Comparative Fingerprint and Composition Analysis of the Three Forms of ^{32}P-Labeled Phenylalanine tRNA from Chloramphenicol-Treated *Escherichia coli*†

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ABSTRACT: Phenylalanine tRNA in *Escherichia coli* (15THU) is comprised of a known primary sequence of nucleotides. When cells are treated with chloramphenicol, three forms of this tRNA appear, each separable by reversed phase chromatography (RPC-5). Using ^{32}P labeling, these forms are compared by fingerprinting of their ribonuclease T_1 digestion products with three different systems differing in the second dimension: high voltage electrophoresis, polyethylenimine-cellulose thin-layer chromatography, and homochromatography. Further compositional analysis shows that the three forms are identical, but that differences exist in the extent of modification of specific minor bases.

In a previous paper (Mann and Huang, 1973), evidence was presented suggesting that the appearance of two new forms of phenylalanine tRNA ($tRNA^{Phe}_{I}$ and $tRNA^{Phe}_{II}$) during chloramphenicol treatment of *Escherichia coli* could have resulted from incomplete modification of the precursor of normal $tRNA^{Phe}$.[1] The new forms arise by *de novo* synthesis instead of by conversion from preexisting cellular RNA through demodification. This was shown by examining the kinetics of accumulation during chloramphenicol treatment and of the disappearance of these forms following the removal of chloramphenicol. Thus, the new forms are related to the normal ($tRNA^{Phe}_{N}$) *via* a sequential modification pathway.

$$tRNA^{Phe}_{I} \longrightarrow tRNA^{Phe}_{II} \longrightarrow tRNA^{Phe}_{N}$$

Barrell and Sanger (1969) have established that $tRNA^{Phe}$ normally consists of a unique primary sequence of 76 nucleotides, nine of which are modified. Based on this knowledge, it was of interest to us to study the state of modification in the new forms. In this paper, comparative fingerprints were performed by three methods: the standard two-dimensional ionophoresis (Sanger *et al.*, 1965), homochromatography (Brownlee and Sanger, 1969), and polyethylenimine-cellulose thin-layer chromatography (Griffin, 1971). By analysis of the extent of biosynthesis of several of the minor nucleosides present in $tRNA^{Phe}$, it has been possible to classify these modification steps as being either sensitive or resistant to inhibition by chloramphenicol.

Experimental Section

Methods

Preparation of ^{32}P-Labeled tRNA from Chloramphenicol-Treated E. coli. A culture of exponential phase *E. coli* THU grown in Tris-glycerol–1 mM P_i (Mann and Huang, 1974) was pelleted by centrifugation and resuspended at 3.5×10^8 cells/ml in 1 l. of Tris-glycerol minus P_i containing 200 μg/ml of chloramphenicol. This Tris-buffered minimal medium contains per liter: tris(hydroxymethyl)aminomethane base, 12 g; potassium chloride, 2 g; ammonium chloride, 2 g; magnesium chloride hexahydrate, 0.5 g; sodium sulfate, 20 mg; and sufficient concentrated HCl to bring the pH to 7.2. To this was added L-histidine, 20 μg/ml; thymidine, 10 μg/ml; uracil, 20 μg/ml; 0.2% vitamin-free casein hydrolysate (General Biochemicals Corp.); and glycerol, 0.5%. P_i signifies inorganic orthophosphate ion supplied as KH_2PO_4. Incubation was carried out at 37° with rapid rotary shaking at 250 rpm in a New Brunswick Gyrotary Shaker. After 2 hr, 50 mCi of carrier-free [^{32}P]orthophosphate (Amersham–Searle) was added and incubation continued for an additional 3 hr. The culture was chilled in ice and collected by centrifugation. Uptake of $^{32}P_i$ into acid-insoluble material was about 75%. The tRNA fractions were extracted and purified initially by sucrose density gradient centrifugation and charged according to procedures described earlier (Mann and Huang, 1973).

Preparative Charging of [^{32}P]tRNA with Phenylalanine. The amount of purified [^{32}P]tRNA was determined spectrophotometrically. Preparative charging was carried out with unlabeled phenylalanine at a concentration of 100 μM. The concentration of [^{32}P]tRNA was maximized (2 mg/ml of reaction mixture). The reaction was incubated at 37° for 15 min in a 250-ml erlenmeyer flask containing a single layer of autoclaved glass beads (3 mm diameter) as recommended by Demushkin *et al.* (1971). The reaction mixture was diluted and the [^{32}P]tRNA purified by DEAE-cellulose chromatography as described earlier (Mann and Huang, 1973).

N-Phenoxyacetyl Derivatization of [^{32}P]tRNA (Phenylalanine Charged). The method was a modification of the original work of Gillam *et al.* (1968), as devised by Case and Mahler, and cited by Marmor *et al.* (1971). The [^{32}P]tRNA was obtained as an ethanol precipitate and diluted to a final concentration of 10 mg/ml in a volume of 0.64 ml. One-fourth volume (0.16 ml) of 2.5 M triethanolamine-HCl buffer (pH 8.2) was added and quickly followed by 1.25 vol (0.80 ml) of *p*-dioxane containing approximate-

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[1] Abbreviations used are: $tRNA^{Phe}$, phenylalanine transfer ribonucleic acid, with subscript N for normal cells and subscript I and II for chloramphenicol-treated cells eluting at 0.60 and 0.62 M NaCl, respectively (in figures the subscript CM for chloramphenicol is used with I and II); PhaPhe-tRNA, *N*-phenoxyacetylphenylalanyl transfer ribonucleic acid; ms^2-i^6A, 2-methylthio-6-isopentenyladenosine.

ly 25 mg of *N*-hydroxysuccinimide ester of phenoxyacetic acid. The reaction was allowed to proceed at 0° for 15 min with constant stirring. Sufficient glacial acetic acid (0.02 ml) was then added to reduce the pH to 5.0 and stop the reaction. This was followed by 0.1 vol (0.16 ml) of 20% KOAc (pH 5.0) and 2 vol (3.2 ml) of 95% ethanol. The mixture was kept at −80° for 1 hr and the precipitated tRNA was collected by centrifugation.

Benzoylated DEAE-Cellulose (BD-Cellulose) Chromatography. The derivatized [^{32}P]tRNA was dissolved in 2 ml of starting buffer consisting of 0.3 M NaCl in 0.01 M MgOAc buffer, neutralized with concentrated HCl to pH 4.8, and 0.001 M Na_2EDTA. The sample was applied to a 1.2 × 48 cm BD-cellulose column. The tRNA was eluted with a 500-ml linear gradient beginning with starting buffer and ending with buffer containing 1.5 M NaCl and 30% ethanol. The position of *N*-phenoxyacetylphenylalanyl-[^{32}P]tRNA (PhaPhe-[^{32}P]tRNA) was determined from the elution position of a marker compound, Pha-[^{3}H]Phe-tRNA. The relevant fractions were pooled and the tRNA recovered by ethanol precipitation and Millipore filtration before rerun on RPC-5. Radioactivity was monitored as previously described (Mann and Huang, 1973).

Reversed-Phase Chromatography (RPC-5). RPC-5 was performed as described in Mann and Huang (1973). The fractions corresponding to the three forms of *N*-PhaPhe-[^{32}P]tRNA were pooled and the RNA recovered as previously described (Mann and Huang, 1973).

Fingerprint Analysis of ^{32}P-Labeled Phenylalanine tRNA ([^{32}P]tRNA^{Phe}). Two-dimensional separation of the oligonucleotide fragments generated by enzymatic digestion of individual [^{32}P]tRNA^{Phe} was conducted according to the method of Sanger *et al.* (1965) as described by Barrell (1971). Briefly, the method involved a microscale digestion of a purified [^{32}P]tRNA preparation with either ribonuclease I producing fragments having a 3′-terminal pyrimidine ribonucleoside phosphate, or with ribonuclease T1 producing fragments having a 3′-terminal guanosine phosphate. The digest was separated in the first dimension by high voltage electrophoresis on a cellulose acetate strip in 7 M urea (pH 3.5). The radioactive oligonucleotide fragments were then transferred to a sheet of DEAE-cellulose paper and separated in the second dimension also by high voltage electrophoresis. The second dimension separation was also achieved by a second method with thin-layer chromatography (tlc) using 40 × 20 cm plastic sheets coated with polyethylenimine impregnated cellulose and developed with 7 M urea–pyridine–formate buffer (pH 3.5), at 60° by a two-step procedure (Griffin, 1971).

A third method, namely homochromatography of Brownlee and Sanger (1969), was used in the second dimension after separation of the T1 digests in the first dimension by high voltage electrophoresis on cellulose acetate. For this, a 3% solution of alkaline hydrolysate of yeast ribonucleic acid (Sigma) in 7 M urea was used. In all cases, the separated ^{32}P-labeled nucleotide fragments were located by autoradiography. Spots from the chromatogram were excised and eluted for subsequent analysis. Fragments produced initially by ribonuclease T1 digestion were further digested with ribonuclease I. These digestion products were separated by one-dimensional high voltage electrophoresis on DEAE-cellulose paper and then autoradiographed. The identity of the digestion products was determined from known R_F values. Total composition of the original ribonuclease T1 digestion products was achieved by further digestion with ribonuclease T2 which gave rise to the individual 3′-ribonucleotides. The digest was separated by one-dimensional thin-layer chromatography on cellulose-coated plates. The solvent system, consisting of 2-propanol–HCl–H_2O (Rubin, 1973), permitted the resolution of the 2′,3′-monophosphates of uridine (U), cytidine (C), adenosine (A), guanosine (G), ribothymidine (T), pseudouridine (ψ), dihydrouridine (D), and 2-methylthio-6-isopentenyladenosine (ms^2-i^6A).

Materials

Materials used in this study were obtained from the following sources: cellulose acetate strips, 55 × 3 cm, from Schleicher & Schuell, Dassel, Germany; DEAE-cellulose paper, DE-81, from Whatman; polyethylenimine-impregnated cellulose thin-layer chromatograms, Polygram CEL 300 PEI, 40 × 20 cm, and cellulose powder, MN 300 HR, from Macherey-Nagel & Co., Duren, Germany; cellulose thin-layer plates, 0.1 mm, from E. Merck, Darmstadt, Germany; electrophoresis indicator dyes, Xylene Cyanol F.F. (blue), Acid Fuchsin (red), and Methyl Orange (Yellow), from George T. Dunn, London; and RP-54 X-ray film from Kodak.

Ribonuclease I (pancreatic RNase; RNase A) and ribonuclease T1 were purchased from Sigma Chemical Co., St. Louis, Mo.; ribonuclease T2 was a gift from G. G. Brownlee. The *N*-hydroxysuccinimide ester of phenoxyacetic acid was from Schwarz/Mann. All other chemicals were reagent grade.

Electrophoresis buffers had the following compositions: (a) pH 3.5 buffer, 0.5% pyridine and 5% acetic acid; (b) 7 M urea (pH 3.5) buffer, above buffer containing 7 M urea; (c) 7% formic acid.

Chromatography buffers had the following compositions: (a) 1.0 M pyridine formate (pH 3.5), 10 ml of formic acid and 5 ml of pyridine in 250 ml of 7 M urea; (b) 2.0 M pyridine formate (pH 3.5), 20 ml of formic acid and 10 ml of pyridine in 250 ml of 7 M urea; (c) 3% homomix, 20 g of yeast RNA (hydrolyzed 10 min at room temperature in 200 ml of 1 M KOH, neutralized to pH 7.5 with concentrated HCl, and dialyzed against 4 l. of distilled water for 2 hr) in a final volume of 667 ml of 7 M urea; (d) 2-propanol (680 ml), concentrated HCl (176 ml), and H_2O to 1 l.

Results

Purification of [^{32}P]tRNA$^{Phe}_{I,II,N}$. The purification of [^{32}P]tRNA$^{Phe}_{I,II,N}$ was achieved by a two-step chromatographic procedure using phenoxyacetyl-derivatized, phenylalanine-charged [^{32}P]tRNA (PhaPhe-[^{32}P]tRNA) from chloramphenicol-treated *E. coli*. Figure 1 shows the BD-cellulose chromatographic elution profile of this material mixed with Pha[^{3}H]Phe-tRNA from chloramphenicol-treated cells as a marker. The amount of PhaPhe-[^{32}P]tRNA was calculated from the area under the late peak which eluted with the tritiated marker. The area was determined by doubling the radioactivity (counts per minute) summed over the second half of the peak (fractions 28–38). The resulting value amounted to 3.8% of the total [^{32}P]RNA eluted from the column. The material in this late peak was collected and subsequently separated by RPC-5 chromatography. The results in Figure 2 show a relatively complex pattern which nevertheless provided adequate separation of the three individual forms of Phe-tRNA. The identities of the three peaks designated by the arrows were corroborated as follows: each peak was pooled and the PhaPhe-[^{32}P]tRNA^{Phe} recovered by ethanol pre-

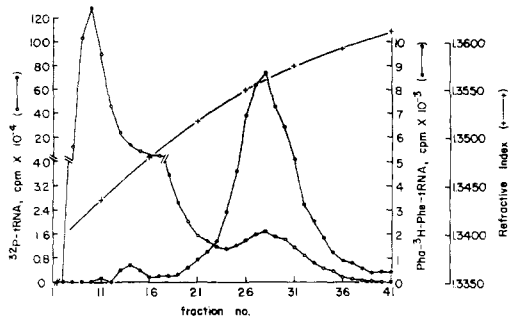

FIGURE 1: BD-Cellulose chromatography of a mixed sample containing phenoxyacetyl-derivatized Phe-[^{32}P]tRNA (PhaPhe-[^{32}P]tRNA) and Pha-[^{3}H]Phe-tRNA from chloramphenicol-treated *E. coli*. A sample of [^{32}P]tRNA from chloramphenicol-treated *E. coli* was preparatively charged with unlabeled phenylalanine and phenoxyacetylated as described under Methods. As a marker, unlabeled tRNA from chloramphenicol-treated cells was charged with [^{3}H]phenylalanine and derivatized. The two samples were mixed (^{3}H, 6.4 × 10^{4} cpm; ^{32}P, 4.5 × 10^{6} cpm) and chromatographed on BD-cellulose as described under Methods. Radioactivity was determined by scintillation counting of 100-μl samples in 10 ml of Triton-X Fluor, described earlier (Mann and Huang, 1973). The flow rate of the column decreased continuously during development with the NaCl–ethanol gradient. Since fractions were collected on a time basis, the volume per fraction decreased with time. The refractive index determinations have not been corrected for the change in volume, and, as a result, the linear gradient has a slightly convex appearance. However, correction was made for the fraction volume in determining the radioactivity profile, and the counts per minute as plotted represent the total eluted radioactivity.

TABLE I: Expected Ribonuclease T1 and Ribonuclease A Digestion Products from *E. coli* tRNA^{Phe}.[a]

From Ribonuclease T1	No.	From Ribonuclease A	No.
(a)		(b)	
AAms^{2}-i^{6}AAψCCCCG	1	AGGGGψ	1
Um^{7}GXCCUUG	1	GAAms^{2}-i^{6}AAψ	1
AUUCCG	1	AGAGC	1
CUCAG	1	GAGU	1
CACCA$_{OH}$	1	GGAs^{4}U	1
TψCG	1	GGGC	1
AψUG	1	GGT	1
As^{4}UAG	1	GGD	1
UCCG	1	AGD	1
CCCG	1	GAU	1
DCG	1	AGC	1
DAG	1	m^{7}GXC	1
CAG	1	pGC	1
AG	2	AC	1
G	8	GU	1
pG	1	U	5
		C	15
		ψ	1

[a] Rearranged from Barrell and Sanger (1969).

cipitation. The tRNA was deacylated by exposure to pH 8.8 for 30 min. Determination of the release of acid-insoluble [^{3}H]phenylalanine showed the extent of deacylation to be greater than 95%. The tRNA samples were preparatively charged with unlabeled phenylalanine, mixed with an appropriate amount of [^{3}H]Phe-tRNA from chloramphenicol-treated cells, and rechromatographed on RPC-5. Each individual ^{32}P-labeled sample was seen to coelute with one of the three [^{3}H]Phe-tRNA peaks, characterized previously (see Figure 2). The order of elution of the three ^{32}P-labeled phenoxyacetylated forms was identical with that of the three charged forms.

Late eluting material, sometimes amounting to 50% of the input [^{32}P]tRNA, appeared in some of the preparations, but it has not been characterized. It could possibly represent hyperphenoxyacetylated Phe-[^{32}P]tRNA^{Phe}, since *E.*

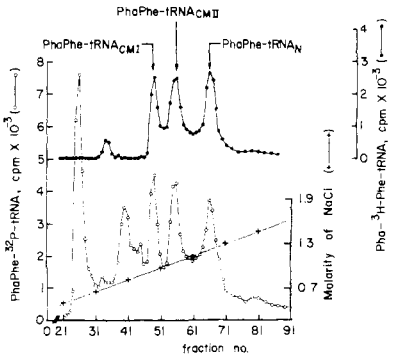

FIGURE 2: RPC-5 elution profile of a mixed sample containing partially purified PhaPhe-[^{32}P]tRNA and Pha-[^{3}H]Phe-tRNA. Fractions 20–38 from the BD-cellulose column (Figure 1) were pooled, ethanol precipitated, and collected by Millipore filtration as described under Methods. Without further treatment, the sample was loaded onto RPC-5 and chromatographed, and the fractions were counted as in Figure 1. The method of identifying the three peaks designated by the arrows is given in the text; see Results section. Fractions 1–20 contained no radioactivity.

coli tRNA^{Phe} is known to undergo stable phenoxyacetylation at positions within the tRNA molecule, and that the extent of reaction is as high as 30% for a 60-min reaction (Friedman, 1972). This fraction is eluted with a much higher ionic strength (2.5 M NaCl) than that required for the three forms of Phe-tRNA being studied here.

Comparative Fingerprint Analysis and Nucleotide Composition Determination of Purified [^{32}P]*tRNA*$^{Phe}_{I,II,N}$. As a first step in determining the nature of the relationship between the three forms of Phe-tRNA from chloramphenicol-treated *E. coli*, two-dimensional fingerprinting was carried out on each of the purified ^{32}P-labeled forms. The method involved ribonuclease T1 digestion followed by electrophoresis (7 M urea, pH 3.5) in the first dimension and homochromatography on PEI-cellulose in the second dimension. Since the principle of homochromatography is to separate molecules according to their size, it was apparent that the three tRNA^{Phe} forms were similar, as there was a basic likeness between them in regard to size and number judged by their fingerprints. For reference, Table I, part a gives the sequence and frequency of the 16 ribonuclease T1 digestion products from *E. coli* tRNA^{Phe} as determined by Barrell and Sanger (1969).

Also a comparison of the ribonuclease I digestion products was performed on each of the three tRNA^{Phe}'s. The digests were fingerprinted by high-voltage electrophoresis (7 M urea, pH 3.5) in the first dimension, and by thin-layer chromatography on PEI-cellulose (pyridine formate, pH 3.5) in the second dimension. In both experiments the high degree of similarity between the three forms was found (results in file). The 17 expected fragments are listed in Table I, part b.

In order to determine the sequence of the individual fragments produced by ribonuclease T1 digestion, a large scale labeling (50 mCi) was performed and the purified tRNA^{Phe}'s were fingerprinted. The first dimension was high

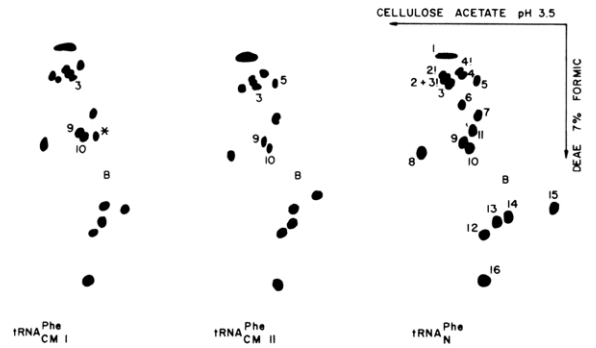

FIGURE 3: Two-dimensional fingerprints of [^{32}P]tRNA$^{Phe}_{I,II,N}$ after ribonuclease T1 digestion. A sample of each of the three purified forms of [^{32}P]tRNA^{Phe} (approximately 10^7 cpm) was digested with ribonuclease T1 and subjected to two-dimensional fractionation. The first dimension was high-voltage electrophoresis on cellulose acetate in 7 M urea (pH 3.5). The second dimension was high-voltage electrophoresis on DEAE-cellulose paper in 7% formic acid. Autoradiographic exposure was for 8 hr. The spots have been numbered arbitrarily. For [^{32}P]tRNA$^{Phe}_N$ there occurred extensive production of fragments with cyclic 2′,3′-phosphodiester termini due to slight underdigestion. Such spots are indicated by an exclamation mark. B indicates the position of the blue tracking dye. The position of the unique fragment in tRNA$^{Phe}_I$ is indicated by an asterisk. The direction of electrophoresis is from right to left in the first dimension and from top to bottom in the second. The originals have been examined by the reviewers and editors. Only a tracing is presented for clarity.

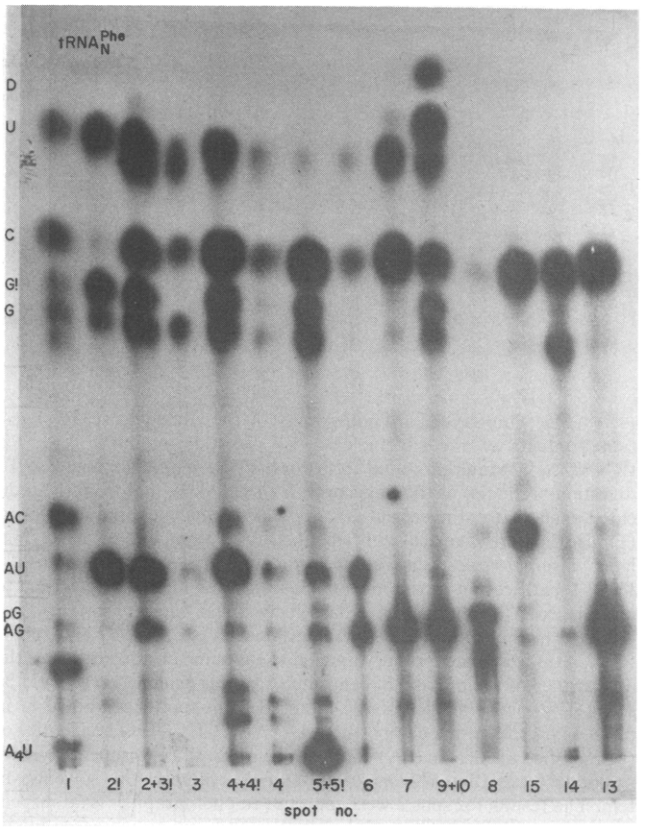

FIGURE 4: High-voltage electrophoresis of the ribonuclease A digestion products of the ribonuclease T1 digestion products of [^{32}P]-tRNA$^{Phe}_N$. Material eluted from the spots (ribonuclease T1 digestion products) of the fingerprint in Figure 3 for [^{32}P]tRNA$^{Phe}_N$ was digested with ribonuclease I and subjected to high-voltage electrophoresis in DEAE paper in pH 3.5 buffer (no urea). The identification of the fragments was made by reference to the known migration values (Barrell, 1971).

voltage electrophoresis (7 M urea, pH 3.5) and the second dimension was also by electrophoresis on DEAE-cellulose paper in 7% formic acid; see Figure 3. For each fingerprint, the individual spots were eluted and split into two portions. One set was digested with ribonuclease I and electrophoresis in one dimension on DEAE-cellulose (pH 3.5 buffer) (Figure 4). The second set was digested further with ribonuclease T2 to completely recover the mononucleotides which were subsequently resolved by thin-layer chromatography (Figure 5).

Table II gives the sequence of each of the ribonuclease T1 fragments as deduced by (1) position of the fragment in the two-dimensional fingerprint (from Figure 3); (2) analysis of the ribonuclease I digestion products (Figure 4); and (3) determination of the total composition of each spot (from Figure 5). In all cases, the three methods gave consistent results.

For tRNA$^{Phe}_N$, 15 of the 16 expected fragments could be identified. The remaining spot, no. 1, corresponding to Um^{7}GXCCUUG, was tentatively identified solely by its position in the fingerprint. Its actual composition, however, could not be determined since in the tlc system used the R_F values of m^{7}G and X were not known. The fingerprint pattern was identical with that of Barrell and Sanger (1969) with the exception of spot 5 (AAms^2-i^{6}AAψCCCCG) which migrated slightly ahead of rather than slightly behind spot 4 (AUUCCG). Two spots containing minor nucleosides were shown to be partially undermodified; *i.e.,* the analysis of these spots was consistent with their being a mixture of two fragments, one containing the modified nucleoside, the other containing the unmodified parent compound. These were spot 10, DCG + UCG, and spot 3, TψCG + TUCG. Only in tRNA$^{Phe}_{II}$ were spots 9 and 10 resolved completely. They were identified as DAG and UCG, respectively. In tRNA, the two fragments were not well separated, as shown by subsequent analysis. The unexpected presence of U in this mixture (spots 9 + 10 in Figures 4 and 5) indicated that one of the two fragments was only partially modified. Since DAG appears to be more stable than DCG, the latter was considered to be the partially unmodified fragment. The close correspondence between the ribonuclease T1 digest products of tRNA$^{Phe}_N$ and those of tRNA^{Phe} as described by Barrell and Sanger (1969) makes it highly likely that the species of tRNA$^{Phe}_N$ isolated from ^{32}P-labeled, chloramphenicol-treated *E. coli* indeed represents the normal form of *E. coli* tRNA^{Phe}.

A similar analysis of the two chloramphenicol-induced forms of [^{32}P]tRNA^{Phe} revealed that most of the ribonuclease fragments were identical with their counterparts in normal tRNA^{Phe}, as judged by their position in the fingerprints. However, some of the fragments which ordinarily contained minor nucleosides were shown to be unmodified in the chloramphenicol-induced forms. The extent of modification was determined for the minor nucleosides, ψ, T, and ms^2-i^{6}A (Table II). Ribothymidine located 54 nucleotides from the 5′ end (T-54) in spot 3 (see Figure 3) was present in all three forms as was D-20 (in spot 9). Pseudouridine-32 (in spot 2), ψ-39 (in spot 5), and D-16 (in spot 10), however, although present in normal tRNA^{Phe}, were absent in both of the chloramphenicol-induced forms. Pseudouridine-55 (in spot 3) appeared to be partially modified in the chloramphenicol-induced forms in a manner similar to that previously described for spot 3 in tRNA$^{Phe}_N$.

Only one distinct difference between tRNA$^{Phe}_I$ and tRNA$^{Phe}_{II}$ was found. It involved the fragment AAms^2-i^{6}AAψCCCCG (Figure 3). In tRNA$^{Phe}_I$, this spot (normally spot 5) was missing, and another spot, designated spot *,

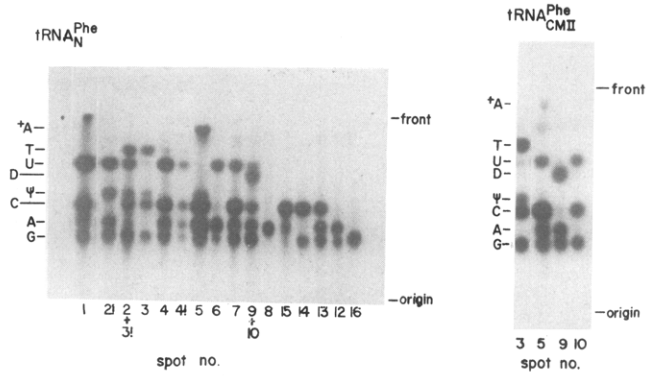

FIGURE 5: Thin-layer chromatography of the ribonuclease T2 digestion products of the ribonuclease T1 digestion products of [^{32}P]-tRNA$^{Phe}_{II,N}$. Material eluted from each of the spots (ribonuclease T1 digestion products) of the fingerprint for tRNA$^{Phe}_{N}$ and from the indicated spots of the fingerprints for the chloramphenicol-induced forms (Figure 3) were digested with ribonuclease T2 and subjected to thin-layer chromatography on cellulose in 2-propanol–concentrated HCl–H_2O. Identification of the mononucleotides was made by reference to established R_F values (B. G. Barrell, personal communication). For tRNA$^{Phe}_{II}$, the samples for spot 5 (AAms^2-i^{6}AΨCCCCG) and spot 7 (CUCAG) were inadvertently applied to the same origin. Although the appearance of the modified adenosine (*A) was evident, no Ψ could be seen. It was concluded that the material from spot 5 contained U in place of Ψ and had the composition AA*AAUCCCCG. The identity of the modified adenosine (*A) could not be made since 6-isopentenyladenosine (i^{6}A) and ms^2-i^{6}A have the same R_F value in this solvent system. The results for spot 2 were too faint to photograph. Visual inspection, however, clearly showed the absence of Ψ and an increased intensity of the U spot, indicating that the fragment was AUUG instead of AΨUG. The results for tRNA$^{Phe}_{I}$ were identical with those for tRNA$^{Phe}_{II}$ except that spot 5 was missing and spots 9 and 10 were not resolved. The details for spot 5 are given in the text. The mixture of spots 9 and 10 revealed the presence of both D and U. Since tRNA$^{Phe}_{II}$ was shown to contain, unambiguously, DAG for spot 9 and UCG for spot 10, it is most likely that the same sequences occur in tRNA$^{Phe}_{I}$. The results of spot 11 for tRNA$^{Phe}_{N}$ are not shown here, but identification from a subsequent experiment gave UCCG.

was present. Although a relationship between spot 5 and spot * has not been shown it seems likely that the loss of spot 5 in the fingerprint of tRNA$^{Phe}_{I}$ is due to an undermodification of the only remaining residue in the fragment which is susceptible to undermodification, namely, ms^2-i^{6}A.

Discussion

Comparative two-dimensional fingerprinting is a useful method for determining sequence similarities between samples of polymeric material such as tRNA. The method has been used here to study the three forms of [^{32}P]tRNA^{Phe} obtained from chloramphenicol-treated *E. coli*. For each of the separation systems used it was apparent that both of the chloramphenicol-induced forms were very similar to normal tRNA^{Phe}. Composition analysis was carried out on the spots from the fingerprints in Figure 3. In the case of normal tRNA, good agreement was obtained between the observed fragments and those expected from the sequence reported by Barrell and Sanger (1969).

The extent of the differences for several minor nucleosides has been compiled in Table II. The conclusions to be drawn from these data are (1) the absence of several minor nucleosides does not abolish phenylalanine acceptor function; (2) the biosynthesis of those minor nucleosdes which were present in all three forms of tRNA^{Phe} (T-54, D-20, and possibly Ψ-55) is not dependent on the prior biosynthesis of those minor nucleosides which were shown not to be present in all three forms (*viz.*, D-16, Ψ-32, Ψ-39, and possibly ms^2-i^{6}A-36); (3) the two dihydrouridine residues of tRNA^{Phe} (D-16 and D-20) are not synthesized simultaneously, whereas two of the pseudouridine residues (Ψ-36 and Ψ-39) may undergo coordinated synthesis: the third (Ψ-55) is synthesized independently; and (4) the synthesis of ribothymidine (T-54) and one of the dihydrouridines (D-20) appears to be unaffected by chloramphenicol treatment. Figure 6 expresses these results in terms of the familiar cloverleaf structure of tRNA.

TABLE II: Comparison of Differences of the Ribonuclease T1 Digestion Products between Normal tRNA^{Phe} and tRNA$^{Phe}_{I,II,N}$ from Chloramphenicol-Treated *E. coli*.

Spot	tRNA$^{Phe}_{I}$	tRNA$^{Phe}_{II}$	tRNA$^{Phe}_{N}$	tRNA^{Phe} [a]
1			Likely	Um^{7}GXCCUUG
2	AUUG	AUUG	AΨUG	AΨUG
3	TΨCG + TUCG	TΨCG + TUCG	TΨCG + TUCG	TΨCG
4			AUUCCG	AUUCCG
5	Absent	AA*AAUCCCCG	AA*AAΨCCCCG	AAms^2-i^{6}AAΨCCCCG
6			A*UAG	As^{4}UAG
7			CUCAG	CUCAG
8			pG	pG
9	DAG	DAG	DAG	DAG
10	UCG	UCG	DCG + UCG	DCG
11			UCCG	UCCG
12			AG	AG
13			CAG	CAG
14			CCCG	CCCG
15			$CACCA_{OH}$	$CACCA_{OH}$
16			G	G
*	Present	Absent	Absent	Absent

[a] From Barrell and Sanger (1969). Definite identification of *U (spot 6) as being 4-thiouridine (s^{4}U) was not made. The identification of *A (spot 5) as being either 2-methylthio-6-isopentenyladenosine (ms^2-i^{6}A) or 6-isopentenyladenosine (i^{6}A) was not made.

The lack of modification of D-16 as well as the normal synthesis of T-54 as found in the chloramphenicol-induced forms is in agreement with the findings of Jacobson and Hedgcoth (1970) who reported that bulk tRNA from chloramphenicol-treated *E. coli* contained a reduced level (50%) of D and a normal level of T. However, the authors also claimed that the level of ψ was unaffected by chloramphenicol treatment. That observation was not substantiated by the work presented here, since the synthesis of two of the three ψ residues in $tRNA^{Phe}$ was shown to be blocked by chloramphenicol treatment. This difference may simply be due to the limits of resolution when using bulk tRNA.

Also using bulk tRNA from chloramphenicol-treated *E. coli* cells, a 30–40% reduction in the contents of four thiouridine and dihydrouridine was observed recently by Waters *et al.* (1973).

Attempts to modify tRNA extracted from chloramphenicol-treated cells were made by Waters (1969). The chloramphenicol-induced $tRNA^{Phe}$ did not accept any exogenous methyl group from *S*-adenosylmethionine. This is indicative that these tRNA's are not undermethylated. Further studies show that methylation of total tRNA during chloramphenicol treatment is quantitatively and qualitatively normal (Waters *et al.*, 1973). Our observation in this study that ribothymidine (T54) is present in all these forms of $tRNA^{Phe}$ is consistent with this finding. Methylthiolation of the isopentenyladenosine, a reaction suspected to take place during the conversion of form II to N, has not been studied here. By inference from other work on $tRNA^{Tyr}$ (Gefter and Russell, 1969), this modification apparently takes place in steps, first by the addition of the isopentenyl group to adenosine (I → II), followed by methylthiolation of the same moiety at the C-2 position (II → N). The degree of modification of this adenosine (A-36) determines, then, the relative affinity of each of the $tRNA^{Phe}$ forms to the RPC-5 column as shown by increasing ionic strength of the buffer required to elute each form.

During other studies with modification *in vitro* it was shown that the minor bases i^6A, T, and ψ varied in amount in precursor $tRNA^{Tyr}$, and i^6A was persistently lower (Shaefer *et al.*, 1973). Since all nucleotide modifications are catalyzed by enzymes at the polymeric state of tRNA precursor (Söll, 1971), a difference in the affinity of these enzymes with the substrate would be one plausible explanation to this observation. It is interesting to point out that in the preparation of $tRNA^{Tyr}$ precursor coded by phage $\phi 80$, chloramphenicol was introduced at 50 μg/ml to prevent cell lysis. If various modification enzymes have different half-lives, the accumulation of the abnormal forms may also be explained by the differential effect of chloramphenicol on the activity of the various modification enzymes in the tRNA maturation pathway. Kinetic analysis of the appearance of chloramphenicol-induced forms of $tRNA^{Phe}$ during chloramphenicol treatment and during recovery from such treatment (Waters, 1969; Mann and Huang, 1973) has lent support to this alternative explanation.

While the present study rests on the supposition of a blockage by chloramphenicol in the conversion of the immediate precursors, undermodified but functional, to the mature fully modified form of $tRNA^{Phe}$, we have not excluded other explanations. One further explanation, although unlikely for lack of precedents, is that chloramphenicol induces a new structural gene for $tRNA^{Phe}$ and turns off in the mean time the first structural gene. The answer to this would require complete sequence analysis of all three forms

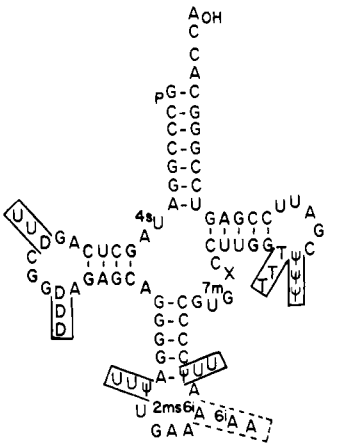

FIGURE 6: Primary nucleotide sequence of *E. coli* $tRNA^{Phe}$ indicating the positions of unmodified nucleosides in $tRNA^{Phe}_{I,II}$. The primary nucleotide sequence of *E. coli* $tRNA^{Phe}$ is reproduced here from the work of Barrell and Sanger (1969). The outlined residue positions indicate those minor nucleosides which have been identified in the three forms of $tRNA^{Phe}$ from chloramphenicol-treated *E. coli*. In each box, the outermost symbol represents the nucleoside identified for $tRNA^{Phe}_{I}$; the middle symbol, for $tRNA^{Phe}_{II}$; and the innermost symbol, for $tRNA^{Phe}_{N}$. At position 36 (dashed outline) only the suspected changes are designated. Chemical verification has not been obtained.

of $tRNA^{Phe}$ comparatively examined here. However, results unpublished in this laboratory have shown that this effect of chloramphenicol on the induction of new forms of tRNA is not restricted to the *E. coli* strain used here. This makes less likely the possibility of the induction of a new gene, of, for example, plasmid or viral origin. Also, a variety of nutritional conditions have been tested and many give a similar effect. These include notably amino acid starvation and uracil deprivation, but not thymine starvation (Huang and Mann, 1973). Low levels of phosphate coupled to slow generation time also induce the formation of form II (Mann and Huang, 1974). These observations make a general defect in specific modification processes more appealing than a second gene hypothesis. Recently, Chase *et al.* (1974) reported the appearance of no less than nine additional forms of *E. coli* $tRNA^{Val}$ as a result of adverse growth conditions. They proposed that all were undermodified forms of the three principal isoacceptors of $tRNA^{Val}$. Mutants of *Salmonella typhimurium* exist which display differential impairment in the ability of modification enzymes for carrying out synthesis at different sites on $tRNA^{His}$ (Singer *et al.*, 1972). A similar situation occurs also in $tRNA^{Leu}$ (Allauden *et al.*, 1972) in the same organism.

In summary, the evidence presented in this study reveals that the chloramphenicol-induced forms of $tRNA^{Phe}$ are undermodified in dihydrouridine, pseudouridine, and presumably 2-methylthio-6-isopentenyladenosine. The data are consistent with a precursor–product relationship between the three forms as suggested earlier (Mann and Huang, 1973).

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References

Allauden, H. S., Yang, S. K., and Söll, D. (1972), *FEBS*

(*Fed. Eur. Biochem. Soc.*) *Lett. 28,* 205.
Barrell, B. G. (1971), *Procedures Nucleic Acid Res. 2,* 780.
Barrell, B. G., and Sanger, F. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3,* 275.
Brownlee, G. G., and Sanger, F. (1969), *Eur. J. Biochem. 11,* 395.
Chase, R., Tener, G. M., and Gillam, I. C. (1974), *Arch. Biochem. Biophys. 163,* 306.
Demushkin, V. P., Nelidova, O. D., and Budovskii, E. I. (1971), *Mol. Biol. USSR 5,* 689.
Friedman, S. (1972), *Biochemistry 11,* 3435.
Gefter, M., and Russell, R. (1969), *J. Mol. Biol. 39,* 145.
Gillam, I., Blew, D., Warrington, R. C., von Tigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7,* 3459.
Griffin, B. E. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 15,* 165.
Huang, P. C., and Mann, M. (1973), *Biophys. J. 13,* 283a Abstr.
Jacobson, M., and Hedgcoth, C. (1970), *Biochemistry 9,* 2513.
Mann, M. B., and Huang, P. C. (1973), *Biochemistry 12,* 5289.
Mann, M. B., and Huang, P. C. (1974), *J. Bacteriol. 118,* 209.
Marmor, J. B., Dickerman, H. W., and Peterkofsky, A. (1971), *J. Biol. Chem. 246,* 3464.
Rubin, G. M. (1973), *J. Biol. Chem. 248,* 3860.
Sanger, F., Brownlee, G. G., and Barrell, B. G. (1965), *J. Mol. Biol. 13,* 373.
Shaefer, K. P., Altman, S., and Söll, D. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 3626.
Singer, C. E., Smith, G. R., Cortese, R., and Ames, B. N. (1972), *Nature (London), New Biol. 238,* 72.
Söll, D. (1971), *Science 173,* 293.
Waters, L. C. (1969), *Biochem. Biophys. Res. Commun. 37,* 296.
Waters, L. C., Shugart, L., Yang, W. T., and Best, A. N. (1973), *Arch. Biochem. Biophys. 156,* 780.

DNA Photoreactivating Enzyme from Placental Mammals. Origin and Characteristics†

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ABSTRACT: DNA photoreactivating enzyme in human leukocytes is concentrated in the phagocytotic monocytes and polymorphonuclear cells. Lymphocytes, erythrocytes, spleen, and serum contain little if any enzyme activity. Since bone marrow, which contains immature, nonphagocytotic monocytes and polymorphonuclear cells, also contains high levels of enzyme, it is unlikely that the enzyme in the mature cells resulted from bacteria engulfed by these cells. Photoreactivating enzyme is also found in murine, and in human cells in culture; confluent murine cells have higher specific activity than do rapidly growing cells. The leukocyte enzyme, which requires ultraviolet-irradiated DNA as substrate and visible light for catalysis, converts pyrimidine dimers in DNA to the corresponding monomers in the light-dependent reaction. The action spectrum for photoreactivation extends from 300 to 600 nm, with a peak at about 400 nm.

Ultraviolet light (220–300 nm) produces cyclobutylpyrimidine dimers[1] between adjacent pyrimidines on the same DNA strand. These dimers have been shown to be a major cause of death and mutation in prokaryotes and in the simple eukaryote *Paramecium* (Setlow and Setlow, 1973; Kimball, 1969; Sutherland *et al.*, 1967). The photoreactivating enzyme repairs dimer-containing DNA in a multistep reaction: the enzyme binds to the DNA, presumably at the dimer, forming a metastable complex (Rupert, 1962). On absorption of a photon in the range 300–600 nm, the enzyme catalyzes the photolysis of the cyclobutane ring, thus producing two monomer pyrimidines, and restoring biological integrity to the DNA (Setlow and Setlow, 1963).

Although the enzyme had been found in all groups of all phyla except the placental mammals (Cook and McGrath, 1967), a photoreactivating enzyme has recently been isolated from human leukocytes (Sutherland, 1974). This discovery is of particular interest as a potential analytical tool: the photoreactivating enzyme acts specifically and exclusively on pyrimidine dimers (Setlow and Setlow, 1963). Thus, if ultraviolet light (uv) induced biological damage can be reversed in a true photoenzymatic reaction, dimers were a major contributor to the production of the damage. In human cells a case of interest is the induction of skin cancer by ultraviolet light (Epstein, 1971).

We show here that the enzyme from leukocytes is of mammalian origin (rather than from bacteria engulfed by the cells), that the activity represents a genuine photoreactivating enzyme, and that the action spectrum for photoreac-

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[1] The name cyclobutadipyrimidine has also been suggested for the cyclobutylpyrimidine dimer (Madden *et al.*, 1973; Cohn *et al.*, 1974).