The Nucleotide Sequence of the GGG-specific Glycine Transfer Ribonucleic Acid of Escherichia coli and of Salmonella typhimurium*

C. W. HILL, GABRILE COMBRIATO, AND WILLIAM STEINHART
From the Department of Biological Chemistry, The Pennsylvania State University, Hershey Medical Center, Hershey, Pennsylvania 17033

DONALD L. RIDDLE* AND JOHN CARBON
From the Section of Biochemistry and Molecular Biology, Department of Biological Sciences, University of California, Santa Barbara, California 93106

SUMMARY
The primary nucleotide sequence of tRNA\textsubscript{Gly} (glycine tRNA specific for the codon GGG) from Escherichia coli and Salmonella typhimurium has been determined. The E. coli tRNA sequence is pG-C-G-G-G-C-G-6-A-G-U-U-C-A-A-U-G-G-D-A-G-A-G-C-U-U-A-U-C-C-C-A-A-G-C-U-C-C-C-G-U-C-U-A-U-A-C-G-A-G-G-G-C-U-C-C-A, where 6 at position 8 is most likely 4-thiouracil. The sequence of the S. typhimurium tRNA\textsubscript{Gly} is identical with that of E. coli except that no evidence for the modification of U at position 8 has been found. In addition, a portion of the S. typhimurium tRNA\textsubscript{Gly} has a 2'-O-methyl modification of the G at position 17. Some unusual features of the tRNA structure are discussed.

EXPERIMENTAL PROCEDURE
Bacterial and Bacteriophage Strains—To facilitate specific labeling and isolation of tRNA\textsubscript{Gly} a λ transducing phage (λ\textsuperscript{gly}+) carrying the E. coli gly U+ gene was isolated. This transducing phage was derived from a λ lysogen, KS72, which has the λ prophage integrated near lysA (8). Details of the isolation and characterization of the transducing phage will be published elsewhere. The transducing phage and the helper phage both carry the λ mutations, ~1857 (thermal inducibility) and S (lysis inhibition), and the defective transducing phage bears the E. coli lysA+ gene as well as glyV. For the tRNA labeling and preparation, strain CH719 (lysA- gly U+ lydI30 argH-) was lysogenized with both helper phage and λ\textsuperscript{gly}+ to produce strain CH722. The S. typhimurium strain used as a source of wild type gly U+ tRNA was TR1526 (hisO1Z?@Z isc57d serA790 @s-554), a derivative of strain LT2.

Growth and Thermal Induction of E. coli Lysogen—CH722 was grown at 34°C in 200 ml of medium containing 12.1 g of Tris (adjusted to pH 7.4 with HCl), 2 g of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.2 g of MgSO\textsubscript{4} 7H\textsubscript{2}O, 0.5 mg of FeSO\textsubscript{4}, 200 mg of arginine, 100 mg of lysine, 100 mg of isoleucine, 100 mg of valine, and 5 g of glucose per liter, to which KH\textsubscript{2}PO\textsubscript{4} was added to 0.4 mM. When the optical density,
measured at 590 nm, reached 1.2, the culture was shifted to 40°
by adding an equal volume of preheated (45°) medium of the
same composition, but containing no phosphate. Incubation
was continued at 40° with shaking. Twenty minutes after the
temperature shift, 50 mCi of carrier-free [32P]phosphoric acid
(New England Nuclear Corp.) were added. Forty-five minutes
after the temperature shift, chloramphenicol was added to a
concentration of 50 µg per ml. The cells were harvested 3 hours
after the temperature shift. At this time, about 40% of the
labeled phosphate was incorporated into acid-precipitable ma-
terial. If the phage does not have the S° mutation, lysis occurs
60 min after thermal induction.

**Growth of S. typhimurium**—Low phosphate medium contained,
per liter: 1.5 g of KCl, 5.0 g of NaCl, 1.0 g of NH4Cl, 2.5 g of
MgSO4, 2.0 g of glucose, and 4 g of low phosphate peptone (9).
An inoculum grown in the above medium was diluted 1:50 into
80 ml of fresh medium containing 10 to 25 µCi of carrier-free
[32P]phosphoric acid (International Chemical and Nuclear
Corp.), and the cells were grown at 37° with shaking. Growth
ceased upon incorporation of 90 to 95% of the [32P]phosphate,
and the cells were harvested by centrifugation.

**Extraction of tRNA**—E. coli tRNA was prepared by the method
of Zubay (10) and was carried through the isopropl alcohol
precipitation step. S. typhimurium tRNA was prepared as
follows. The cell pellet was suspended in 5 ml of TM buffer
(1 mM Tris-chloride, 10 mM MgCl2, pH 7.5), and 2 mg of un-
fractionated tRNA from TR1526 were added as carrier. RNA
was extracted from the cells by shaking with 5 ml of buffer-
saturated phenol (1 part TM buffer to 5 parts phenol) for 1 hour
at room temperature. The mixture was centrifuged to separate
the phases and the aqueous layer was collected. The phenol
was washed with 5 ml of fresh buffer, and the combined aqueous
fraction (10 ml total) was placed on a DEAE-cellulose (Whatman
DE23) column (1.0-ml column volume) equilibrated with TM
buffer. All salt solutions used with this column contained TM
buffer. After application of the RNA sample, the column was
washed with 10 ml of 0.02 M NaCl buffer, followed by 10 ml of
0.2 M NaCl buffer. The tRNA was eluted from the column in 10
ml of 1.0 M NaCl buffer and precipitated by the addition of 2
volumes of ethyl alcohol. The DEAE-cellulose column step is
necessary because direct ethyl alcohol precipitation of the tRNA
was not quantitative. The column step also removes labeled
protein, DNA, and high molecular weight RNA from the pre-
paration. The tRNA was deaminacylated by suspension in 2
ml of 0.1 M Tris-chloride, pH 9.0, for 20 min at 37°; then precipi-
tated by the addition of NaCl to 0.2 M and 2 volumes of ethyl
alcohol. The tRNA pellet was washed by suspension in 2 ml of
ethyl alcohol, then 2 ml of ethyl ether, and dried in a vacuum
desiccator.

**Purification of [32P]tRNA**—E. coli tRNA was aminocacyl-
ated as follows. The 4.8-ml reaction mixture contained 6 µg of
[32P]tRNA, 10 mM MgCl2, 1 mM ATP, 10 mM KCl, 0.1 mM sodium
cacodylate (pH 7.0), 4 mM glutathione, 0.05 mM [3H]glycine (4
µCi per µmole, New England Nuclear Corp.), and dialyzed E.
coli 100,000 X g supernatant fraction containing 80 units of
glycyl-tRNA synthetase. The mixture was incubated for 20
min at 37° and extracted with phenol; 0.1 volume of 20% sodium
acetate (pH 4.5) was added, and the tRNA was precipitated by
adding 3 volumes of ethyl alcohol. A similar procedure was
used for the aminocacylation of S. typhimurium tRNA.

The most satisfactory chromatographic system for the puri-
fication of tRNA was found to be the RPC-5 reversed phase chro-
matography system (11, 12). Advantages of this system
over the benzoylated DEAE-cellulose system (13) were faster
column operation and better material recovery, and the columns
could be reused repeatedly. The major disadvantage was
slightly poorer separation of the isoaccepting glycyl-tRNA
species. [3H]Glycyl-tRNA was fractionated on a RPC-5 column
as follows. All buffers contained, in addition to NaCl, 0.01 M
sodium acetate (pH 4.5), 0.01 M MgCl2, and 0.005 M mercapto-
ethan-10. The first column had dimensions of 0.9 X 55 mm
and was equilibrated with 0.3 M NaCl buffer. The aminocacylated
tRNA was dissolved in 0.5 ml of buffer containing 0.3 M NaCl;
to this solution, 0.5 ml of 50% sucrose was added. This was
applied to the top of the column and overlayed with the same
buffer without sucrose. The sample was washed onto the column
under pressure, and the wash was continued until 30 ml of eluate
had been collected. The jacketed column was maintained at 26°
throughout. The column was eluted with a 400-ml, 0.5 to 0.6 M
NaCl linear gradient. Two-milliliter fractions were collected
every 2.5 min; 0.05 ml of each fraction was either precipitated
with 2 x 1 MCl and collected on 25 µM GF/C glass filters or
dried directly on the filters. Results are shown in Fig. 1. To the
pooled fractions, 2 mg of carrier tRNA were added, and the mix-
ure was precipitated with 3 volumes of ethyl alcohol. The
aminocacylated tRNA was phenoxacyetlated as described by Gillam
et al. (14) and reapplied to a smaller RPC-5 column (0.9 X 25 cm)
as above. Elution was carried out with a 400-ml, 0.5 to 0.7 M
NaCl linear gradient (Fig. 2). After the addition of 0.2 mg of
carrier tRNA, the pooled fractions were precipitated as above,
dissolved in 1 M Tris (pH 9.0), incubated at room temperature for
1 hour to effect deaminacylation, and reprecipitated. In an
alternate procedure, the pooled glycyl-tRNA from the second
column was concentrated by means of a DEAE-cellulose column
as follows. A column (0.4-ml volume) was constructed by remov-
ing the top 0.6-ml segment of a 1.0-ml pipette. The column
was packed with 100 µl of DEAE-cellulose (Whatman DE23) in
1.0 M NaCl, 0.01 M MgCl2, and 0.01 M sodium acetate (pH 4.5),
equilibrated with the same buffer containing 0.2 M NaCl.
The tRNA pool from the RPC-5 column was diluted by the addi-
tion of 2 volumes of buffer in order to lower the NaCl concentra-
tion to approximately 0.2 M, and it was pumped onto the column
at 40 ml per hour. The tRNA was eluted from the column in
1.0 ml of 1.0 M NaCl buffer and precipitated in 2 volumes of
ethyl alcohol overnight at -20°. The tRNA was centrifuged
and resuspended in 0.1 ml of water for storage at -20° and use
in fingerprint analysis.

**Sequencing Techniques**—The protocols of Barrett (15) were
followed for the digestion and fingerprinting of the [32P]tRNA,
as well as for characterization of the resulting oligonucleotides
by alkaline hydrolysis, dephosphorylation with bacterial alkaline
phosphatase, snake venom phosphodiesterase digestion, pan-
eric RNase digestion, T1-RNase digestion, U2-RNase diges-
tion, and carboximide blocking. In addition to the procedures
described by Barrett (15), polynucleotide phosphorylase was used
as described by Madison et al. (16) to determine the 5'-trinucleo-
tide diphasophate of certain fragments. In this case, the oligo-
nucleotide was treated with HCl, as described by Barrett, to
decyclize cyclic 2',3'-phosphate and then dephosphorylated with
alkaline phosphatase (1 µg of enzyme with 10 µg of carrier tRNA
in 10 µl of 0.1 M Tris chloride, pH 8.2, for 30 min at 37°); this
mixture were added 10 µl of 0.04 M KH2PO4 (pH 7.2) and 0.008 M
MgCl2 containing 40 µg of polynucleotide phosphorylase, and
digestion was continued for 2 hours. Under these conditions,
we found the major degradation product to be the trinucleoside
diphosphate from the 5' end of the oligonucleotide; in some cases
a small amount of the S'-terminal dinucleoside monophosphate was also obtained. For the partial digestion to obtain overlaps, 0.5 to 1 μCi of [32P]tRNA was digested with T1-RNase for 10 min at 0°, with an enzyme to tRNA weight ratio of about 1:600. The material was fractionated by electrophoresis and thin layer homochromatography with Homomixture b as described by Barrell (15), except that, in some of the work on the E. coli species, homochromatography was carried out at 37° instead of 60°. Modified nucleotides were characterized by descending chromatography on Whatman No. 1 paper using Systems a and b as described by Barrell (15).

Materials—Materials were obtained from the following sources: pancreatic RNase A, snake venom phosphodiesterase, spleen phosphodiesterase, and E. coli alkaline phosphatase (BAP-F) from Worthington Biochemical Corp.; T1-RNase from both Worthington and Calbiochem; U2-RNase from Ustilago, a gift of Dr. H. Okazaki of the Sankyo Co.; polynucleotide phosphorylase from Miles Laboratories, Inc.; the carbodiimide blocking reagent, N-cyclohexyl-N'-2-(4-morpholino)ethyl carbodiimide methyl-p-toluene sulfonate, from both Fluka and Aldrich Chemical Co., Inc.; yeast RNA from Sigma Chemical Co.; RP-14 (1314 x 17 inch) medical x-ray film from Eastman Kodak Co.; Whatman DE51 DEAE-cellulose paper (50-m roll) from Fisher, and cellulose acetate strips, No. 2500 (30 mm x 570 mm), from Schleicher and Schuell, Inc. The toluene-based scintillation fluid contained Liquifluor (New England Nuclear Corp.).

RESULTS

Purification of E. coli tRNA

We estimate that tRNA constitutes less than 1% of the total E. coli tRNA. Thus, isolation of the pure species in sufficient quantities for sequence determination is difficult. Since the work on the amber suppressor strain of tRNA had demonstrated the value of utilizing a transducing phage for the amplification and specific labeling of a tRNA species (17), a similar approach was adopted for this work. A defective λ phage which carried the lggA+ glyU+ region of the E. coli host was derived from a lysogen, K979, which has the λ prophage integrated near lggA (8). A lysogen carrying the glyU+ transducing phage was used to obtain selective labeling of tRNA with [32P]phosphate, as described under "Experimental Procedure." The tRNA was isolated from the cells and amnonoacylated with [3H]glycine. The tRNA was then fractionated on a reversed phase column (RPC-5) as shown in Fig. 1. The large peak of [3H]glycyl-[32P]tRNA from Fractions 56 to 60 is tRNA. The size of the peak reflects an 8- to 10-fold amplification of tRNA derived from evenely labeled, nonlysogenic E. coli. The second large peak of [3H]glycyl-[32P]tRNA (Fig. 1) is the major portion of the S. typhimurium tRNAGly and is 85 to 90% pure. As is discussed below, the smaller peak in Fractions 35 to 38 is also tRNA, but it contains a 2'-O-methyl-G modification.

The material in Fractions 56 to 60 was pooled for further purification. O, [3H]; •, [32P].

Fig. 1. Escherichia coli strain CH72 [3H]glycyl-[32P]tRNA was applied to RPC-5 column (0.9 x 55 cm) as described under "Experimental Procedure." The tRNA was eluted using a 400-ml, 0.5 to 0.6 M NaCl gradient in 0.01 M MgCl2, 0.01 M sodium acetate (pH 4.5), and 0.005 M β-mercaptoethanol. Fractions (190) of approximately 2.0 ml were collected, one fraction every 2.5 min. The material in Fractions 56 to 60 was pooled for further purification. O, [3H]; •, [32P].

Purification of S. typhimurium [32P]tRNA—S. typhimurium [3H]glycyl-[32P]tRNA was initially fractionated by either RPC-5 chromatography or benzoylated DEAE-cellulose chromatography. The appropriate fractions were pooled, phenoxacyetylated, and applied to a RPC-5 column. Chromatography of [32P]tRNA pooled from a benzoylated DEAE-cellulose column is shown in Fig. 3. The major peak of [3H]glycyl-[32P]tRNA (Fractions 41 to 47) is the major portion of the S. typhimurium tRNA and is 85 to 90% pure. As is discussed below, the smaller peak in Fractions 35 to 38 is also tRNA, but it contains a 2'-O-methyl-G modification.

Fig. 2. The pooled material from Fig. 1 was phenoxacyetylated as described under "Experimental Procedure" and applied to a RPC-5 column (0.9 x 25 cm). The material was eluted with a 400-ml, 0.5 to 0.7 M NaCl gradient in 0.01 M MgCl2, 0.01 M sodium acetate (pH 4.5), and 0.005 M β-mercaptoethanol. Fractions (79) of approximately 5.0 ml were collected, one fraction every 6 min. The material in Fractions 36 to 40 was pooled and concentrated for use in tRNA sequence determination. O, [3H]; •, [32P].

Purification of S. typhimurium [32P]tRNA—S. typhimurium [3H]glycyl-[32P]tRNA was initially fractionated by either RPC-5 chromatography or benzoylated DEAE-cellulose chromatography. The appropriate fractions were pooled, phenoxacyetylated, and applied to a RPC-5 column. Chromatography of [32P]tRNA pooled from a benzoylated DEAE-cellulose column is shown in Fig. 3. The major peak of [3H]glycyl-[32P]tRNA (Fractions 41 to 47) is the major portion of the S. typhimurium tRNA and is 85 to 90% pure. As is discussed below, the smaller peak in Fractions 35 to 38 is also tRNA, but it contains a 2'-O-methyl-G modification.
Fig. 3. Salmonella typhimurium [3H]glycyl-[32P]tRNA, derived by fractionation on benzoylated DEAE-cellulose, was phenoxy-acetylated and chromatographed on a RPC-5 column (0.9 X 25 cm) using a 400-ml, 0.5 to 0.8 M NaCl gradient for elution. The major peak (Fractions 41 to 47) and the minor peak (Fractions 35 to 37) were pooled separately and concentrated for use in tRNA sequence determination. •, 3H; □, 32P.

T1-RNase Fingerprints—Purified E. coli [32P]tRNA$^{\text{Gln}}$ was digested with T1-RNase and subjected to two-dimensional electrophoresis as described by Barrel (15); the result is shown in Fig. 4. In all, 14 T1-RNase fragments were found. Fragments t13 and t14, as shown in Fig. 4, are not well resolved, but they may be resolved by prolonged electrophoresis in 7% formic acid or by homochromatography. As is described later, the sequence of each of the fragments has been determined and is as designated in Table I. As anticipated from the specificity of T1-RNase, all but one of the fragments have G- as the 3'-terminal residue. The exception is Fragment t1 (C-U-C-C-A), which must be the 3'-terminal sequence of the tRNA molecule. When fingerprints were made from tRNA which had not been deaminoacylated, Fragment t1 carried the [3H]glycine label. Table I also lists the molar yields of the fragments. With the exception of the di- and mononucleotides, each occurs in approximately one molar yield. Fragment t9 streaks during the DEAE-7% formic acid electrophoresis, probably due to the presence of a modified base. The fingerprint of the major peak of S. typhimurium tRNA$^{\text{Gln}}$ was virtually identical with that of E. coli, except that Fragment t9 did not streak.

Nucleotide Composition and Pancreatic RNase Digestion of T1-RNase Fragments—Each T1-RNase fragment was hydrolyzed with NaOH, the products were separated, and the nucleotide composition of each fragment was determined (Table II). Fragment t7 gave only one product, and this product moves faster than does U- during paper electrophoresis at pH 3.5. The same nucleotide is present in pancreatic Fragment p15 (see below), where it has been shown to be pGp. Electrophoresis of nucleotides produced by alkaline hydrolysis gave evidence of modified bases in only Fragments t8 and t10. Fragment t8 contains D-
Venom phosphodiesterase digestion was carried out on oligonucleotides which had been dephosphorylated with bacterial alkaline phosphatase. Base composition after venom phosphodiesterase digestion was determined as described above. The products of pancreatic RNase digestion of the T1-RNase fragments were separated by DEAE-paper electrophoresis at pH 3.5, and the resulting di- and trinucleotides were characterized by alkaline digestion as above.

Identified by the electrophoretic and chromatographic properties of its alkaline decomposition product (15). Fragment t10 contains both T- and W- , which were also identified by their electrophoretic and chromatographic properties (15). The probable presence of a modified base in Fragment t9 is discussed below.

Several of the T1-RNase fragments were digested with pancreatic RNase, and the products were separated by electrophoresis on DEAE-paper at pH 3.5. The resulting di- and trinucleotides were elucidated and the nucleotide compositions were determined after alkaline hydrolysis. The pancreatic RNase products found in each case are listed in Table II. The combined data from nucleotide composition analysis and pancreatic RNase digestion were sufficient for the complete sequence determination of Fragments t2 through t9 (Table II).

Sequence of Fragment t1—The nucleotide composition of Fragment t1, as determined by alkaline hydrolysis, is C-1,U- (Table II). Complete digestion with snake venom phosphodiesterase gave 2 moles of -C-1, 1 of -U-, and 1 of -A- (Table II). The gain of an A and the loss of a C in the venom phosphodiesterase digestion establishes the 3’ end of Fragment t1 as an unphosphorylated A and the 5’ end as C-. Partial digestion with venom phosphodiesterase produced four products which were further characterized by alkaline and complete venom phosphodiesterase digestion. These four products were C-(C-1,U-2)A, C-(C-1,U-2)C, C-U-C, and C-U (Table III). Since venom phosphodiesterase sequentially removes 3’-mononucleotides from the 3’ end of an oligonucleotide, we conclude that the sequence of Fragment t1 is C-U-C-C-A.

Sequence of Fragment t11—Nucleotide composition and pancreatic RNase digestion of Fragment t11 (Table II) established that this fragment is (U-2,C-3)A-A-G- . Complete venom phosphodiesterase digestion of the dephosphorylated fragment proved that the 5’ residue is C (Table II). Fragment t11 was derivatized with the carbodiimide reagent, as described by Darrell (15), to make the U residues resistant to pancreatic RNase digestion. Two products were obtained after pancreatic RNase digestion in addition to free C. They were shown to be U-U-C- and A-A-G- by alkaline hydrolysis (Table III). The additional information necessary for the complete analysis of Fragment t11 was obtained by digestion with polynucleotide phosphorylase as described under “Experimental Procedure.” The limit products obtained on digestion of a dephosphorylated oligonucleotide are the trinucleoside diphosphate and the dinucleoside monophosphate derived from the 5’ end of the oligonucleotide. When this procedure was applied to Fragment t11, the major product obtained was C-U-U (Table III). Evidence for this structure was that alkali digestion produced equal amounts of C- and U-, whereas venom phosphodiesterase digestion gave only -U-. From this it is concluded that the complete sequence of Fragment t11 is C-U-C-C-A-A-G-.

Sequence of Fragment t10—Fragment t10 has the nucleotide composition (T-,W-,C-)G- (Table II). Partial digestion of this fragment with spleen phosphodiesterase leads to the accumulation of a product containing 1 residue each of W-, C-, and G- (Table III). From this result, we can conclude that the 5’ residue is T-. A small amount of C-G- was also found after partial spleen phosphodiesterase digestion. Venom phosphodiesterase digestion of dephosphorylated Fragment t10 produced three products in equimolar amounts. These were C-, C-, and a third product (Table III) which was concluded to be T-W- since alkaline digestion of this product produced T-. Harada et al. (18) reported finding these same products after the digestion of T-W-C-G with venom phosphodiesterase. These results show that the sequence of Fragment t10 must be T-W-C-G. This conclusion was verified by the finding that carbodiimide blocking followed by pancreatic RNase digestion results in two products: (T-,W-)C- and G- (Table III).

Sequence of Fragment t12—As described in Table II, Fragment
Oligonucleotides eluted from T1-RNase or pancreatic RNase fingerprints were treated as indicated. Polynucleotide phosphodiesterase digestion was performed as described under “Experimental Procedure”; other enzymatic digestion procedures and the carbodiimide derivatization procedures were as described by Barrall (15). Electrophoretic mobilities are expressed in relation to the mobility of the blue dye marker (xylene cyanole FF). Nucleotide composition was determined as shown in Table IV. Table IV presented in approximately single molar yields except Fragment p4 also shows that all fragments larger than mononucleotides are present in approximately single molar yields except Fragment p4 (G-C), which is present twice, and Fragments p7 (G-U-) and p7α, which are always found in very low yield. As is discussed below, Fragments p7 and p7α are probably related and derived from the same position in the total sequence. Similar results are obtained for S. typhimurium tRNA, except that G-U-

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a = electrophoresis was on DEAE paper at pH 3.5
b = electrophoresis was on DEAE paper in 7% formic acid
c = electrophoresis was on DEAE paper at pH 1.9
d = electrophoresis was on DEAE paper at pH 3.5

t12 has the nucleotide composition of (C-3,A-2,U-3,G-3) and contains A-C- and A-U- sequences. Complete venom phosphodiesterase digestion of the dephosphorylated oligonucleotide established C as the 5' residue (Table II). Digestion with phosphodiesterase digestion of dephosphorylated Fragment t13 showed that the single A residue occurs at the 5' end of the fragment (Table II). Pancreatic RNase digestion of Fragment t14 after derivatization with the carbodiimide reagent produced, in addition to C- and G-, two products of nucleotide composition (A-,U-,U-A-), namely U-U-C- (Table III). The final information necessary for deducing the sequence was obtained by partial digestion of Fragment t14 with spleen phosphodiesterase. Among the products obtained was the trinucleotide (C-,U-)G- (Table III). The sequence of all Fragments t1-RNase fragments are found. The sequence of all Fragments t1-RNase fragments is identical with those of E. coli as determined by essentially identical procedures. Pancreatic RNase fingerprint of tRNA2Gm—Fig. 5 is a pancreatic RNase fingerprint of E. coli tRNA2Gm. Sixteen pancreatic RNase fragments are found. The sequence of all fragments has been determined as shown in Table IV. Table IV also shows that all fragments larger than mononucleotides are present in approximately single molar yields except Fragment p4 (G-C), which is present twice, and Fragments p7 (G-U-) and p7α, which are always found in very low yield. As is discussed below, Fragments p7 and p7α are probably related and derived from the same position in the total sequence. Similar results are obtained for S. typhimurium tRNA, except that G-U- is
present in one molar yield. Yields of Fragments p14 and p16 are variable; the most likely reason for this variability is difficulty in transferring these G-rich fragments from the cellulose acetate strips.

Sequence of Pancreatic RNase Fragments—The nucleotide composition of each pancreatic fragment, determined after alkaline hydrolysis, is listed in Table V. D- in Fragment p3 and T- in Fragment p16 were identified as described above for these same modified bases in Fragments t8 and t10, respectively. Alkaline or T1-RNase hydrolysis of Fragment p8 produced two products: C- and a product containing 2 32P eq and moving faster (with streaking) than did U- during paper electrophoresis at pH 3.5. Dephosphorylated Fragment p8 had a mobility identical with authentic dephosphorylated G-C. Therefore, Fragment p8 is G-C-. T1-RNase digestion was performed on all fragments containing both A- and G-, and the resulting products were characterized by their electrophoretic mobility on DEAE-paper at pH 3.5 (15) and by their nucleotide composition (Table V). From the data given in Table V, the sequence of all of the pancreatic RNase fragments except Fragments p15 and p16 can be deduced.

Fragment p15 (G-A-G-G-C-) was dephosphorylated and the nucleotide composition of the fragment was found to be G-A-G by alkaline hydrolysis and -G-A-G-C- by venom phosphodiesterase hydrolysis. This shows that G is the 5' residue of Fragment p15. The sequence must be G-A-G-A-G-C-.

Alkaline hydrolysis of Fragment p16 suggested that the nucleotide composition of the fragment was (G-A-T-) when results were calculated with the assumption of 1 T- per oligonucleotide (Table V). However, the yield of A- was consistently low, ranging from 0.7 to 0.9 mole per mole of T- in various determinations. This low yield of A- casts some doubt on the assignment of 0 A- residues to the oligonucleotide. T1-RNase digestion produced 0.9 mole of A-G- and 3.0 moles of G- per mole of T-; this unequivocally shows the composition of Fragment p16 to be (G-A-G-G-T-). Fragment p16 was dephosphorylated and digested with polynucleotide phosphorylase. The major product was G-A-G (Table III) as shown by alkaline and snake venom phosphodiesterase digestion. A very small amount of G-A was also found. Since the G-A-G must be derived from the 5' end of the fragment, the complete sequence of Fragment p16 must be G-A-G-G-G-T-.

As was found for the T1-RNase fragments, the sequences of S. typhimurium pancreatic fragments are identical with those of E. coli.

Determination of Total Sequence—Once the sequence of each T1-RNase and pancreatic RNase digestion product is known, the total tRNA sequence may be determined from overlapping oligonucleotides obtained by partial enzymatic digestion. The overlaps obtained by partial digestion of the tRNA with T1-RNase are shown in Fig. 6. The oligonucleotides were fractionated by electrophoresis and thin layer homechromatography as described under “Experimental Procedure.” The oligonucleotides were eluted from the thin layer plates; one half was digested completely with T1-RNase and the other half was digested with pancreatic RNase. The products were fractionated by electrophoresis on DEAE-paper and characterized by position, nucleotide composition, and enzymatic digestion. The results of these procedures are shown in Table VI.

Analysis of the fragments listed in Table VI allows deduction of the complete sequence of tRNA$^\text{e}$ (Fig. 6). The deductive reasoning is as follows. Complete T1-RNase digestion of Fragment a produces three products: A-U-U-C-C-C-U-U-C-G-,
TABLE V
Composition of pancreatic RNase fragments

Each pancreatic RNase fragment was hydrolyzed with alkali, and the products were separated by electrophoresis on paper at pH 3.5, and the spots were counted using liquid scintillation counting. The pyrimidine nucleotide was assumed to be in one molar yield, and the yields of the purine nucleotides were calculated accordingly. Those fragments indicated were also digested with Tt-RNase, and the products were separated by electrophoresis on DEAE-paper at pH 3.5. Each di- and trinucleotide obtained was further characterized by alkaline hydrolysis and electrophoresis as above. D- and T- were identified by acidic and basic chromatography (15); -G- was identified as described in the text.

<table>
<thead>
<tr>
<th>Fragment number</th>
<th>Alkali</th>
<th>Other</th>
<th>T1-RNase products</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1</td>
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</tr>
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</tr>
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</tr>
<tr>
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<td>1.1</td>
<td>(-G-) 0.9</td>
<td></td>
</tr>
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<td>1.1</td>
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</tr>
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</tr>
<tr>
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<td>1.7</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>p15</td>
<td>1</td>
<td>0.7</td>
<td>3.6 (T-) 1</td>
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</tr>
<tr>
<td>p16</td>
<td></td>
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</tr>
</tbody>
</table>

Fig. 6. Summary of overlapping T1-RNase fragments. Sequences of T1-RNase partial digestion fragments of *Escherichia coli* tRNA<sub>60</sub> were deduced from the data in Table VI as indicated in the text.

C-C-C-G-, and C-U-C-C-A. As discussed above, C-U-C-C-A must be the 3' end of the intact tRNA and consequently the 3' end of Fragment a. Of the two possible ways of assembling the other two Tt-RNase components of Fragment a, only the sequence A-U-C-C-U-C-U-G-C-G-C-G-C-G-C-G-A must result in the one A-U- and two G-C- dinucleotides that are found when Fragment a is digested with pancreatic RNase (Table VI). This establishes the sequence of residues 56 to 74 in Fig. 6. Fragment b contains all of Fragment a plus G-, A-G-, T-G-C-G-, and C-U-C-U-A-G-C-G. Pancreatic digestion of Fragment b produces G-A-G-G-G-T- and G-A-U-, along with other oligonucleotides. G-A-G-G-G-T- must overlap T-G-C-G-, since these fragments contain the only T- in their respective catalogs. Fragment c has two sequences in common with Fragment b: A-G- and C-U-C-U-A-G-C-G. The A-G- of Fragment b must come from within the pancreatic fragment G-A-G-G-G-T- and cannot be the 5' end of Fragment b. Therefore, we can conclude that C-U-C-U-A-U-A-C-G- is at the 5' end of Fragment b; the only way to assemble the Tt-RNase fragments to produce G-A-U- after pancreatic RNase digestion is as in the sequence given for residues 39 to 74 as shown in Fig. 6. Partial Fragment c, in addition to C-U-C-U-A-U-A-C-G-, contains C-U-C-C-C-A-A-G- and 2 eq of A-G-.

In order to yield the pancreatic RNase products A-G-A-G-C- and A-A-G-C-, the complete sequence of Fragment c must be that shown in Fig. 6, establishing the sequence of residues 26 to 74. Partial Fragment d contains all of Fragment c plus A-A-C-G-, whereas partial Fragment e contains all of Fragment d plus D-A-G-. This extends the established sequence to residues 18 to 74. Fragment h has A-A-C-G- and D-A-G- in common with Fragment e, as well as G-, U-A-G-, and U-U-C-A-U-G. The G-G-D- found in the pancreatic digest of Fragment h places...
Table VI

Fragments produced by limited T1-RNase digestion

<table>
<thead>
<tr>
<th>Partial fragment</th>
<th>Products of complete T1-RNase digestion</th>
<th>Products of complete RNase digestion a</th>
</tr>
</thead>
</table>

* excluding U- and C- mononucleotides

The G-residue in the sequence G-G-D-A-G-, whereas the A-G-U- could be produced only if U-A-G- is on the 3' side of U-U-C-A-A-U-G-. This establishes the sequence of residues 8 to 74. Other than G-, Fragment m has only U-A-G- in common with Fragment h, while adding 2 eq of C-G-, 2 of G-, and pG- to the 5' end of the tRNA. The occurrence of pG-C- and G-G-G-C- in the pancreatic digest is compatible only with the sequence indicated for Fraction m in Fig. 6. Therefore, the entire sequence is as illustrated in Fig. 6. All of the other partial fragments obtained are consistent with this sequence. The sequence of 74 nucleotides depicted in Fig. 6 is in agreement with the molar yields of T1-RNase fragments (Table I) and pancreatic RNase fragments (Table IV) actually found.

Nature of U- at Position 8—The T1-RNase Fragment t9 (U-A-G-) and the pancreatic Fragment p7 (G-U-) have in common the U-residue at position 8 in the complete sequences (Fig. 7). Several observations suggest that this U-residue is modified, at least in part, in the mature tRNA. First, Fragment p7 is obtained in considerably less than molar yield (Tables I and IV), while t9 tends to streak during the 7% formic acid electrophoresis (Fig. 4). Second, variable amounts of a G-U- fragment are found at position 8 in a large variety of E. coli tRNA species, it seems likely that U- in Fig. 7 is 4-thiouridylic acid. No evidence for a modified U- at this position has been found for the tRNA derived from S. typhimurium.

Modification of G at Position 17—About 25% of the S. typhimurium tRNA carries a 2'-O-methyl modification of the G at position 17 of the sequence (Figure 7). tRNA with this modification chromatographed as a distinct peak (Fig. 3, Fractions 35 to 38), eluting from the final RPC-5 column just ahead of the major peak of glycyl-tRNA. T1-RNase and pancreatic RNase fingerprints obtained from the minor peak differed from fingerprints obtained from the major species as follows. On the T1-RNase fingerprint, Fragment t13 was absent from its normal position. A new spot was present which had migrated just ahead of Fragment t14 in the first dimension and failed to move ahead of Fragment t13 in the second dimension. A 2'-O-methyl modification is known to have this mobility of this product and its chromatographic mobility in solvent System a is identical with that described by Barrett (15) for the dinucleotide 2'-O-methyl-G-G-. The 2'-O-methyl modification prevents enzymatic or alkaline hydrolysis of the dinucleotide. On a pancreatic RNase fingerprint, Fragment p13 (G-G-D-) moved just ahead of its normal position in the second dimension. A 2'-O-methyl modification is known to have this
effect on the mobility of G-G-D- on pancreatic RNase fingerprints (21). Alkaline hydrolysis of the faster moving fragment produced D- and the dinucleotide (2'-O-methyl-G-G-) which migrates slightly more slowly than U- upon electrophoresis at pH 3.5. A small amount of Fragment p13 was detected at the normal position on the fingerprint, probably due to contamination of the minor peak material with the major peak (Fig. 3).

We conclude that the minor peak of tRNA<sub>Gly</sub><sup>G66</sup> differs from the major peak by a modification which affects both Fragments t13 and p13. No other differences were detected. Based on the effect of the modification on the electrophoretic mobility of these oligonucleotides, and the results of electrophoresis and chromatography of the alkaline digestion products, we conclude that the G at position 17 carries a 2'-O-methyl modification and that Fragments t13 and p13 are both derived from the sequence U-U-C-A-A-U-G,-G-D-. The 2'-O-methyl-G modification is commonly found at such a position in tRNA sequences. The observation that only a portion of the tRNA population carries the modification is similar to that reported for tRNA<sub>Met</sub> of E. coli (21).

The 2'-O-methyl modification was detected only in S. typhimurium tRNA<sub>Gly</sub> whereas the U modification was detected only in E. coli tRNA<sub>Gly</sub>. This may be due to differences in the method of purifying the tRNA for sequence analysis, or it may reflect species or strain differences in tRNA modification.

Other Modifications—All of the fragments were eluted from a T<sub>1</sub> RNase fingerprint of E. coli tRNA<sub>Gly</sub> and digested with a combination of pancreatic RNase and spleen phosphodiesterase; the products were fractionated by pH 3.5 electrophoresis. All products were eluted and characterized by descending paper chromatography as in Systems a and b of Barrell (18). These procedures revealed the presence of D- in Fragment t8 and W- and T- in Fragment t10 as noted above. No indication of the presence of any other modified nucleotide was found.

![Cloverleaf model of tRNA<sub>Gly</sub> from E. coli](image_url)

**DISCUSSION**

The nucleotide sequence of tRNA<sub>Gly</sub><sup>G66</sup> is arranged in the cloverleaf pattern in Fig. 7a. The anticodon is C-C-C, as expected for a tRNA which recognizes G-G-G but not G-G-A (23). The structure drawn in Fig. 7a has several unusual features. First, it is possible to make two additional AU base pairs in the anticodon loop of tRNA<sub>Gly</sub><sup>G66</sup> as well as in tRNA<sub>Gly</sub><sup>C66</sup> (Ref. 4; Fig. 7b). As is customary, the anticodon arm has been drawn with five base pairs, leaving an anticodon loop of seven bases. However, hairpin loops containing as few as three bases are possible in RNA chains (24). Second, the dihydrouridine loop of tRNA<sub>Gly</sub><sup>G66</sup> as drawn, is comprised of seven unpaired bases, and is one of the smallest such loops yet reported. Although the size of this loop is quite variable, the dihydrouridine loops of most other tRNAs thus far sequenced contain eight or more bases (25). The only other exception is tRNA<sub>Thr</sub><sup>Gly</sup> of *Staphylococcus epidermidis*, which also has only seven unpaired bases in this loop (26). The *S. epidermidis* tRNA<sub>Thr</sub> does not function in protein synthesis, but it is able to participate in peptidoglycan synthesis.

Third, the bases at positions 15 and 46 in tRNA<sub>Gly</sub><sup>G66</sup> are not complementary. In most tRNA sequences there is a correlation between the purine nucleotide at position 15 and the pyrimidine nucleotide in the extra arm adjacent to the TΨC arm. When the purine is G, the pyrimidine is C, but when the purine is A, the pyrimidine is U. The structural significance of this correlation has been suggested by Levitt (27) in a model for the structure of tRNA<sub>Gly</sub> in which hydrogen bonding between the purine at position 15 and the pyrimidine adjacent to the TΨC arm was proposed. There is evidence for such base pairing in *Sw<sub>11</sub>* tRNA<sub>Thr</sub> (28). A mutational alteration which disrupts this base pair leads to a substantial loss of *Sw<sub>11</sub>* activity (29). However, in tRNA<sub>Gly</sub><sup>G66</sup> such base pairing cannot occur, since the purine at position 15 is A, whereas the pyrimidine in the extra
arm adjacent to the TΨC arm is C (position 46). We conclude that base pairing between these positions, although possible in nearly all tRNA sequences reported thus far, is not a universal feature of tRNA structure. In addition to tRNA$^{Gly}$, yeast tRNA$^{Ala}$ also lacks complementary bases at these positions (30).

Sequence comparison of two tRNA$^{Gly}$ isoacceptors (Fig. 7) suggests that the structure of the amino acid arm might be important in recognition of tRNA$^{Gly}$ by the glycyl-tRNA synthetase. Although over-all sequence homology is low, the amino acid arms of these isoacceptor species are strikingly similar. The amino acid arm is one of the few regions in which tRNA$^{Glu}$ differs from tRNA$^{Glu}$ of E. coli (4, 31). Crotthers et al. (32) have suggested that the 4th nucleotide from the 3' end of tRNA molecules may be a “discriminator” site recognized by aminoacyl-tRNA synthetases, serving to divide all tRNAs into four major groups (30).

In addition to tRNA$^{Gly}$, the anticodons of the G-G-G-specific tRNAs from E. coli (4, 31). Crothers et al. (32) have suggested that the 4th nucleotide from the 3' end of tRNA molecules may be a “discriminator” site recognized by aminoacyl-tRNA synthetases, serving to divide all tRNAs into four major groups (30).

As reported here, the sequences of the G-G-G-specific tRNAs from E. coli and S. typhimurium are identical except for modifications at positions 8 and 17. This finding parallels the finding that both histidine and valine tRNAs from these organisms are recognized by the glutaminyl-tRNA synthetase, and becomes a glutamine acceptor. The sequences of tRNA$^{Gly}$ and tRNA$^{Ala}$ reveal a striking similarity in the sequence of both the amino acid arms adjacent to the TΨC arm are C (position 46). We conclude that base pairing between these positions, although possible in nearly all tRNA sequences reported thus far, is not a universal feature of tRNA structure. In addition to tRNA$^{Gly}$, yeast tRNA$^{Ala}$ also lacks complementary bases at these positions (30).

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C. W. Hill, Gabriele Combriato, William Steinhart, Donald L. Riddle and John Carbon


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