with venom diesterase (*F*) showed adenosine to be the 5′ terminus.

PEAK 14. The assignment of the pentanucleotide sequence A-S-C-A-Gp is tentative for a number of reasons. Although the A_{310} due to 4-thiouridine is quantitatively recovered on chromatography at neutral pH, the necessary purification by rechromatography or electrophoresis at low pH results in a fragmentation of the single A_{310} peak and loss of the oligonucleotide. With the preparations discussed in this paper, the recovery of 4-thiouridine from the oligonucleotide has not exceeded 20%, but the nucleoside itself is completely stable to our analytical procedures. The above pentanucleotide has been isolated from other *E. coli* B $tRNA^{Phe}$ preparations (not discussed here) where the loss of sulfur has been balanced by the appearance of a uridine residue (M. Uziel, R. D. Kelmers, and G. D. Novelli, unpublished observation). Thus, although the analytical values in Table III for A-S-C-A-Gp are not very convincing, they represent the sulfur content of preparations 13-104.

On the other hand, the -A-G- sequence and two adenosine residues help in the material balance of the oligonucleotides isolated from RNase A and RNase T_1 hydrolysates. The sequence was determined by the following experiments. Since snake venom diesterase (*F*) released one-half of the adenylic acid, this residue occupies the free 5′-OH terminus. Sequential hydrolysis (*C*) resulted in one free cytidine, indicating an -S-C- linkage. Since no free guanosine or thiouridine (or uridine) was observed, these must be linked to the two adenylic acid residues, giving the sequence A-S-C-A-Gp.[4]

PEAK 15. A-A-A-ψ-C-C-C-C-Gp. This nonanucleotide was isolated in better than 90% yield from RNase T_1 hydrolysates and its sequence determined by a combination of RNase A and phosphatase treatment (*C*), analysis of the nucleosides released (four cytidines and one guanosine), and recovery of the remaining oligonucleotide, $A_3\psi$. The terminal A was identified after venom diesterase treatment (*F*). Synthetic A-A-A and the related oligonucleotide, isolated after one cycle of procedure *P* on $A_3\psi$, cochromatograph on gradient 7A (Figure 3). Two cycles of procedure *P* followed by identification of the 3′-terminal group as A proved the sequence A-A-A (Figure 2).

RNase A Oligonucleotides. Two RNase A hydrolysate patterns are illustrated in Figures 6 and 7. Because of the larger quantity of 13-105 available to us, we used this material to isolate the various oligonucleotides for sequence work. Hydrolysis was performed on 690 A_{260} units of preparations 13-105 (50% $tRNA^{Phe}$) (Figure 6). The evidence supporting the individual sequences is listed in Table IV. A hydrolysate of the 65%

[4] Recent experiments utilizing $tRNA^{Phe}$, which has been stabilized with thiosulfate, yields an oligonucleotide containing 4-thiouridine, 2 adenosine, 1 guanosine, and *two* cytidines in the sequence A-S-C-C-A-Gp.

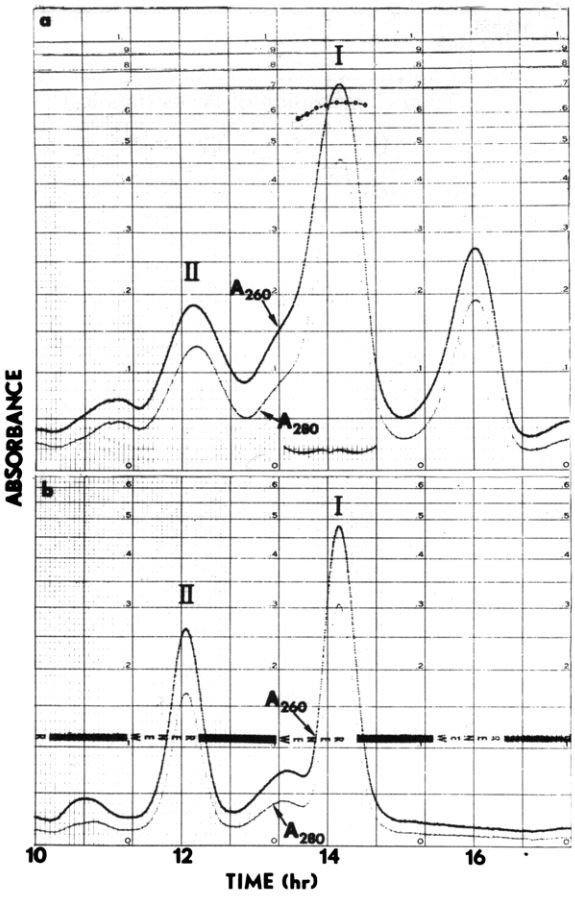

FIGURE 5: The oligonucleotide containing 7-methylguanosine was isolated in quantity from a partial hydrolysate of $tRNA^{Phe}$ (13-104). (a) The isolated peak was desalted, concentrated, and chromatographed on gradient 11A. (b) The central portion of the main peak was desalted on G-25, concentrated by evaporation *in vacuo*, and chromatographed again on gradient 11A. The appearance of substantial quantities of the earlier peak (5a-II) together with a corresponding loss of the main peak (5a-I) partially explains the low recoveries of this oligonucleotide.

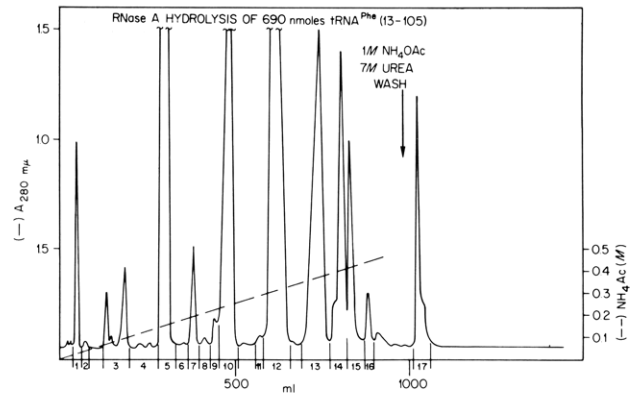

FIGURE 6: Based upon terminal adenosine content, 690 nmoles of tRNA (13-105) containing approximately 350 nmoles of $tRNA^{Phe}$ was hydrolyzed with RNase A (50 nmoles of RNase A in 2.2 ml, pH 7.2, in a pH-Stat at 37°). Solid urea was added to bring the final concentration to 7 M, and the whole sample was separated with gradient 1 (Table II). Analyses were performed as indicated in Table IV.

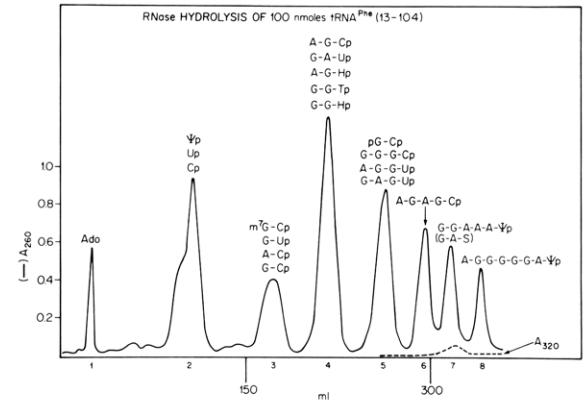

FIGURE 7: One-hundred nanomoles of tRNA (13-104, 65% $tRNA^{Phe}$) was hydrolyzed as described in Figure 6 and chromatographed on gradient 2. The peaks were analyzed as described in Table IV, and Table V lists the molar ratios of the isolated oligonucleotides. Rechromatography of peak 7 in gradient 5 at pH 3.3 resulted in the appearance of a number of 320 absorbing peaks, none of which were recoverable. This low pH has been found to destroy our thiouridine-containing oligonucleotides.

$tRNA^{Phe}$ (13-104) was made in order to identify which of the smaller oligonucleotides present in 13-105 were actually part of $tRNA^{Phe}$ [5] and to measure accurately the recoveries of certain oligonucleotides (Figure 7). Table V lists the molar ratios present based on the fraction of the tRNA accepting phenylalanine. Except where noted, the following results are taken from the experiment described under Figure 6.

PEAKS 2, 4, 6, AND 8 were too small to be identified.

PEAK 1. This peak is the column breakthrough and contains no nucleotide material.

PEAK 3. This region contained all four nucleosides in small quantities.

PEAK 5. The mononucleotide peak contained cytidylic, uridylic, and pseudouridylic acids. The pseudouridylic acid was characterized by its ultraviolet spectrum at pH 7, 12, and 14. The amounts present were determined by analysis with phosphatase (*O*) and chromatography (*K*). Color tests for dihydrouridine (*R*-1) were negative.

PEAKS 7, 9, AND 10. Of the five dinucleotides identified in these peaks, four were present in suficient quantity to be part of the phenylalanyl-tRNA. A-Up is not derived from $tRNA^{Phe}$. m^7G-Cp was characterized by its composition. After alkaline hydrolysis (*A* and *K*) and nucleoside analysis, only cytidine and an equivalent amount of the alkaline hydrolysis products of 7-methylguanosine were found (Figure 8). Under milder assay conditions (*B* and *K*) the 7-methylguanosine itself is

[5] With the higher probability that $tRNA^{Phe}$ and contaminating tRNAs will yield the same dinucleotides, trinucleotides, and tetranucleotides, the presence of greater than one residue per mole of $tRNA^{Phe}$ is not unexpected. On the other hand, the low probability of larger oligonucleotides being common to all the tRNAs in the preparation makes a 100% recovery the maximum expected and, because of inevitable mechanical losses, makes 90% a reasonable yield for large oligonucleotides present in $tRNA^{Phe}$. See also Table III footnote *d*.

TABLE IV: Oligonucleotides Isolated from 13-105[a] after RNase A Hydrolysis.

Peak No.	Oligonucleotide	nmoles Isolated	Analysis[b] and Remarks	Total Composition of Peaks (nmoles) ψ	U	T	G	A	C
5			*O, K, I, J*	450	2700				7800
7			8.6 units (A_{260}) of a mixture of dinucleoside phosphates						
9–10	m^7G-Cp	153	*N, A, B, K, L*				153[c]		150
	A-Cp	296	*N, A, B, K, L*		32			286	296
	G-Cp	400	*N, A, B, K, L*		41		401	5	420
	A-Up		*N, A, B, K, L*		80			80	
	G-Up	460	*N, A, B, K, L*		466		472		
12	A-A-Cp				10			160	68
	A-G-Cp	420	*N, A, B, K, L*				420	500	480
	A-G + C[d]		*D, K, L*				85		492
	G-G-Cp				11		230	16	80
	A-G-Hp	400	*N, A, B, K, L*				450	400	
	(A-G) + H[d]		*D, L, R*				68		
	G-A-Up	440	*N, A, B, K, L*		440		450	460	
	G + (A-U)[d]		*D, L, R*		85		400	20	
	G-G-Tp	480	*N, A, B, K, L*			480[e]	2010[e]		
	G-G-Hp	440							
13	pG-Cp	380	*N, A, B, K, L*				415	115	380
	$(A_2G)C$		*N, A, B, K*				43	95	45
	G_3C	80	*N, A, K*		25		360	32	87
	A-G-G-Up[f]	120	*N, A, K; D, K; F, K*		257		720[g]	294[g]	
	G-A-G-Up[f]	120							
14–15	A-G-A-G-C	250	*N, A, B, K*				580	540	252
	G-G-A-A-A-ψ	260	*N, A, B, K*	260			394	830	
16			*N, A, K*		26		150	80	
17	A-G-G-G-G-G-A-ψ	90	*N; A, B, K; P^3; D-1, K*	86			586	230	48

[a] This material was only 50% $tRNA^{Phe}$, *i.e.*, only 345 nmoles of the total 690 nmoles of tRNA are $tRNA^{Phe}$. The oligonucleotides were separated on gradient 1. See Figure 7 for details of the hydrolysis. [b] Letters in the central section refer to procedures described in the Experimental Section, and superscripts on the *P* analyses refer to the number of periodate cycles. [c] This value includes 138 nmoles of 7-methylguanosine. See also Figure 5. [d] Products of the reaction *D, L, K*. [e] Summation of G-G-T and G-G-H. [f] Rechromatography and quantitation of A-G-G-U and G-A-G-U were performed on preparations 13-104 (Figure 7). The oligonucleotide recovery values reported in Table V have been taken from this hydrolysis and normalized for the differences in the amount of starting material. The values for these tetranucleotides are low in the above table because of mechanical losses. [g] Summation of A-G-G-U and G-A-G-U.

quantitatively recovered. The remaining dinucleotides were similarly characterized by their composition and charge as determined by thin layer electrophoresis. The main components were all found in peak 7 and are presumed to be cyclic phosphate derivatives of the various dinucleotides.

PEAK 11. was not characterized.

PEAK 12. Of the seven trinucleotides found in the 50% pure $tRNA^{Phe}$ sample (13-105), only A-G-Cp, A-G-Hp, G-A-Up, G-G-Up, and G-G-Tp were present in sufficient quantity to be part of the phenylalanine tRNA. Upon rechromatography in gradient 3, three major peaks were found. The first, A-G-Cp, was identified by analysis (*K*) after sequential treatment with RNase T_1 and phosphatase (*D*-1). Free cytidine was released. The sequence A-G was confirmed by electrophoresis of the RNase T_1 products (*D*-2). The second peak was a mixture of A-G-Hp and G-A-Up. These were separated by rechromatography in gradient 6 (pH 9.6) and the sequences determined after *D*-1 and *D*-2 treatments. No ultraviolet-absorbing nucleoside was found after *D*-1 on A-G-H. However this trinucleotide gave a positive dihydrouridine test (*R*-1). The electrophoretic mobility was consistent for a trinucleotide of this composition. G-A-Up, on the other hand, released guanosine after *D*-1, and the dinucleotide A-Up was found after *D*-2.

Similarly, G-G-T and G-G-H were separated by rechromatography with gradient 6 and the sequence confirmed by treatments *D*-1 and *D*-2. One of these two trinucleotides gave a positive dihydrouridine test (*R*-1). Both released all their guanosine after *D*-1 or *D*-2.

PEAK 13. Four tetranucleotides and the dinucleoside triphosphate pG-Cp were found in this peak. Analysis

TABLE V: Oligonucleotides Obtained from a RNase A Digest of 13-104.[a]

Peak	Composition	Molar Ratio[b]
1	Adenosine	1.4
2	Cp	16.0
	ψp	1.2
	Up	9.3
3	m^7G-Cp	0.35
	A-Cp	1.6
	G-Cp	1.3
	G-Up	1.4
4	A-G-Cp	1.4
	G-A-Up	1.2
	A-G-Hp	1.2
	G-G-Tp	1.4
	G-G-Hp	1.3
5	pG-Cp	0.9
	G-G-G-Cp	1.0
	A-G-G-Up	0.9
	G-A-G-Up	0.9
6	A-G-A-G-C	1.2
7	G-G-A-A-A-ψ	1.2
7a	G-A-S[c]	
8	A-G-G-G-G-G-A-ψ	0.5

[a] One-hundred nanomoles of 13-104 (65% $tRNA^{Phe}$) was hydrolyzed with RNase A, then separated on gradient 2 (Table II, Figure 7). [b] The ratio of moles of oligonucleotide found to moles of $tRNA^{Phe}$ present. See footnote *d* of Table III. [c] Although not isolated in these experiments, the oligonucleotide containing 4-thiouridine has been shown to contain the sequence G-A-S.

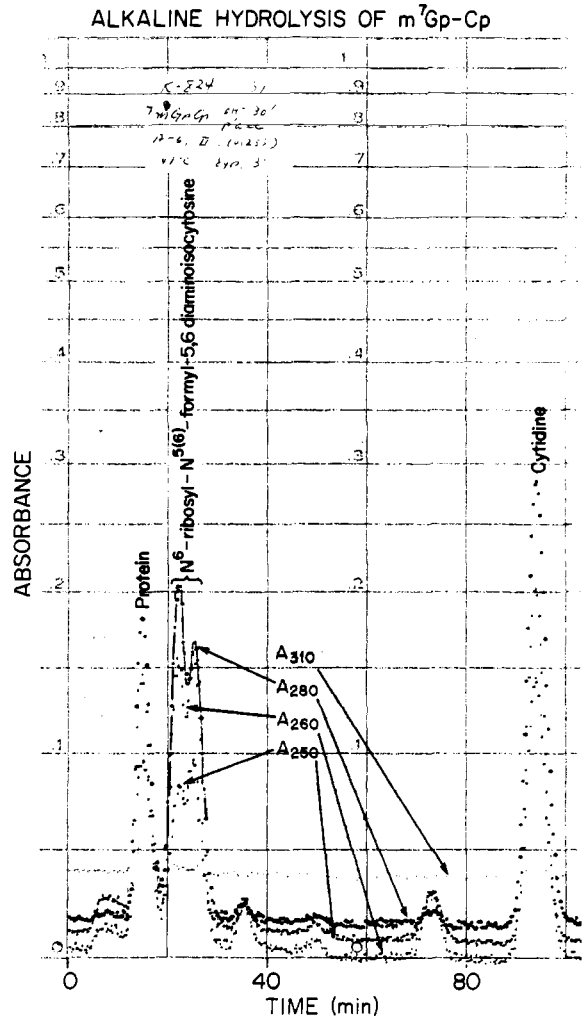

FIGURE 8: The breakdown of 7-methylguanosine in alkali is illustrated by the treatment of m^7GpCp with alkali (*A*) followed by nucleoside analysis. The absorbancy at 280 nm has been emphasized with heavy lines. Absorbancy at four wavelengths is sequentially recorded on the analyzer (Uziel *et al.*, 1968). The first peak is the phosphatase protein excluded from the resin. The second and third peaks are the isomeric N^6-ribosyl-$N^{5(6)}$-formyl-5,6-diaminoisocytosine derivatives.[6] The fourth peak is cytidine. Using an ϵ_M^{260} 10,000 for the isomers, there is an equimolar quantity of cytidine and the sum of the isocytosine derivatives. In this chromatographic system, 7-methylguanosine elutes at 108 ml (about 6 hr). No ultraviolet absorbance was eluted in this region.

(*B* and *K*), electrophoretic mobility, and a negative dihydrouridine test (*R*-1) proved the sequence pG-Cp. This oligonucleotide is expected in this chromatographic region because of its charge. Of the remaining tetranucleotides, only G-G-G-Cp, A-G-G-Up, and G-A-G-Up were found in sufficient quantity to have been derived from $tRNA^{Phe}$. This conclusion is drawn from analysis of 13-104 (65% $tRNA^{Phe}$), where the three tetranucleotides were recovered in greater than 90% yield. In the present experiment, they were recovered in very low yield.

G-G-G-Cp was readily separated on gradient 4 and its sequence was established by composition (*B* and *K*).

The remaining tetranucleotides were poorly separated on DE-32 chromatography, but the total analysis from this region of 13-104 indicated that there were sufficient uridines to represent two oligonucleotides. Procedure *D*-2 yielded A-Gp, Gp, and Up in equimolar amounts. Treatment with venom diesterase (*F*) released equal amounts of adenosine and guanosine, the sum of which equaled the uridine content. Thus, two isomeric tetranucleotides were present, A-G-G-Up and G-A-G-Up.

PEAKS 14 AND 15. Rechromatography of these peaks with gradient 5 gave the pentanucleotide A-G-A-G-Cp and the hexanucleotide G-G-A-A-A-ψp. The first sequence was proven by sequential RNase T_1 and phosphatase treatment (*D*-1), which yielded only cytidine, and by identification of the terminal A by venom diesterase (*F* and *K*). The second oligonucleotide, G-G-A-A-A-ψp, was similarly characterized with *D*-1; only guanosine was released. Some preparations gave a low guanosine release, based on the pseudouridine content, but these were all less active (phenylalanine acceptance) or came from chemically damaged $tRNA^{Phe}$. The tetranucleotide remaining after procedure *D*-2

[6] W. E. Cohn (unpublished observations) has observed a similar distribution of the isomeric alkaline hydrolysis products of 7-methylinosine.

was recovered; the pseudouridylic acid was isolated from it after alkali or venom diesterase hydrolysis; and the spectrum was measured at pH 12 and 14 to identify the pyrimidine.

PEAK 16. was not characterized.

PEAK 17. Peak 17, upon rechromatography, showed one major component, which proved to be an octanucleotide (*A*, *B*, and *K*). Digestion with venom diesterase (*F*) showed that the 5′ nucleoside was adenosine. T_1 hydrolysis followed by electrophoresis (D-2) resulted in A-Gp, three to five guanylic acids, and a low A-ψp. To check the assumed structure A-G-G-G-G-G-A-ψ, the octanucleotide was degraded stepwise with three cycles of periodate (*P*). The results showed that the pseudouridylic acid, which was not recovered, is followed by adenosine and guanosine, thus confirming the above sequence. We have no explanation for the low recovery (50%) of this oligonucleotide other than technical losses. However, the corresponding T_1 oligonucleotide A-ψ-U-Gp is isolated in >90% yield indicating that the A-G-G-G-G-G region is contributing to the low recovery. The conclusion that this oligonucleotide came from $tRNA^{Phe}$ is dependent upon the increased yield from the higher purity preparations.

Discussion

Because of the unique nature of several of the isolated oligonucleotides, it is possible to reconstruct the sequences of several regions of the $tRNA^{Phe}$ molecule.

Of the regions common to all known tRNA structures, the sequence adjacent to the aminoacylation site has been identified as G-C-A-C-C-A. The pentanucleoside tetraphosphate C-A-C-C-A, isolated from RNase T_1 hydrolysates, fulfills the requirements of the aminoacylation site, since it has a terminal adenosine with free vicinal hydroxyl groups and is attached to the tRNA molecule through a C-C-A linkage. The Gp is placed at the 5′-hydroxyl end because the C-A-C-C-A was isolated from an RNase T_1 hydrolysate. The other region common to all the known tRNA structures is the T-ψ-C-G region, which for $tRNA^{Phe}$ can be extended to G-G-T-ψ-C-G by permitting overlap of the G-G-T isolated from RNase A hydrolysates.

A third region, including the potential anticodon, may be reconstructed on the basis of overlaps. The oligonucleotide A-A-A-ψ-C-C-C-C-Gp contains the sequence with the necessary adjacent adenines. The corresponding oligonucleotide, isolated from the pancreatic RNase hydrolysates, was G-G-A-A-A-ψ, which permits placement of the two sequences to give G-G-A-A-A-ψ-C-C-C-C-Gp.

The sequence G-G-A-A-A contains the only adjacent adenines in this tRNA molecule. The presence of three adjacent adenines would fulfill the predicted anticodon requirements from the original poly U dependent incorporation of phenylalanine (Nirenberg and Matthaei, 1961). However, this sequence is inadequate to explain the response to the codon UUC (Söll *et al.*, 1966).

With yeast (RajBhandary and Chang, 1968) and wheat germ (Dudock *et al.*, 1968) $tRNA^{Phe}$, the assignment of the anticodon was simplified by the presence of an unknown base ("Y"), and the assumption of wobble in the third position, permitting GAA to fulfill all the anticodon requirements.

The sequence G-A-A is also present in *E. coli* B $tRNA^{Phe}$. This would easily satisfy the ribosome bindind data for both UUU and UUC codons. However, because of the unique placement of the potential anticodons in an overlapping structure, the possibility of a reversible conformational shift permitting the use of either AAA or GAA as anticodons cannot be ruled out. All tRNA sequences known at this time contain a uridine linked to the 5′ end of the anticodon. If G-A-A is the anticodon, then the sequence GGAAA represents a departure from the usual structure of the anticodon region.

Although the total requirements for a functional anticodon are as yet not known, structural and binding studies indicate that the conformation of the anticodon region as well as the sequence are important factors (Clark *et al.*, 1968; Fuller and Hodgson, 1967).

Added in Proof

The $tRNA^{Phe}$ used in these experiments has been separated into two chromatographic peaks. The more purified material contains 6-(3 methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine in the sequences A-A-N-A-ψ and G-A-A-N-ψ.

Acknowledgments

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The Fructose 1,6-Diphosphatase of *Dictyostelium discoideum**

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ABSTRACT: Fructose 1,6-diphosphatase from the slime mold *Dictyostelium discoideum* was found to have an alkaline pH optimum (9.0–9.5). Little or no activity occurred at physiological pH unless EDTA was added. In the presence of 0.2 mM EDTA an additional peak of activity, equal to that at pH 9.5, appeared at pH 7.5–8.0. EDTA stimulated activity at each pH tested (7.5, 8.5, and 9.5). Stimulation of activity was greatest at pH 7.5 and progressively decreased at high pH. The reaction was completely dependent upon the presence of Mg^{2+} or Mn^{2+}. At optimum concentrations the reaction rate with Mg^{2+} was about three times higher than with Mn^{2+}. In addition to EDTA, mercaptoethanol, cysteine, dithiothreitol, KCN, and histidine stimulated the reaction rate at pH 7.5. Adenosine monophosphate, deoxyadenosine monophosphate, 3′,5′-cyclic adenosine monosphosphate, adenosine diphosphate and adenosine triphosphate did not stimulate nor inhibit enzyme activity at pH 7.5 in the presence of 0.2 mM EDTA. Fructose 1,6-diphosphate was slightly inhibitory above 0.2 mM. The low activity of the enzyme as well as the lack of regulation of its activity by 3′,5′-cyclic adenosine monophosphate suggests that it may not be important in gluconeogenesis.

Fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) is an enzyme which has been extensively studied by many workers (see reviews by Atkinson, 1966; Wood, 1966). The FDPase from many organisms has an alkaline pH optimum and essentially no activity at physiological pH. Many compounds, such as EDTA and some sulfhydryl reagents, can activate this enzyme at the latter pH. Depending upon the source of the enzyme, activity at physiological pH can be inhibited by AMP, FDP, by both compounds or by neither (Scala *et al.*, 1968). Evidence obtained *in vivo* and *in vitro* in mammalian systems indicates that FDPase is important in the regulation of gluconeogenesis (Krebs *et al.*, 1964; Scrutton and Utter, 1968).

FDPase has been reported in cell free extracts of *Dictyostelium discoideum* by Cleland and Coe (1968). The activity of the enzyme when compared to that found in other organisms was unusually low and did not change during differentiation. A recent study of the phosphofructokinase of *D. discoideum* (Baumann and Wright, 1968) showed that this enzyme had an unusual regulatory pattern, inconsistent with its role as a key enzyme in metabolic regulation of glycolysis and gluconeogenesis. The low level of FDPase found by Cleland and Coe (1968) as well as evidence discussed by Baumann and Wright (1968) suggested that during differentiation of *D. discoideum* the hexose units of glycogen (already present in amoeba) are used as precursors for the synthesis of polysaccharides and trehalose which accumulate during fruiting body formation. In such a system gluconeogenesis from amino acids would not be of great importance, as is also

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