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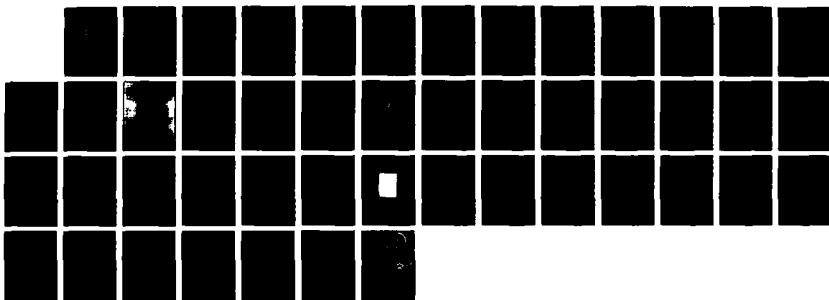
GENETICS STRUCTURE AND FUNCTIONAL FEATURES OF THE RNA
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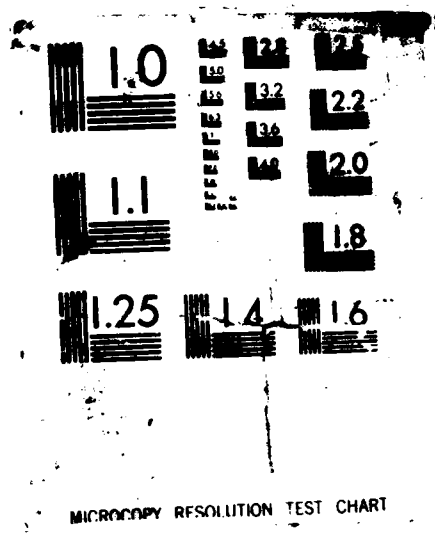
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GENETICS, STRUCTURE AND FUNCTIONAL FEATURES OF THE
RNA MODIFICATION ENZYME, tRNA ψ SYNTHASE I

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Naval Biosciences Laboratory

University of California, Berkeley, CA

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Nucleoside modifications are significant structural components of most classes of cellular RNA. About 50 RNA modifications are known, varying in complexity from simple base or sugar methylations to multifunctional heterocyclic groups. The use of RNA modification enzymes as probes of protein-nucleic acid interaction has been limited by the lack of pure enzymes of this type. Our goal is to develop the pseudouridine modification system for this purpose, by identifying the enzymes, genes and molecular interactions involved in the synthesis of of this nucleoside.

Pseudouridine (5-ribosyl uracil)(ψ) is one of the most abundant RNA modifications and is present in virtually all species of transfer RNA, in the larger ribosomal RNAs of most organisms, and in many eukaryotic nuclear RNAs. These diverse ψ modifications are formed by a family of pseudouridylation enzymes. However, the detailed reaction mechanism, the basis of enzyme specificities and the effects of pseudouridylation on RNA structure are largely unknown. One of these enzymes, tRNA ψ synthase I (PSUI), is the best characterized member of the family and offers a model for the enzyme mechanism, gene organization and expression.

PSUI has been identified as the product of the hisT gene of E. coli and S. typhimurium. hisT mutants lack this modification enzyme and exhibit a variety of growth and regulatory aberrations attributable to reduced rates of protein synthesis. These mutants accumulate a population of "mature" but undermodified tRNAs, which can serve as substrates for quantitation of the enzyme activity. The enzyme assay is based on the obligatory release of a proton from C₅ of the uracil ring when uridine is converted to ψ . By employing hisT [pyrimidin-5-³H]tRNA as the substrate, the reaction is followed by the release of ³H into a water-exchangeable form. This reaction system has allowed the identification of PSUI activity in extracts from bacteria, yeasts, adult, embryonic and tumor tissues and cultured cell lines.

ψ is found at only 7 sites in the tRNA of coliform bacteria. By examining the structures of individual hisT tRNAs, we have identified the site specificity of PSUI. With the exception of residues 38, 39 and 40, all of the other ψ modifications normally found in wild-type tRNAs are present in hisT tRNAs. These include ψ_{32} in the anticodon loop; ψ_{55} in the T ψ loop; ψ_{65} in the T ψ stem; and ψ_{13} in the DHU stem. Thus, the action of PSUI is limited to modification of uridine residues at positions 38, 39 and 40 in the 3'-half of the anticodon loop and stem of tRNA. This enzyme is not involved in the synthesis of ψ in ribosomal RNA in coliform bacteria.

In order to delineate the mechanism in molecular detail, it is necessary to employ purified enzyme and tRNA substrates of known structure. This has become feasible with the isolation and cloning of a 2.3 kb insert containing the E. coli hisT gene into pBR 322.

The resulting plasmid, designated ψ_{300} , expresses the enzyme at 15-20 times the basal level, with no deleterious effect on the host. Extensive restriction mapping of the insert has been carried out and the structural and regulatory regions have been sequenced.

In collaboration with M. Winkler (Northwestern Univ.), it has been found that the *hisT* gene is part of an operon containing a second upstream gene which codes for a 45 kd subunit protein of unknown function. This conclusion stems from the behavior of mutated plasmids and analysis of plasmid-coded proteins in "minicells" and "maxicells". Frameshift mutations and deletions in the upstream gene are highly polar and abolish the production of PSUI. The expression of the two polypeptides is coupled; the 45 kd subunit product protein is synthesized in about 8-fold excess compared to PSUI. The basis for this differential expression may relate to the need for a translational frame shift for initiating the synthesis of PSUI.

Recently, the *hisT* operon has been inserted into a runaway replication plasmid, pBEU50, which can overproduce the enzyme by another 3-5-fold. In this strain, we estimate that PSUI accounts for about 1% and the upstream gene product about 5% of the soluble protein.

PSUI (subunit MW 31,000) has been isolated in more than 90% purity from *E. coli* 294 carrying plasmid ψ_{300} . In common with other tRNA ψ synthases, optimum rates of ^3H release require only a monovalent cation (preferably NH_4^+) and a thiol; no other proteins, cofactors or energy sources are necessary for the reaction. Preincubation with iodoacetate or other sulfhydryl inhibitors irreversibly blocks the activity, pointing to the catalytic role of a cysteine residue. ^3H release is unaffected by pretreatment of the enzyme with micrococcal nuclease, indicating that an RNA component is not involved in the modification reaction. Although the two genes in the *hisT* operon are tightly linked, their products appear to be functionally independent. PSUI can modify all of the *hisT* isoacceptors of tRNA^{His}, tRNA^{Phe} and tRNA^{Leu} to their respective wild-type counterparts. Since this group of tRNAs contains all of the known topological arrangements for the ψ modification of residues 38, 39 and 40, the 45 kd subunit protein is not an accessory functional component of PSUI, or a second synthase needed to modify one of these sites.

We have been investigating possible conformational differences between the wild-type and *hisT* isoacceptors of tRNA^{Phe} and tRNA^{His} from their sensitivity to base-specific and strand-specific nucleases. The wild-type and *hisT* isoacceptor pairs differ significantly in their sensitivity to T_1 , U_2 and S_1 nuclease probes. The differences are not localized to the site of the modification, but extend over much of the anticodon loop and adjacent 3'-region of the tRNA. Thus, the introduction of ψ at residues 38 and 39 appears to produce a general shift in the conformation of the anticodon region. These structural differences may underlie the inefficient translational behavior of *hisT* tRNAs.

Our view of the specific role of ψ is that it provides additional hydrogen bonding sites for the association of an RNA with other nucleic acids or proteins. The formation of ψ may also produce subtle conformational changes in RNA molecules that are necessary to optimize their steric interactions.

Fig 1,2

Introduction: Our laboratory's main interest is the post-transcriptional modifications found in cellular RNAs. Several dozen RNA modifications have been characterized, with structures ranging from simple base or sugar methylations to complex heterocyclic structures which require multiple biosynthetic steps. These modifications probably serve as recognition sites for other nucleic acids or proteins, and are important in various phases of protein synthesis, gene expression and cellular regulation. A surprising portion of the genome in prokaryotes such as *E. coli* -- 1% or more of the coding capacity -- is invested in the structure of RNA modification enzymes.

RNA modification systems could provide excellent models and probes of protein/nucleic acid interaction, in view of their distinctive site specificity and the vast body of RNA structural information. The main barrier to this has been the difficulty in preparing pure modification enzymes. Our purpose is to develop such a model RNA modification system by defining the enzymes, genes and molecular interactions in the biosynthesis of pseudouridine.

Fig. 3

Pseudouridine (Ψ) (5-ribosyl uracil) is one of the most abundant RNA modifications, and is unique in possessing a -C-C- linkage between the base and ribose, in contrast to the usual -N-C- bond. This modification is significant in several respects: (a) RNA sequences containing Ψ have been implicated in attenuation control of bacterial gene expression; in the priming of retroviral reverse transcriptases; and in the action of small nuclear RNAs during mRNA splicing; (b) the enzymes that form Ψ are highly sensitive to fluoropyrimidine anti-cancer agents; (c) the urinary excretion patterns of Ψ are finding practical use in monitoring the presence and treatment of cancer.

Fig. 4

Distribution of Ψ : Ψ is found in virtually all species of transfer RNA, from *Mycoplasma* to mosquitoes, in cytoplasmic, organellar and virally-coded tRNAs. In addition, it is also found in many other types of RNA -- the 16 and 23 S ribosomal RNA of prokaryotes and their eukaryotic homologues; in 5 S RNA of yeasts; in most 5.8 S ribosomal RNAs, all of the small nuclear RNAs (U_1 - U_6) and several nuclear 7-8 S RNAs of less defined function. O-methyl and N_1 -methyl Ψ are found in ribosomal and certain tRNAs, respectively. A more complex modification, N_1 -methyl-3-amino-3-carboxypropyl Ψ is present in yeast and mammalian 18 S RNA; and an amino-alanyl derivative of Ψ , of unknown function, has been detected in plants. It is logical to assume that this group of modifications is formed by a family of pseudouridyl- ation enzymes, but we know little about their specificity or whether there are one or several reaction mechanisms. One of these, tRNA Ψ synthase I, (PSUI) is the best characterized member of this group and offers an excellent model for analysis of an RNA modification reaction. In earlier work, we developed a simple quantitative assay for this activity, based on the fact that obligatory release of a proton from C_5 of the pyrimidine ring when uridine is converted to Ψ . The substrate is a bulk tRNA preparation from *hisT* mutants of *S. typhimurium* which lack the enzyme and accumulate a population of modifiable tRNAs. When these mutants are grown in [5- 3 H]uridine, the tRNAs are specifically tritiated at C_5 of pyrimidines: the formation of Ψ leads to the release of a tritiated proton which is freely exchangeable with water and not absorbable to

Fig. 3

Norit A. Using this assay, PSUI activity was identified in extracts from coliform bacteria and shown to be the product of the hisT gene; analogous activities were detected in yeasts, tissue culture celllines, normal adult, embryonic and tumor tissues. The activity was also extensively purified from S. typhimurium and steer thymus, but sufficient pure protein for mechanistic work could not be obtained because of its low abundance in these sources. The successful cloning of the E. coli gene for PSUI has now made it possible to isolate sufficient protein for molecular studies.

Fig. 5

Site Specificity of PSUI: The specificity of PSUI has been determined by analyzing the structure of individual tRNAs purified from hisT mutants. There are only 7 sites at which ψ is found in coliform tRNAs. The relative 3-dimensional geography of these sites is better defined in Figure 6. Note that some of these (residues 32, 38, 39 and 40) are moderately close together (within ca. 10 Å). The other modifiable sites (residues 13, 55 and 65) are more distant from these and from each other (>20 Å), suggesting that several enzymes are needed for these modifications. In line with this, B. Ames' group at Berkeley discovered that hisT tRNA^{His} lacked the two ψ residues at positions 38 and 39. Similarly, D. Soll et al found that the tRNA^{Leu}1 from a hisT strain lacked the normal ψ ₃₈ and ψ ₄₀ modifications. These findings pointed to positions 38, 39 and 40 as recognition sites for PSUI.

In order to complete the analysis, we have isolated several additional tRNAs containing the remaining ψ modification sites and sequenced their relevant structural regions. The two phenylalanine tRNAs (Phe₁ and Phe₂), and the aspartate and glutamate tRNAs were prepared from both wild-type and hisT strains of S. typhimurium. Nucleoside composition of RNA hydrolyzates was determined by HPLC and sequencing was conducted by chemical, enzymatic and random hydrolysis/post-labeling methods.

Fig. 7

Fig. 8

Fig. 9

Fig. 10

The nucleoside compositions of wild-type and hisT tRNA^{Asp} are identical; both contain ψ at residue 65. ψ ₁₃ is still present in hisT tRNA^{Glu}. ψ ₃₉ is lacking in the anticodon region of hisT tRNA^{Phe}2; ψ ₃₂ is still present in this tRNA. All of the hisT tRNAs also contain ψ ₅₅. Thus, with the exception of residues 38, 39 and 40, all of the ψ modifications normally found in wild-type tRNA are still present in hisT tRNAs. This defines the site specificity of PSUI as limited to these 3 residues (38-40) in the anticodon loop and stem, and indicates that there are from 2-5 enzymes required for the biosynthesis of tRNA in these organisms.

Fig. 11

In addition, the ribosomal RNA of hisT mutants contains a normal complement of ψ . (The 23 S RNA of Salmonella is mostly processed to two fragments of 660 kd and 430 kd; the 16 S RNA does not contain ψ). This indicates that PSUI does not participate in the pseudouridylation of ribosomal RNA in this organism. The varied pleiotropic effects of the hisT mutation must be attributed solely to the structural changes in the affected tRNAs, rather than changes in ribosomal structure.

Fig. 12

In collaboration with Dr. Martin Buck, we have determined the main structural difference between the two Salmonella tRNA^{Phe} isoacceptors. These isoacceptors differ in the modified purine on the 3' side of the anticodon triplet. tRNA^{Phe}2 from both hisT and wild-type cells contains ms²iA at this position, whereas the respective tRNA^{Phe}1 species contain a hydroxylated derivative, ms²io⁶A (Figure).

SUMMARY:

Fig. 15

Structure and Expression of the *hisT* Operon: In order to delineate the mechanism of ψ formation in molecular detail, it is necessary to employ purified enzymes and tRNA substrates of known structure. The isolation of working quantities of PSUI is now feasible, with the isolation and cloning of the *hisT* gene into multicopy plasmid vectors. The genetic and regulatory aspects of this work have been conducted in collaboration with Dr. Malcolm Winkler of Northwestern Univ.

The *hisT* gene was originally identified in the Clarke-Carbon plasmid, 28-44, containing the *purF* gene with which it co-transduces. Subcloning of HindIII fragments into pBR 322 produced a plasmid (ψ_{210}) containing a 10 kb insert with both *purF* and *hisT* genes. A 2.3 kb HindIII-ClaI fragment of ψ_{210} was further subcloned, yielding a plasmid (ψ_{300}) which contained the *hisT* gene, but lacked the *purF* gene. The presence of a *hisT* structural gene in ψ_{300} was confirmed genetically by restoration of PSUI activity after transformation into strains containing *hisT* insertions, missense or amber mutations. The introduction of ψ_{300} into a *hisT* strain also restored the chromatographic properties of tyrosine, histidine and phenylalanine tRNAs to those characteristic of the wild-type isoacceptors. Both ψ_{210} and ψ_{300} express PSUI activity at ca. 20 times the basal level, with no significant effect on the host.

In order to analyze the structure and location of the *hisT* gene, subclones and deletions of plasmid ψ_{300} were constructed and assayed for PSUI activity and for the proteins produced in "minicells" and "maxicells". These experiments showed that: (1) the proximal 1.3 kb region of the gene insert contains the promoter for the *hisT* gene and encodes a 45,000 dalton subunit protein that is not PSUI; (2) the distal 1.0 kb portion of the insert codes for the 31,000 dalton subunit of PSUI; (3) the 45,000 dalton polypeptide is synthesized in about 8-fold excess over that of PSUI; (4) frameshift mutations and deletions in the upstream gene are highly polar and abolish the production of PSUI. The synthesis of the two polypeptides is coupled, suggesting that the two genes are part of a differentially expressed operon. Insertion of mini-MudI(lac Km) phage into various portions of the ψ_{300} insert confirmed that the *hisT* gene is the second (downstream) gene in the operon. The DNA sequence in the region where the two genes abut indicates that the stop codon for the 45,000 dalton polypeptide overlaps the start codon for PSUI. Thus, the differential expression of the two genes may relate to the need for a translational frame shift for initiating the synthesis of PSUI.

We have recently succeeded in cloning the *hisT* operon into a runaway replication plasmid, pBEU 50. At 30°, the plasmid vector is present in a moderate number of copies in the cell. Above 35°, control of plasmid replication is lost and the number of plasmid copies increases continuously. Cell growth and protein synthesis continue for several hours at the higher temperature, usually with great overproduction of plasmid-encoded genes during this period. Eventually, cell growth is inhibited and viability is lost.

The 2.3 kb HindIII-ClaI fragment from ψ_{210} containing the *hisT* operon was cloned into pBEU50 to produce a 10 kb plasmid with the insert disrupting the *tet* gene. When this plasmid was introduced into the *hisT* strain, *E. coli* FB105, a *tet*-sensitive transformant was obtained which showed PSUI activity. The plasmid (designated MJ14/BEU50) was then grown in *E. coli* 294 grown under various conditions. MJ14/BEU50 produced

FIG. 22

ca. 3 times the PSUI activity found with plasmid ψ_{300} at 30° . When the temperature was raised to 39° and kept there for 3 hrs before returning to 30° , another increase of 60% in activity was obtained. This method produces a 100-fold amplification over the basal level in *E. coli*. Even at 30° , there is a significant increase in the plasmid replication and expression of PSUI. SDS-polyacrylamide gel electrophoresis clearly shows the presence of both the 45,000 dalton polypeptide and the 31,000 PSUI polypeptide products of the *hisT* operon in crude extracts. We estimate that these products account for ca. 5% and 1%, respectively, of the crude soluble protein under these optimized conditions. With this approach, the preparation of substantial quantities of these proteins should now be feasible.

FIG. 23

Properties of Purified PSUI: PSUI has been purified 700-fold from *E. coli* 294 bearing plasmid ψ_{300} to about 95% homogeneity, in 10% yield. The pentapeptide sequence, -lys-ile-ala-leu-gly- was determined by for residues 10-14 of the protein, in agreement with the presumptive gene sequence. However, the exact C-terminal sequence is not yet established. The enzyme requires a monovalent cation and a thiol for optimum rates of ^3H release; no external energy source or other cofactors are needed. In the absence of thiol, incubation with DTNB or PCMB progressively inhibits the enzyme. Preincubation with these or iodoacetamide irreversibly blocks the activity, pointing to the role of a cysteine residue in the enzyme mechanism. Incubation of PSUI without tRNA leads to a progressive loss of activity; this occurs even at 25° and accelerates as the temperature is raised. The inactivation is not reversed in the presence of thiol, but can be totally prevented by low levels of a single tRNA, or an unfractionated tRNA mixture.

FIG. 24

FIG. 25

FIG. 26

FIG. 27

Although the two genes of the *hisT* operon are tightly linked and regulated, their products appear to be functionally independent. The purified PSUI can modify all of the *hisT* isoacceptors of tRNA^{His}, tRNA^{Tyr} and tRNA^{Leu} to their respective wild-type counterparts. Since this group of tRNAs contains all of the known topological arrangements for modification of residues 38-40, the 45 kd protein is not an accessory functional component of PSUI, or a second synthase needed to modify one of these sites.

FIG. 28

FIG. 29

Collaborative experiments with Dr. Larry Hardy (UC San Francisco) have shown that fluorouracil (FU)-containing tRNA is a potent inhibitor of the synthase. This inhibition is accompanied by formation of a tight covalent complex between the enzyme and FU-tRNA, which is retained on nitrocellulose filters. The complex is relatively stable to denaturation with SDS and is slowly reversible by chasing with non-labeled FU-tRNA, with a dissociation half-time of about 6 hours. These experiments indicate that relatively stable adducts are formed between the enzyme and FU-tRNAs, in keeping with the proposed mechanism of the reaction (see below), and raise the prospects that they can be used to identify the active center of the enzyme.

FIG. 30

The kinetic features and properties of the enzyme are consistent with a reaction mechanism analogous to that advanced for thymidylate synthetase. In this mechanism, the reaction is an intramolecular rearrangement, involving at least three steps: (a) cleavage of the base-sugar glycosidic bond; (b) a 180° rotation of the base relative to the ribofuranosyl ring and (c) re-formation of the C-C bond of ψ . Each of these steps would be facilitated by the initial nucleophilic

addition of the enzyme to C₆ of the pyrimidine ring. Saturation of the 5,6 double bond by nucleophilic addition would facilitate glycosidic bond cleavage. The covalent bond formed with the enzyme would provide an axis for the ring flip. Finally, an electrophilic substitution at C₅ would be assisted by nucleophilic addition at C₆. The proposed mechanism is consistent with the necessity for a nucleophilic thiol in the reaction, presumably a cysteine residue in PSUI. It also provides an explanation for the enzyme inhibition by FU-tRNA, which could occur via the formation of a highly stable initial adduct, or a rearranged adduct.

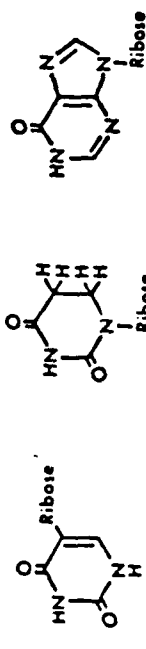
Structural and Functional Consequences of Pseudouridylation: The hisT mutants of E. coli and S. typhimurium show regulatory defects in the expression of several amino acid biosynthetic operons that are best explained by lower rates of translation by hisT tRNAs. While extensive molecular motion appears to be a necessary part of the mechanism, the lack of an external energy requirement suggests that the reaction may produce tRNAs of more stable conformation. Accordingly, we have investigated possible conformational differences between the wild-type tRNAs and their undermodified hisT counterparts based on the susceptibility to structure-specific enzymes (S₁ and mung bean nucleases) and base-specific (T₁ and U₂) nucleases. In these experiments, 3'-end labeled tRNAs were subjected to partial hydrolysis with the enzyme probes under conditions that vary tRNA conformation. The hydrolysis fragments were separated electrophoretically and quantitative estimates were made of the percent of the molecules that cleaved at each site. The results show the following: (1) the anticodon region is generally the most susceptible portion of the molecule to cleavage by single-strand and base-specific nucleases (except for the aminoacyl end). Accessibility of most regions of the tRNA to these nucleases is greatly reduced in the presence of Mg⁺⁺, except for sites in the anticodon region and variable loop. (2) hisT tRNAs, lacking the ψ modifications at positions 38 and 39, are more susceptible than the wild-type tRNAs to the specific nuclease probes, especially in the anticodon region; (3) The differences in nuclease sensitivity are not localized to the sites which lack the ψ modification, but include other residues in the anticodon loop and adjacent stem. The inescapable conclusion is that introduction of ψ at residues 38 and 39 leads to a shift in the conformation of the anticodon loop and adjacent stem. The inefficient translational behavior of hisT tRNAs could result from the suboptimal configuration of the anticodon region.

Fig.
30-35

Supported by Contract # N00014-81-C-0570.

STRUCTURES OF SOME RNA NUCLEOSIDE

MODIFICATIONS

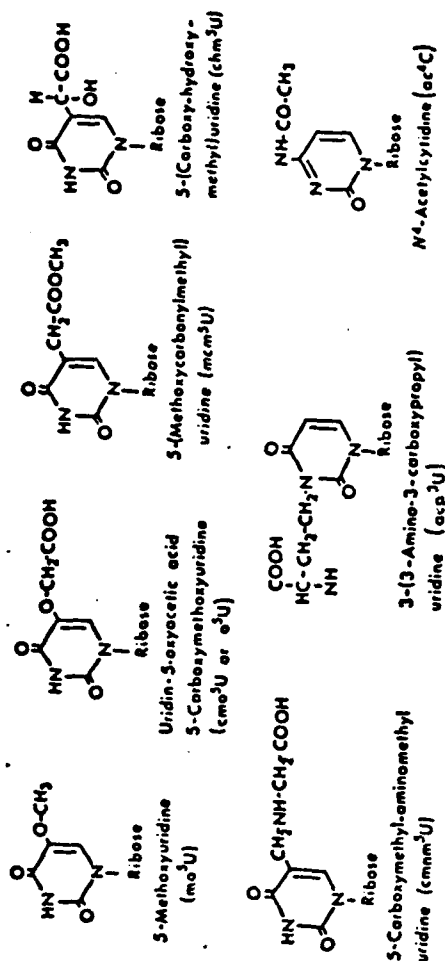


Pseudouridine (U)

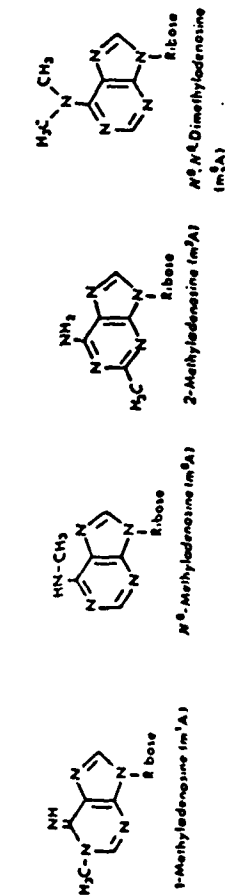
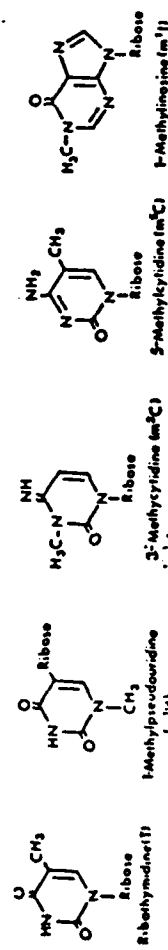
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(ID or HU)

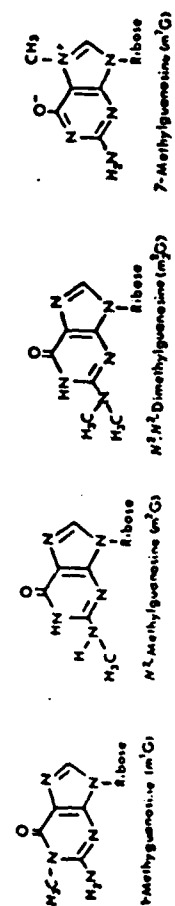
Inosine (I)



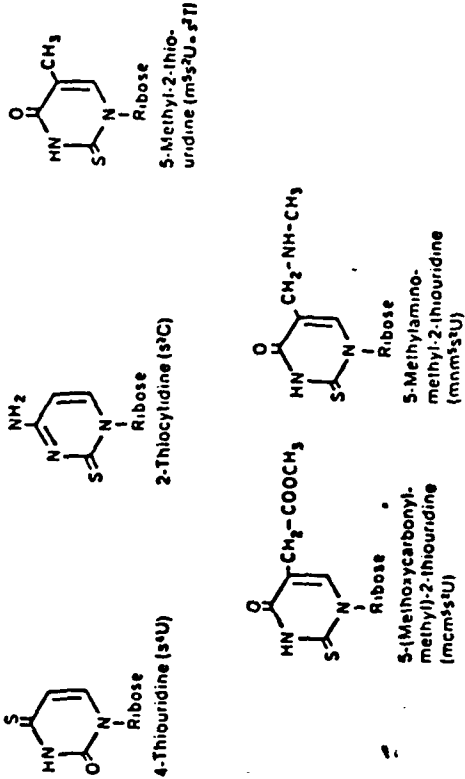
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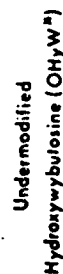
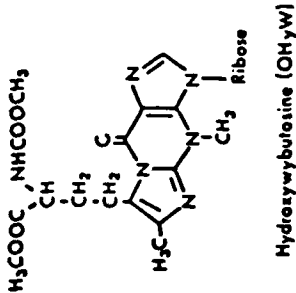
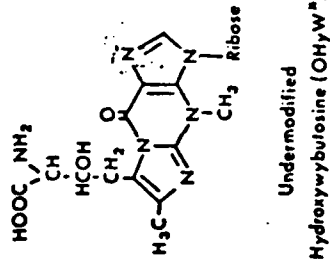
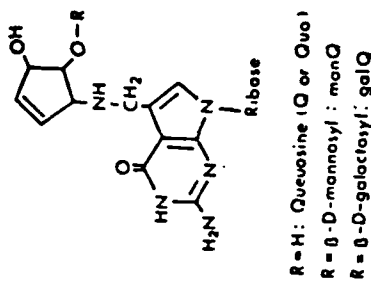
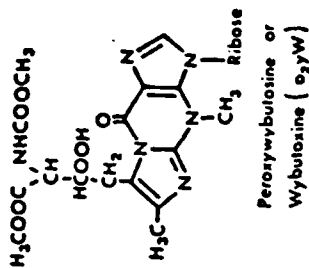
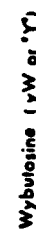
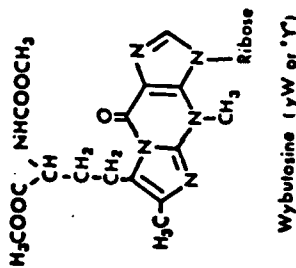
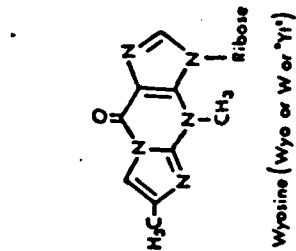
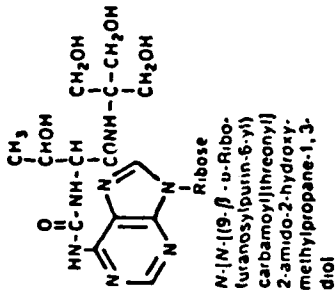
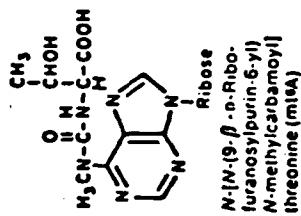
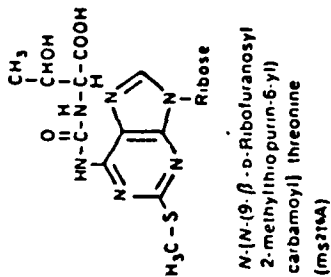
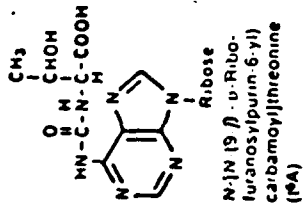


Structures of the base methylated nucleosides found in tRNAs



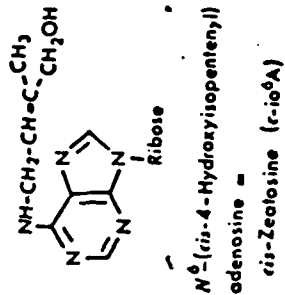
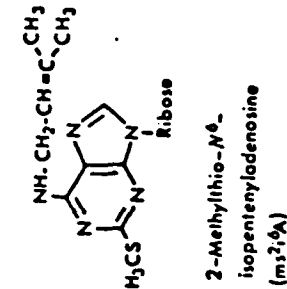
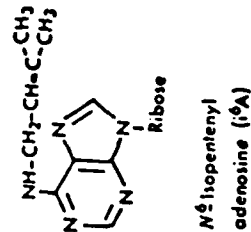
Some thioderivatives of pyrimidic nucleosides





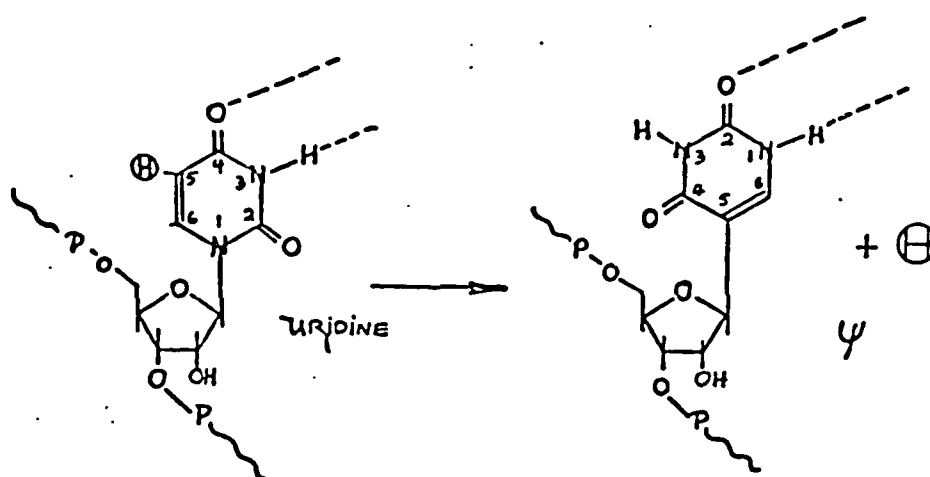
The t⁶A family of purine nucleosides

The W and Q family of hypermodified nucleosides



The i⁶A family of adenosine derivatives

ASSAY FOR tRNA ψ SYNTHASE I



Incubation Mixtures (0.2 ml) contain:

50 mM Tris-HCl, pH 8.0; 100 µg bovine serum albumin;
4.5 mM dithiothreitol; 100 mM NH₄Cl;
0.1 A₂₆₀ unit of [5-³H] hisT or wild-type tRNA (*S. typhimurium*)
(ca. 400,000 cpm);
Enzyme.

Incubation is for 20 min at 37°. Reactions are quenched with 1.0 ml of 15% (w/v) Norit A in 0.1 N HCl. After 30 min at 37°, the mixtures are filtered through 0.45 μ membran filters. 0.5 ml samples of filtrate are mixed with 4.5 ml of "AquaSol" and counted to determine the [^3H] released. Corrections are made for control mixtures, lacking the enzyme.

NATURAL OCCURRENCE OF PSEUDOURIDINE COMPOUNDS

<u>Sources Containing Pseudouridine</u>	<u>Comments</u>
tRNA: from <u>Mycoplasma</u> , <u>Archebacteria</u> , other prokaryotes, bacteriophage-coded tRNAs; protozoans, yeasts, cytoplasmic and chloroplast tRNAs; mitochondrial tRNAs from many sources.	Present in virtually all "elongator" tRNAs, up to 4 or 5 residues/chain.
Ribosomal RNAs: 16S and 23S RNAs and their homologues from <u>Archebacteria</u> , many other prokaryotes, plants and all eukaryotic species examined.	Ψ is absent from the 16S RNA of <u>E. coli</u> and <u>S. typhimurium</u> .
5S ribosomal RNA of several <u>Saccharomyces</u> strains. Absent from most prokaryotic eukaryotic 5S RNAs.	In <u>Saccharomyces</u> , 1 Ψ / chain (121 residues).
5.8S ribosomal RNA of all sources examined, except fungi.	1 or 2 Ψ residues/chain (151-157 residues).
Small Nuclear RNAs:	
U ₁ (nucleoplasmic)	2 Ψ / 165 residues
U ₂ (nucleoplasmic)	12 Ψ / 188-189 residues
U ₃ (nucleolar)	2 Ψ / 210-214 residues
U ₄ (nucleoplasmic)	3 Ψ / 142-146 residues
U ₅ (nucleoplasmic)	3 Ψ / 116-118 residues
U ₆ (perichromatin granules)	3 Ψ / 107-108 residues
7 - 8 S Nuclear RNAs: RNA 7-1, 7-2, 8A, 8B, 7-3	1-2 Ψ / chain in each. Absent from 4.5S and 7S RNAs.

Other Pseudouridine Derivatives:

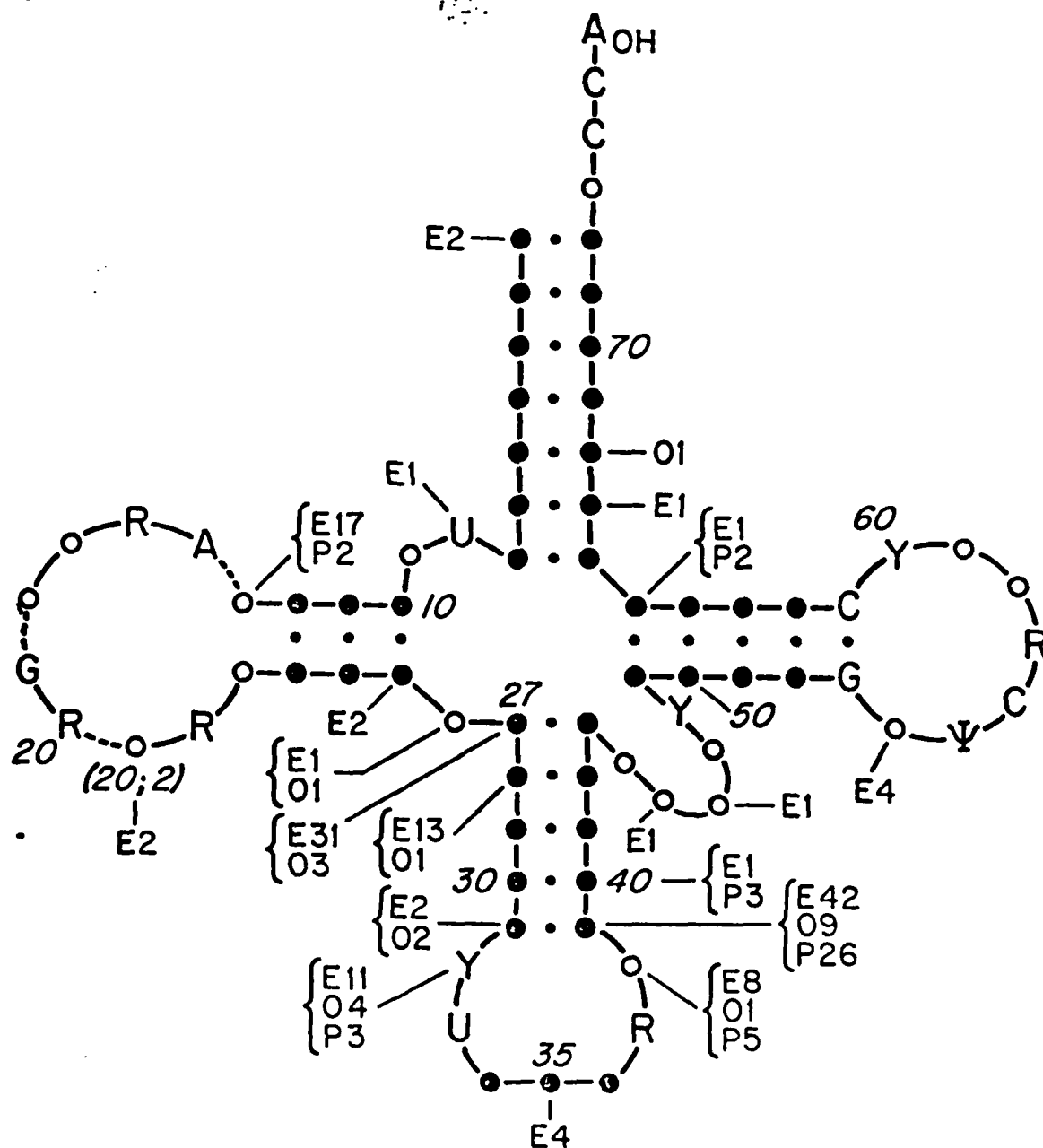
2'-O-methyl- Ψ is found in a number of tRNAs and in many ribosomal RNAs.

m¹ Ψ is an abundant constituent of tRNAs from Halobacterium species, found in place of m⁵U at residue 54.

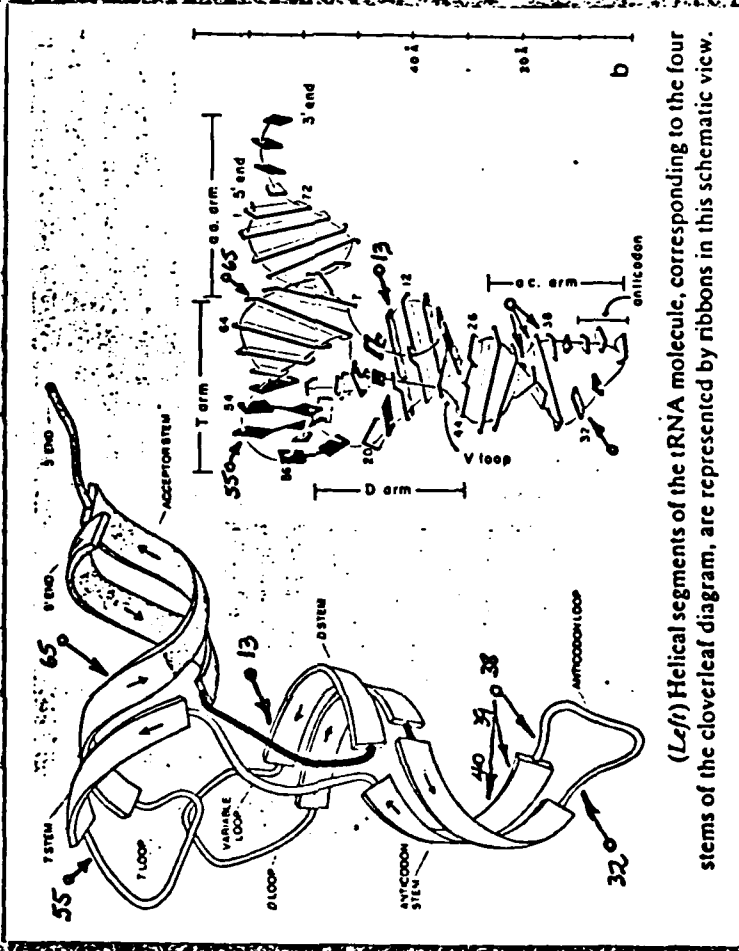
m¹cap³ Ψ is a normal constituent of the 18S ribosomal RNAs of yeasts and mammalian cells.

3-alanyl-6-amino- Ψ has been detected in plants; unknown function.

DISTRIBUTION OF PSEUDOURIDINE RESIDUES IN PROKARYOTIC (P), EUKARYOTIC (E)
AND ORGANELLE (O) TRANSFER RNAs



DISTRIBUTION OF PSEUDOURIDINE IN COLIFORM tRNAs



(Left) Helical segments of the tRNA molecule, corresponding to the four stems of the cloverleaf diagram, are represented by ribbons in this schematic view.

RNA SEQUENCING BY PARTIAL RANDOM HYDROLYSIS METHOD

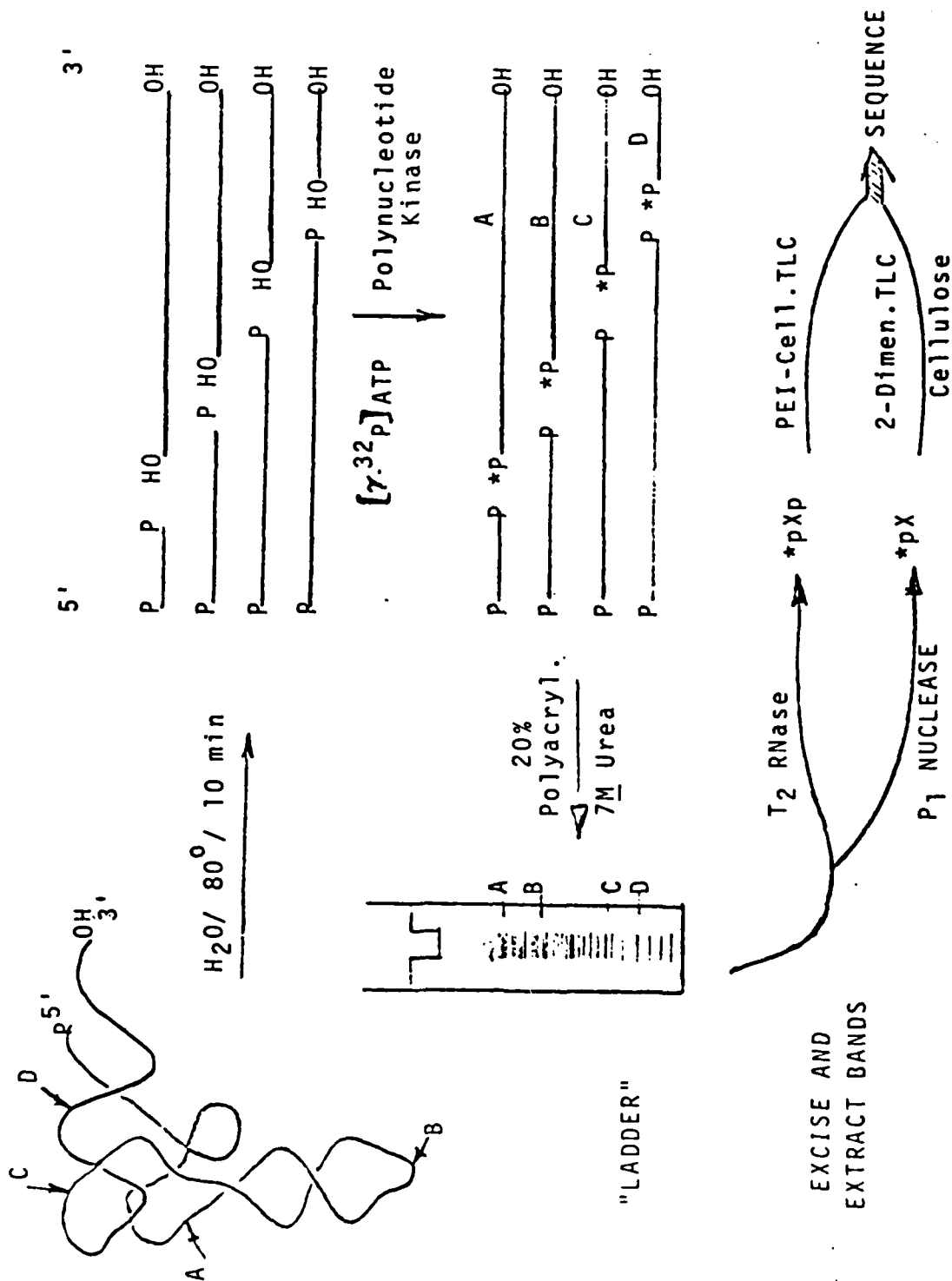


Fig. 7

NUCLEOSIDE COMPOSITION OF S. typhimurium tRNA^{Asp}

NUCLEOSIDE	Relative Molar Ratios:		
	Wild-Type tRNA ^{Asp}	<u>hisT</u> tRNA ^{Asp}	<u>E. coli</u> tRNA ^{Asp*}
ψ	2.2	1.9	2
C	21.3	21.4	22
U	11.3	12.0	10
A	10.1	9.8	10
m ⁷ G	1.2	1.1	1
m ⁵ U	0.7	1.3	1
m ² A	0.9	1.0	1
Q ^{**}	ND	ND	1
s ⁴ U ^{**}	ND	ND	1
D ^{**}	ND	ND	3

* Based on the published sequence for E. coli tRNA^{Asp}.

** Presence identified by 2-dimensional TLC after post-labeling of T₂ ribonuclease hydrolyzates.

NUCLEOSIDE COMPOSITION OF S. typhimurium tRNA^{Glu}

NUCLEOSIDE	Relative Molar Ratios:	
	<u>S. typhimurium</u> <u>hisT</u> tRNA ^{Glu}	<u>E. coli</u> tRNA ^{Glu} ₂
ψ	2.1	2.1 (2)
C	27.3	29.3 (27)
U	9.1	9.9 (9)
A	11.8	13.4 (13)
G	22.6	24.6 (22)
m ⁵ U	1.4	1.0 (1)
m ² A	1.0	1.0 (1)
mm ⁵ s ² U	0.7	0.9 (1)

The values in parentheses are those expected for E. coli tRNA^{Glu}₂, based on its published sequence.

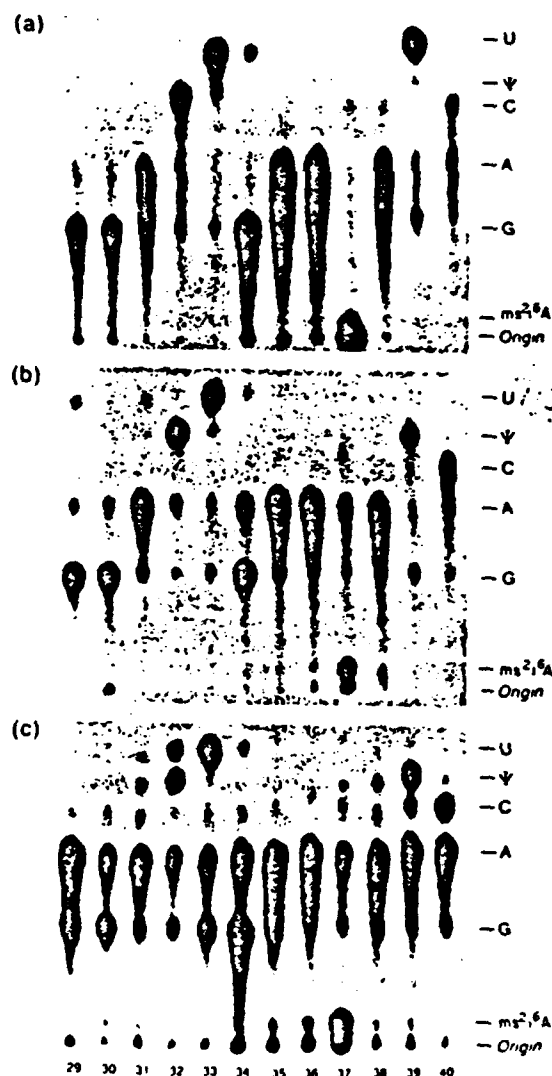


FIG. 7. Nucleotide sequence analysis of the anticodon region of untreated *hisT*⁻ (tRNA)⁺⁺ (a), wild type (tRNA)⁺⁺ (b), and the modified product of *hisT*⁻ (tRNA)⁺⁺ with steer thymus enzyme (c).

NUCLEOSIDE COMPOSITION OF S. Typhimurium

RIBOSOMAL RNA FRACTIONS

Nucleoside	Mole Percent of Nucleoside in:							
	<u>16 S RNA</u>		<u>23 S RNA</u>		<u>660 k RNA</u>		<u>430 k RNA</u>	
	WT	hist	WT	hist	WT	hist	WT	hist
ψ	0.0	0.0	0.39	0.37	0.42	0.38	0.0	0.0
C	23.4	23.5	23.0	22.1	22.5	22.3	23.5	23.2
U	20.5	20.6	21.0	20.6	20.2	21.0	19.5	19.2
A	23.9	23.7	24.9	23.6	23.0	23.9	25.0	25.8
G	32.2	32.2	30.3	33.3	33.9	32.5	32.0	32.9
m ⁵ C	0.22	0.20						
m ⁷ G	0.25	0.22						
m ² G	0.20	0.21						
m ₂ A	0.19	0.19						

FIG. 12

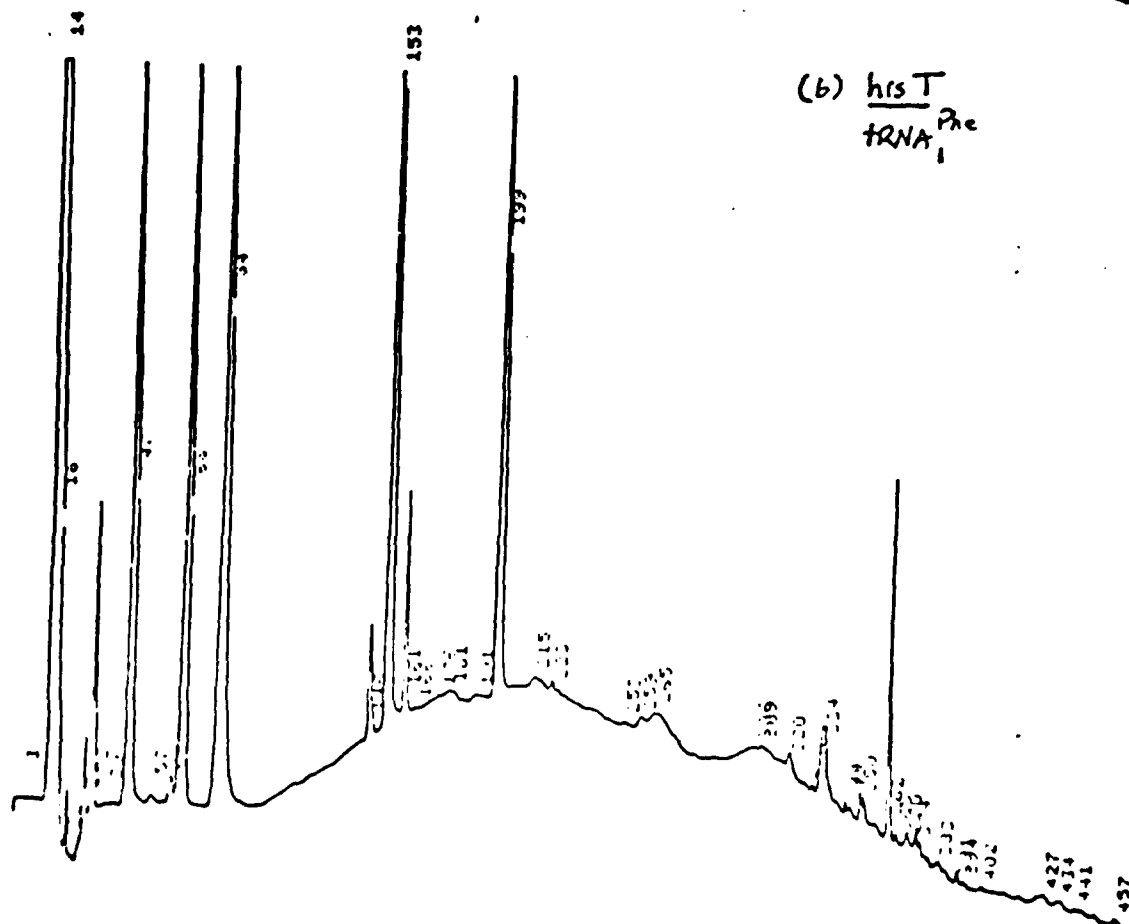
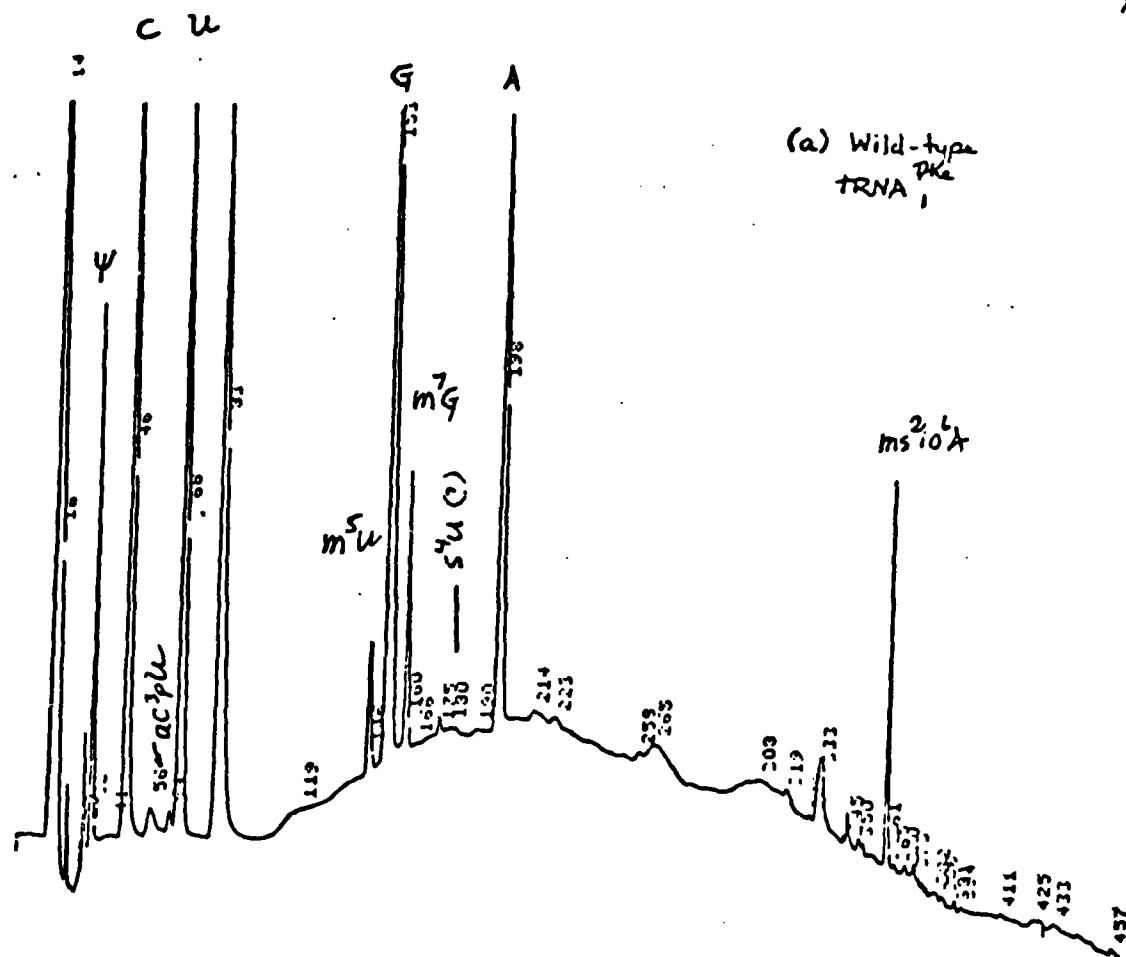
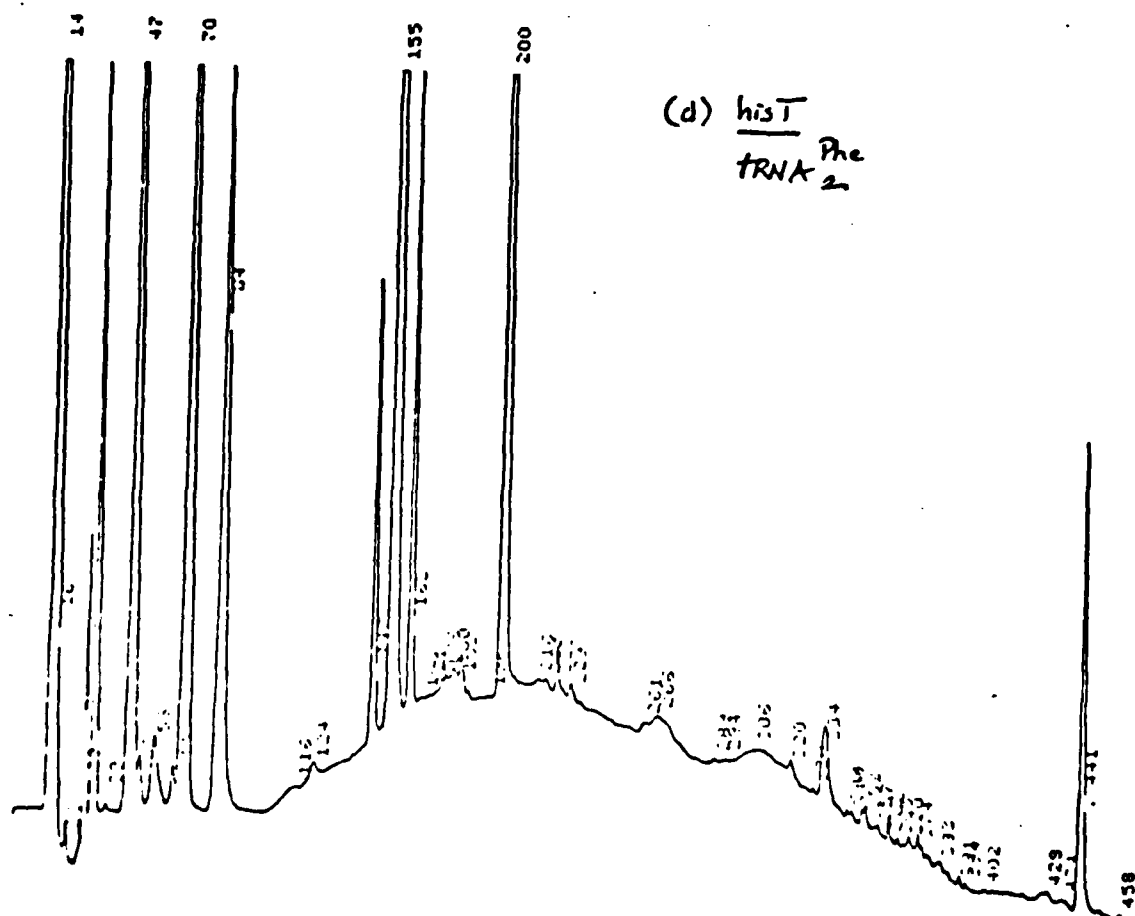
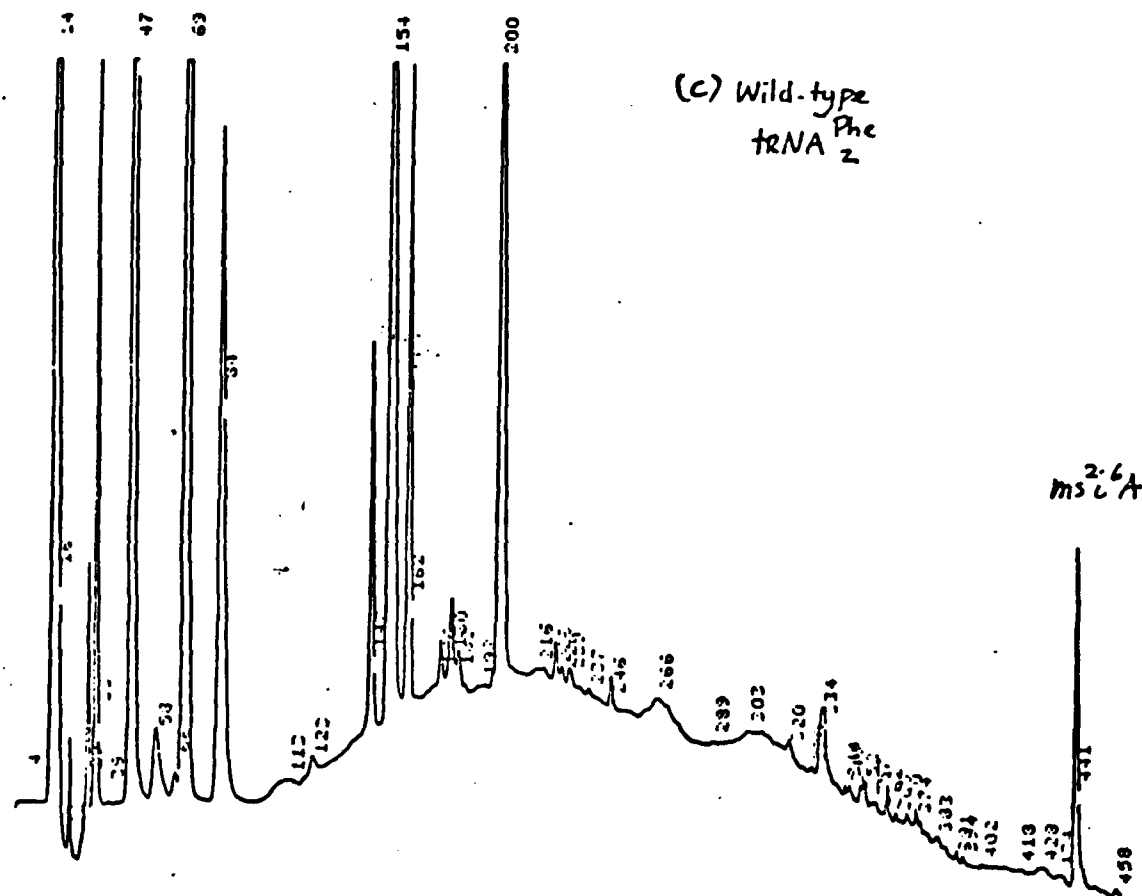


Fig. 13



COMPOSITION OF WILD-TYPE AND hist PHENYLALANINE tRNA ISOACCEPTORS

Relative Molar Ratios	Wild-type tRNA ₁ ^{Phe}		<u>hist</u> tRNA ₁ ^{Phe}		Wild-type tRNA ₂ ^{Phe}		<u>hist</u> tRNA ₂ ^{Phe}	
	Expected ^a	Found	Expected ^a	Found	Expected ^a	Found	Expected ^a	Found
A/ψ	4.7	4.29	7.0	6.62	4.7	4.96	7.0	6.5
G/ψ	7.7	7.88	11.5	11.9	7.7	8.26	11.5	11.4
C/ψ	7.3	7.12	11.0	11.0	7.3	8.26	11.0	10.6
U/ψ	2.7	2.86	4.0	4.85	2.7	3.62	4.0	5.0
⁷ mG/ψ	0.33	0.47	0.5	0.66	0.33	0.55	0.5	0.65
⁵ mU/ψ	0.33	0.29	0.5	ND	0.33	0.39	0.5	0.37
^{2,6} msiA/ψ	0.0	0.0	0.0	0.0	0.33	0.35	0.5	0.62
^{2,6} msioA/ψ	0.33	0.44	0.5	0.72	0.0	0.0	0.0	0.0

^a Based on the assumption that the wild-type isoacceptors contain 3 ψ residues per chain (residues 32, 39 and 55) and that the hist isoacceptors contain 2 ψ residues per chain (residues 32 and 55) (9). The chain length of the tRNA is assumed to be 76 nucleotides.

SUMMARY: SITE SPECIFICITY OF tRNA ψ SYNTHASE I

1. THE SPECIFICITY OF tRNA ψ SYNTHASE I IS LIMITED TO THE FORMATION OF ψ AT RESIDUES 38, 39 AND 40, IN THE 3'-HALF OF THE ANTICODON LOOP AND ADJACENT STEM REGION.

2. ALL OTHER ψ MODIFICATIONS NORMALLY FOUND IN WILD-TYPE tRNAs ARE STILL PRESENT IN hisT tRNAs OF S. TYPHIMURIUM. THESE INCLUDE MODIFICATION AT RESIDUE 55 (T ψ LOOP); RESIDUE 65 (T ψ STEM); RESIDUE 13 (DHU STEM); AND RESIDUE 32 (5'-HALF OF THE ANTICODON LOOP).

3. tRNA ψ SYNTHASE I IS NOT INVOLVED IN THE FORMATION OF ψ IN RIBOSOMAL RNA.

4. SINCE THE PRIMARY LESION IN hisT MUTANTS IS THE DEFECTIVE ψ MODIFICATION OF tRNA, THE PLEIOTROPIC EFFECTS OF THE MUTATION MUST BE THE RESULT OF ALTERED tRNA STRUCTURE, RATHER THAN RIBOSOMAL STRUCTURE.

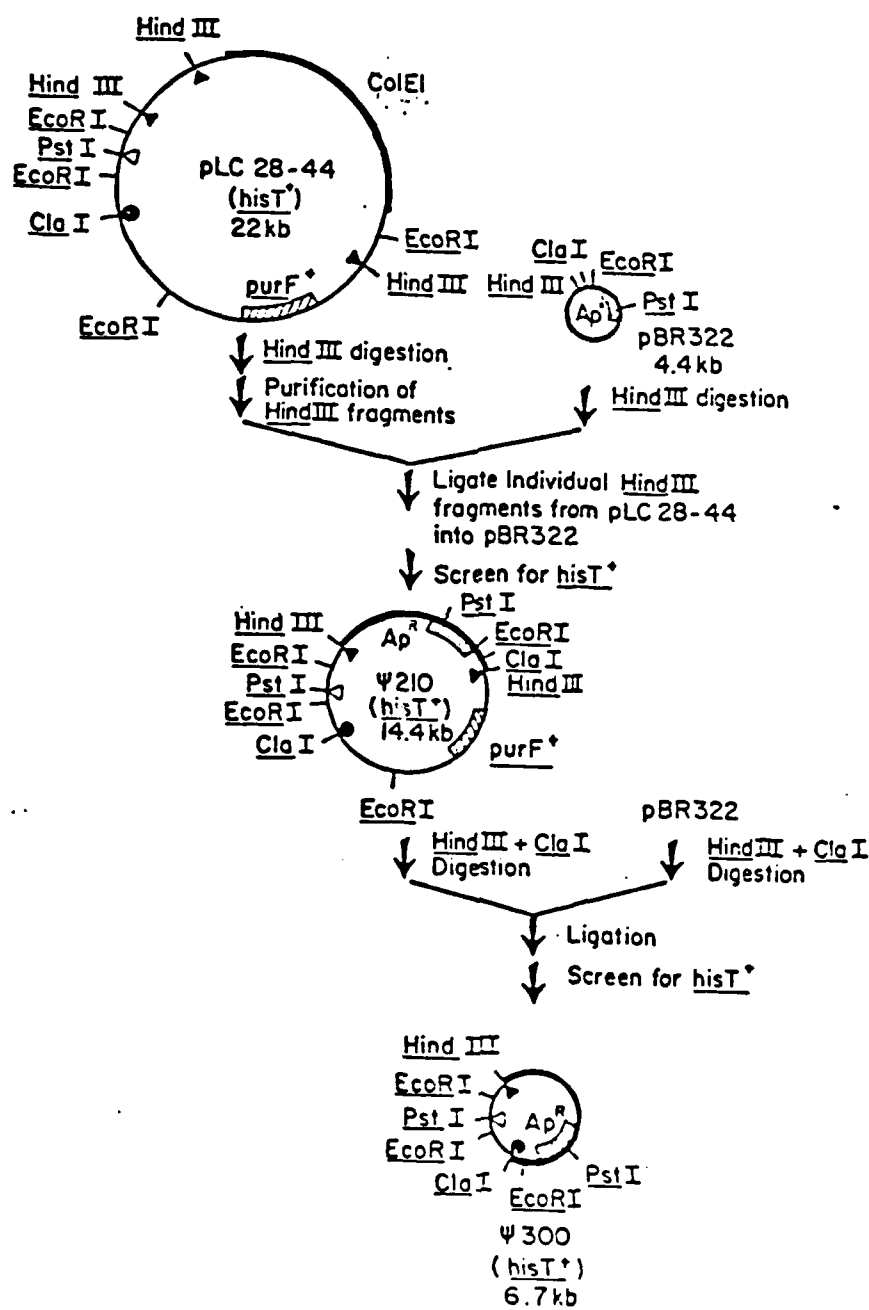


Table 2. Complementation of *hisT* mutations by recombinant plasmids

Strain ^a (type of <i>hisT</i> mutation)	Plasmid	PSUI activity ^b	Colony morphology on minimal + 2% glucose medium ^d
<u><i>E. coli</i></u> :			
FB105 ^c (unspecified)	None	-	S
	ψ210	++	S
	ψ300	++	S
NU90 (φ(<i>hisT</i> '- <i>lacZ</i> ⁺))	None	-	W
	ψ210	++	S
NU91 (φ(<i>hisT</i> '- <i>lacZ</i> ⁺))	None	-	W
	ψ210	++	S
<u><i>S. typhimurium</i></u> :			
TT4242-1 (<i>hisT</i> 1504; unspecified)	None	-	W
	ψ210	++	S
	ψ300	++	S
	pNU41	-	W
TA1309 (<i>hisT</i> 2890; amber)	None	-	W
	ψ210	++	S
	ψ300	++	S
	pNU41	-	W
TA1316 (<i>hisT</i> 2897; amber)	None	-	W
	ψ210	++	S
	ψ300	++	S
	pNU41	-	W
TA1322 (<i>hisT</i> 2890' <i>sup</i> 500; suppressed amber)	None	+	S
	ψ210	++	S
	ψ300	++	S
	pNU41	+	S
TA263 ^e (<i>hisT</i> 1536; temperature sensitive)	None	-	W
	ψ210	+	S
TT5866 (<i>hisT</i> 290::Tn5)	None	-	W
	ψ210	++	S
	ψ300	++	S
	pNU41	-	W

Table 2. (continued)

a Strains were grown in LB + Cys medium at 37°C with shaking for 12 hrs. Strains containing plasmids were grown in the presence of 50 µg ampicillin/ml.

b Extracts were prepared and assayed for PSUI activity as described in the Material and Methods. The amount of protein assayed from each extract was greater than the amount necessary for maximal PSUI activity for E. coli and S. typhimurium wild-type strains containing a single copy of the hisI gene. "++" indicates PSUI specific activities of about 2300 cpm [³H]-released/mg of protein. "+" refers to PSUI levels of at most one-half the "++" level. "-" indicates [³H]-release indistinguishable from control reaction mixtures lacking extract.

c Extracts prepared from strains FB105, FB105/ψ210, and FB105/ψ300 did not cause tritium release in reaction mixtures containing fully-modified [³H]-tRNA substrate isolated from wild-type S. typhimurium strain ara-9 (17).

d S, smooth colonies; W, wrinkled colonies; +W, partially wrinkled colonies.

e Strains were grown and PSUI enzyme assayed at 41°C.

Fig. 18

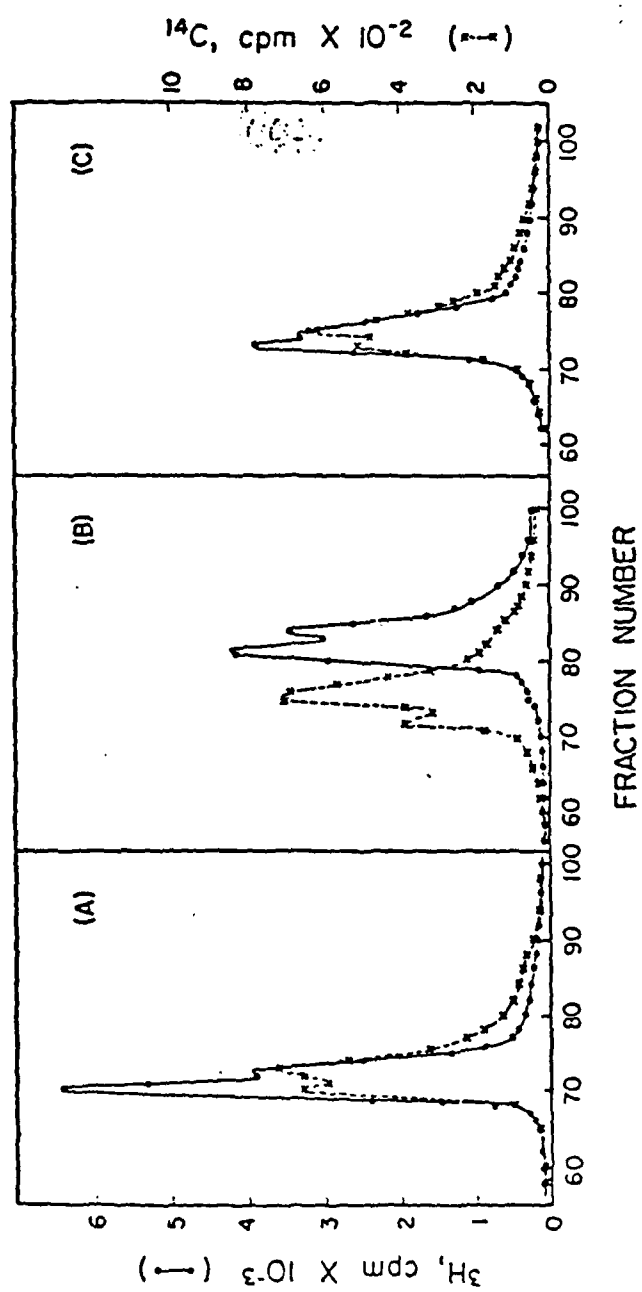
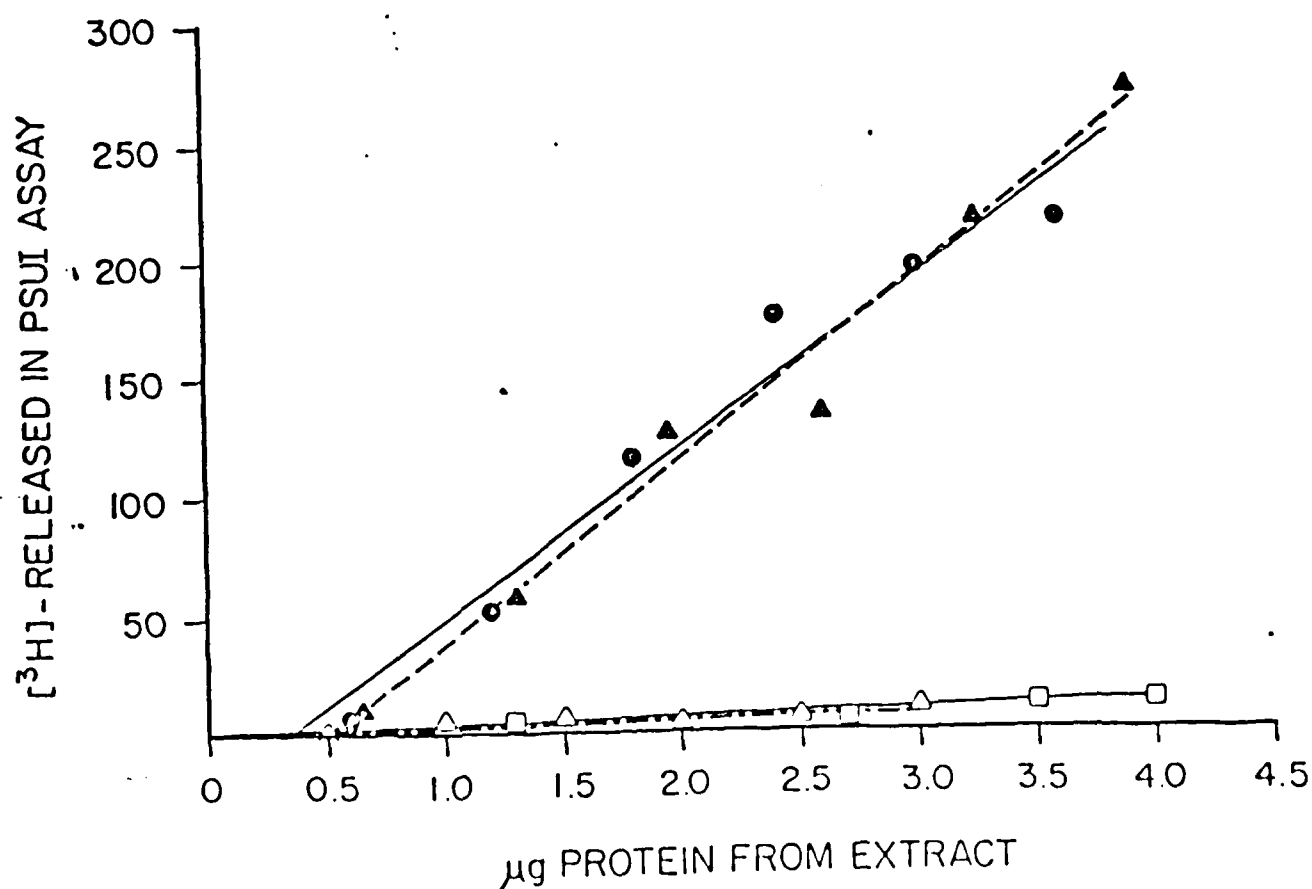
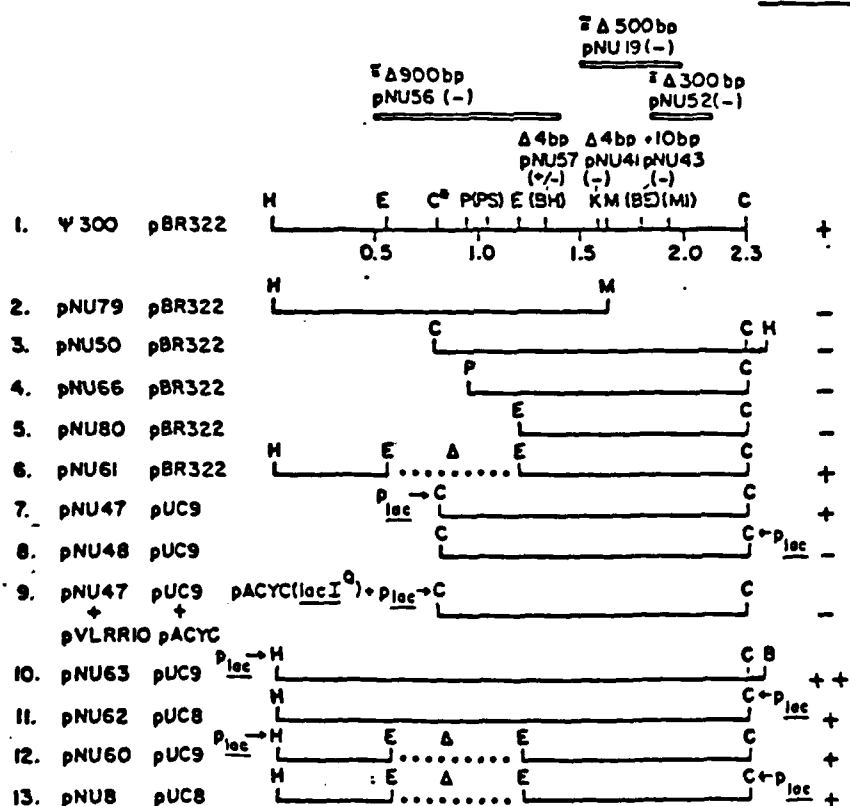


FIG. 2. Restoration of wild-type patterns of tRNA pseudouridine modification by plasmid $\psi 300$. (A) The tRNA fractions isolated from strains TA265 (hisT⁺) or TT4241(hisT⁺) were aminoacylated in vitro with ¹⁴C-Tyr or ³H-Tyr, respectively, mixed together, and co-chromatographed on RPC-5 columns as described in the Materials and Methods. X, ¹⁴C-Tyr-tRNA^{Tyr} from TA265; ●, ³H-Tyr-tRNA^{Tyr} from TT4241. (B) The same experiment as (A) except that tRNA was isolated from strains TA265 (hisT⁺) and TT4242-1 (hisT1504)/pBR322. X, ¹⁴C-Tyr-tRNA^{Tyr} from TA265; ●, ³H-Tyr-tRNA^{Tyr} from TT4242-1/pBR322. (C) The same experiments as (B) except that tRNA was isolated from strains TA265 (hisT⁺) and TT4242-1 (hisT1504)/ $\psi 300$. X, ¹⁴C-Tyr-tRNA^{Tyr} from TA265; ●, ³H-Tyr-tRNA^{Tyr} from TT4242-1/ $\psi 300$.

FIG. 3. Overproduction of PSUI in hisT⁺ strains containing plasmids ψ 210 or ψ 300. Bacterial strains were grown exponentially to $\approx 5 \times 10^8$ cells/ml in LB + Cys medium at 37°C with shaking. Strains containing plasmids were grown in the presence of 50 μ g ampicillin/ml. Extracts were prepared and the [³H] released in the PSUI assay was measured for different amounts of the extracts as described in the Materials and Methods. □, JM83 (hisT⁺); Δ, JM83/pNU66; ▲, JM83/ ψ 210; ●, JM83/ ψ 300.



<u>PLASMID</u>	<u>VECTOR</u>	<u>INSERT</u>	<u>PSUI ACTIVITY</u>
1	2	3	4
5	6	7	8
9	10	11	12
13	14	15	16
17	18	19	20
21	22	23	24
25	26	27	28
29	30	31	32
33	34	35	36
37	38	39	40
41	42	43	44
45	46	47	48
49	50	51	52
53	54	55	56
57	58	59	60
61	62	63	64
65	66	67	68
69	70	71	72
73	74	75	76
77	78	79	80
81	82	83	84
85	86	87	88
89	90	91	92
93	94	95	96
97	98	99	100



B = BamHI (BE) • BsiET (BH) • BssH2 C = CloI E = EcoRI H = HindIII K = KpnI
M = MstI (MI) • MluI P = PvuII (PS) • PstI

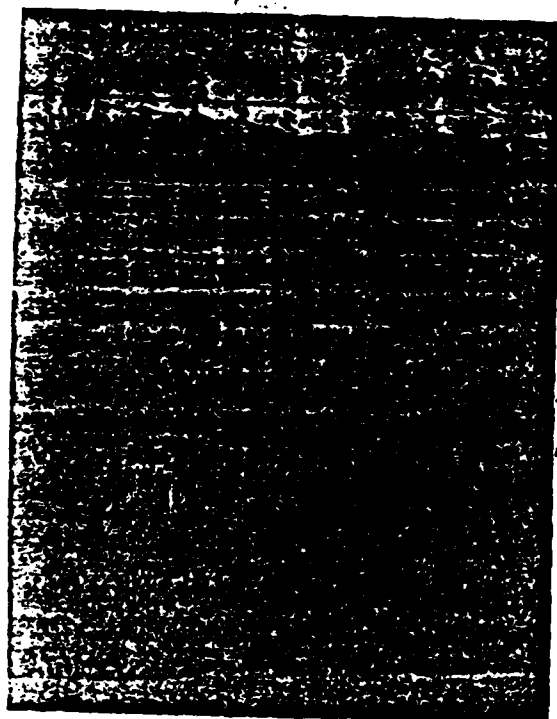
OVERPRODUCTION OF tRNA ψ SYNTHASE I IN PLASMID VECTORS

PLASMID	GROWTH CONDITIONS	ENZYME ACTIVITY
		^3H release, cpm/ μg Prot
ψ_{300} (pBR 322)	30 $^{\circ}$, 7 hr.	29.
"	30 $^{\circ}$, 24 hr.	16.
ψ_{MJ14} (pBEU 50)	30 $^{\circ}$, 7 hr.	92.
"	30 $^{\circ}$, 24 hr.	109.
"	30 $^{\circ}$ --39 $^{\circ}$ (3 hr)--30 $^{\circ}$ (4 hr)	143.
"	39 $^{\circ}$, 24 hr.	41.

Under these conditions, activity of the host strain, E. coli 294, is approx. 0.5-1.5.

Medium: 2X YT + ampicillin, 50 $\mu\text{g}/\text{ml}$.

Cultures grown at 30 $^{\circ}$, transferred as shown. Elapsed time measured from the time of transfer (cell density = 0.1 at 650 nm).



11.5
14.2
45
31
21.5
14.4

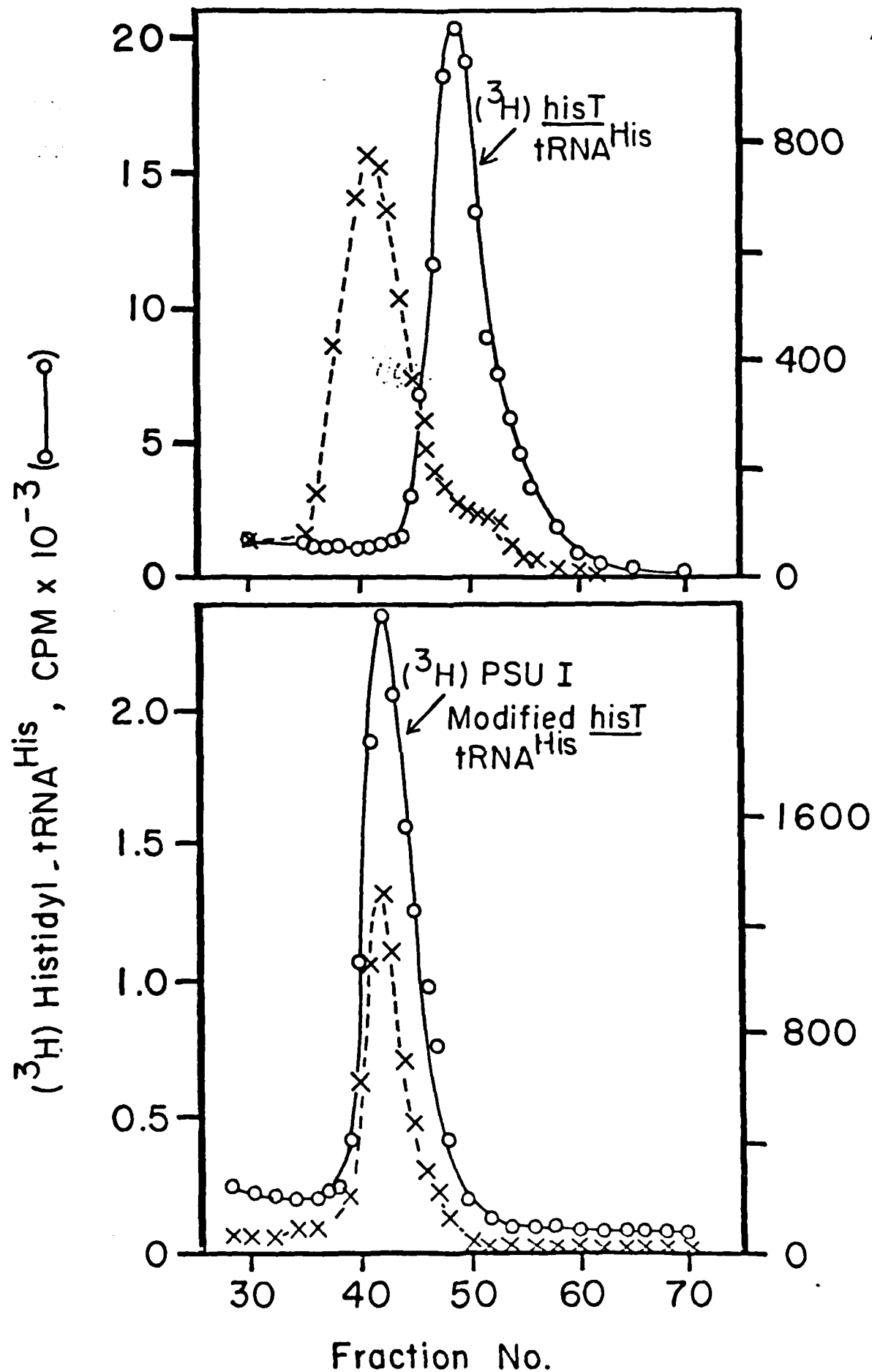
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REQUIREMENTS FOR OPTIMUM RATES OF ^3H RELEASE BY PSU I

REACTION SYSTEM	^3H Released, CPM	% of Control
Complete System (Control)	1489.	100.
Add pA, pC, pU pG, 0.5 mM each	1342.	90.1
Add ψ^{do} , ψ^{p} , 0.5 mM each	1468.	99.1
Add Ura, Udo, pU, UDP, 0.5 mM each	1398.	93.9
Complete System, but [^3H] wild-type tRNA in place of <u>hisI</u> tRNA	3.8	0.3
Omit NH_4Cl	10.0	0.67
Omit Dithiothreitol (0.25 μM from enzyme)	1197.	80.4

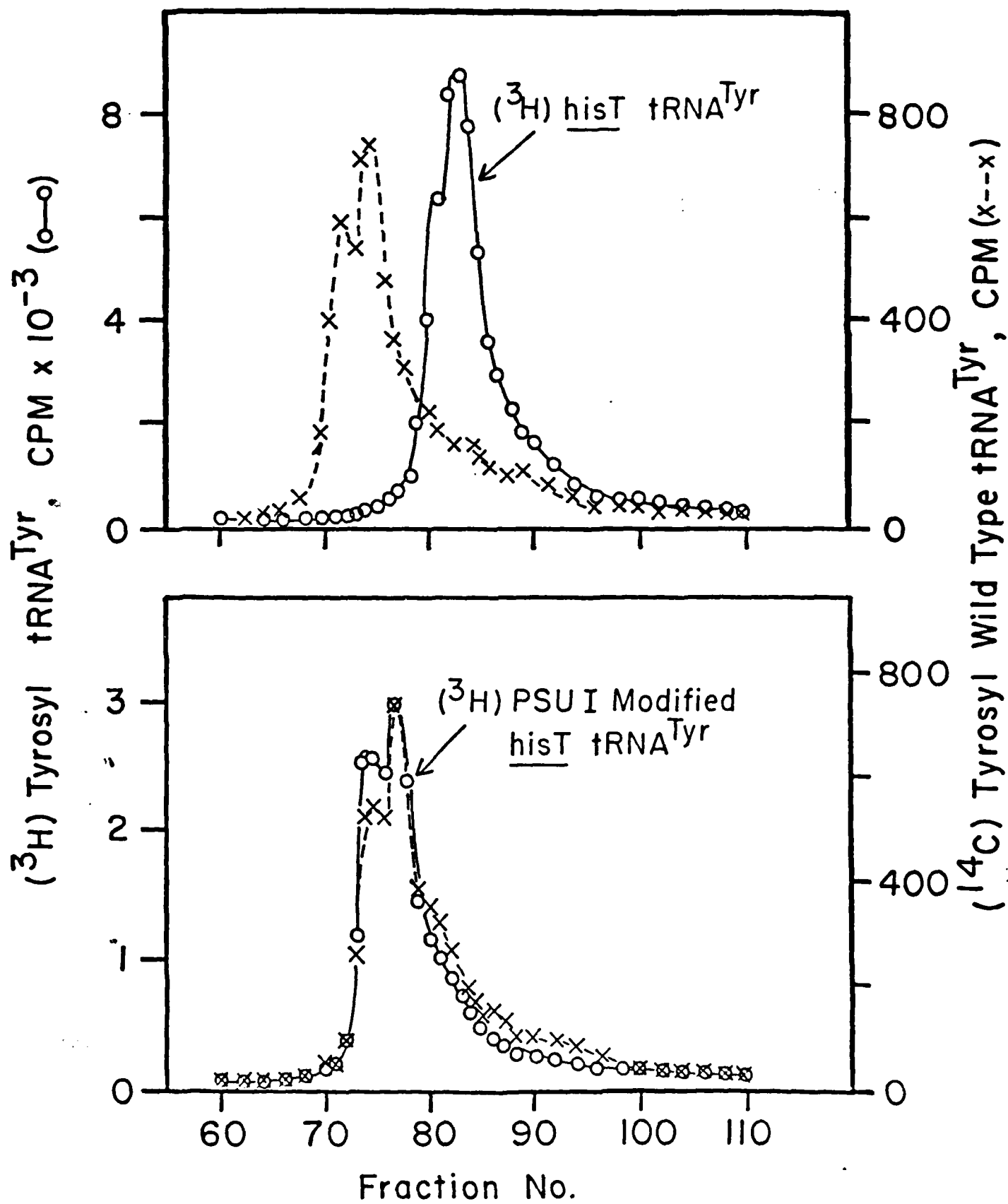
THIOL DEPENDENCE OF tRNA ψ SYNTHASE I

REACTION SYSTEM	^3H Released, cpm/20 min assay	
	Expt. 1	Expt. 2
Complete System (5 mM dithiothreitol)	1144.	1129.
(5 mM Cysteine)	1095.	
(5 mM β -MCE)	1136.	
Omit thiol (mixture includes 0.25 μM β -MCE from enzyme solution)	749.	778.
Omit thiol, add:		
DTNB, 0.01 mM	452.	
" 0.10 mM	103.6	
" 0.25 mM		24.1
PCMB, 0.01 mM	445.	
" 0.10 mM	20.8	
" 0.25 mM		0.0
Preincubate Enzyme (minus thiol) with:		
Buffer only	568.	425.
DTNB (0.1 mM)	100.4	(0.25 mM) 19.5
PCMB (0.1 mM)	100.5	(0.25 mM) 26.8
Iodoacetamide		(0.25 mM) 86.6



EFFECT OF tRNA ON ACTIVITY AND STABILITY OF PSU I

REACTION SYSTEM	³ H RELEASED, cpm/20 min	
	Expt. 1.	Expt. 2
Complete System (no additives)	1136.	690.
Add FU-tRNA (wild-type), 0.1 A ₂₆₀ u.	121.	
Add FU-tRNA (<u>hisT</u>), 0.1 A ₂₆₀ u.	150.	
Add Yeast tRNA ^{Phe} 0.02 A ₂₆₀ u.		634.
Add <u>E. coli</u> tRNA ^{Glu} 0.02 A ₂₆₀ u.		678.
Add bulk <u>Salmonella</u> tRNA (wild-type) 0.02 A ₂₆₀ u.		641.
Add bulk <u>Salmonella</u> tRNA (<u>hisT</u>) 0.02 A ₂₆₀ u.		643.
Preincubate Enzyme (minus thiol) with:		
Buffer only	522.	311.
DTNB, (0.2 mM)		41.4
Dithiothreitol (5 mM)		376.
Yeast tRNA ^{Phe}		622.
<u>E. coli</u> tRNA ^{Glu}		655.
Bulk <u>Salmonella</u> tRNA (wild-type)		649.
Bulk <u>Salmonella</u> tRNA (<u>hisT</u>)	1143.	653.
FU-tRNA (wild-type)	69.0	
FU-tRNA (<u>hisT</u>)	80.8	

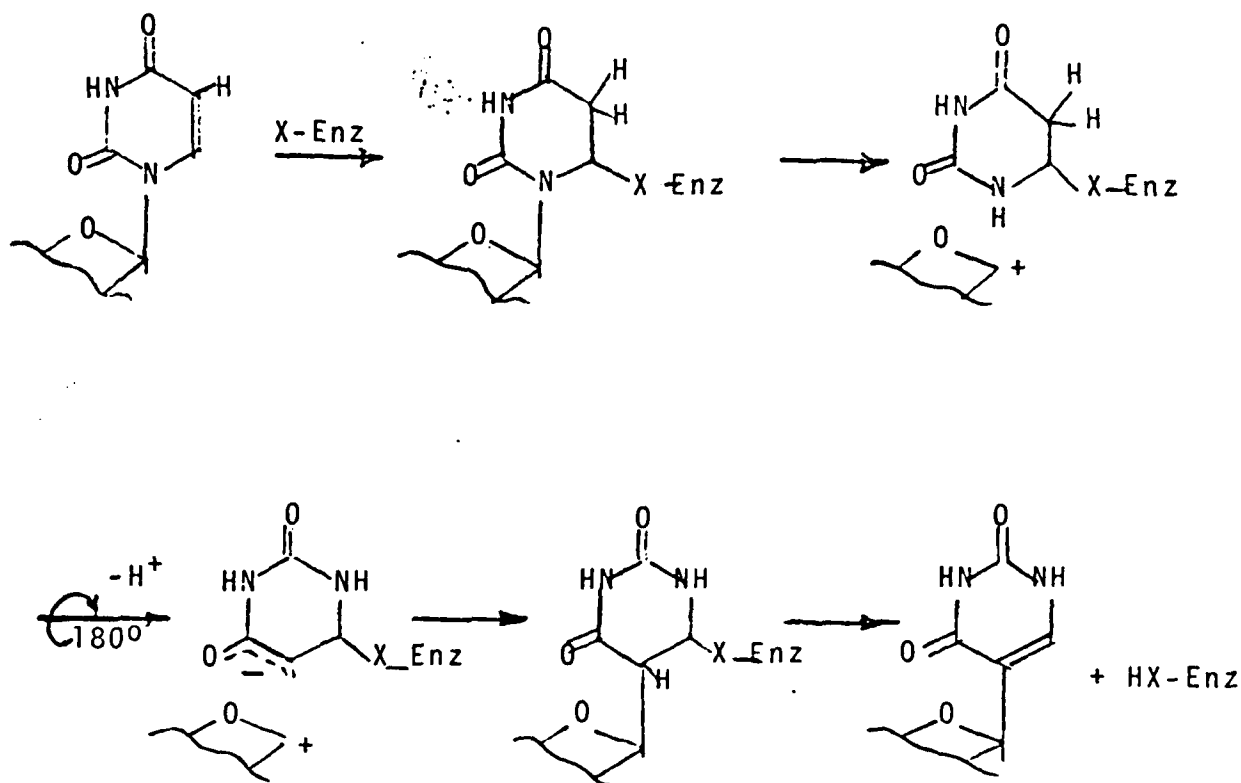
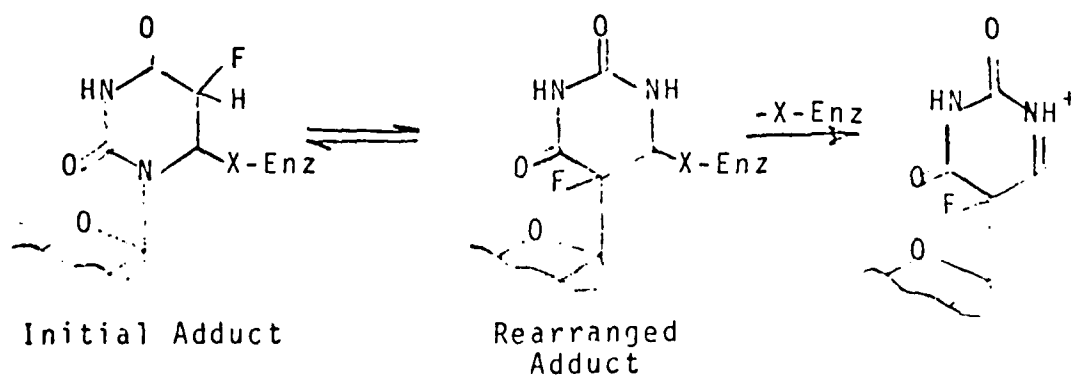


BINDING OF FU-tRNA TO tRNA ³SYNTHASE I

REACTION CONDITIONS	(3H) tRNA Retained on filter (pmoles per 100 μ l aliquot)	
	<u>+ Enzyme</u>	<u>- Enzyme</u>
0.4 μ M (6- ³ H)5-FUra-tRNA		
24 hours	3.0	0.06
34 hours	3.2	0.06
24 hours, chased with unlabeled 5-FUra-tRNA for 10 hours	1.2	N.D.
0.4 μ M (5- ³ H)Urd-tRNA (<u>hisT</u>)		
24 hours	0.18	0.05
34 hours	0.19	0.05

STABILITY OF FU-tRNA: SYNTHASE COMPLEX TO SDS DENATURATION

TREATMENT OF COMPLEX	(6- ³ H)5-FUra-tRNA Retained on filter (pmoles per 100 μ l aliquot)	
	<u>+ Enzyme</u>	<u>- Enzyme</u>
Spin Dialysis	2.9	0.11
1% SDS (15 min, 40°); spin dialysis	1.2	0.18
Pretreatment of Enzyme (before adding FU-tRNA) with 1% SDS (15 min, 40°); then spin dialysis.	0.20	0.23

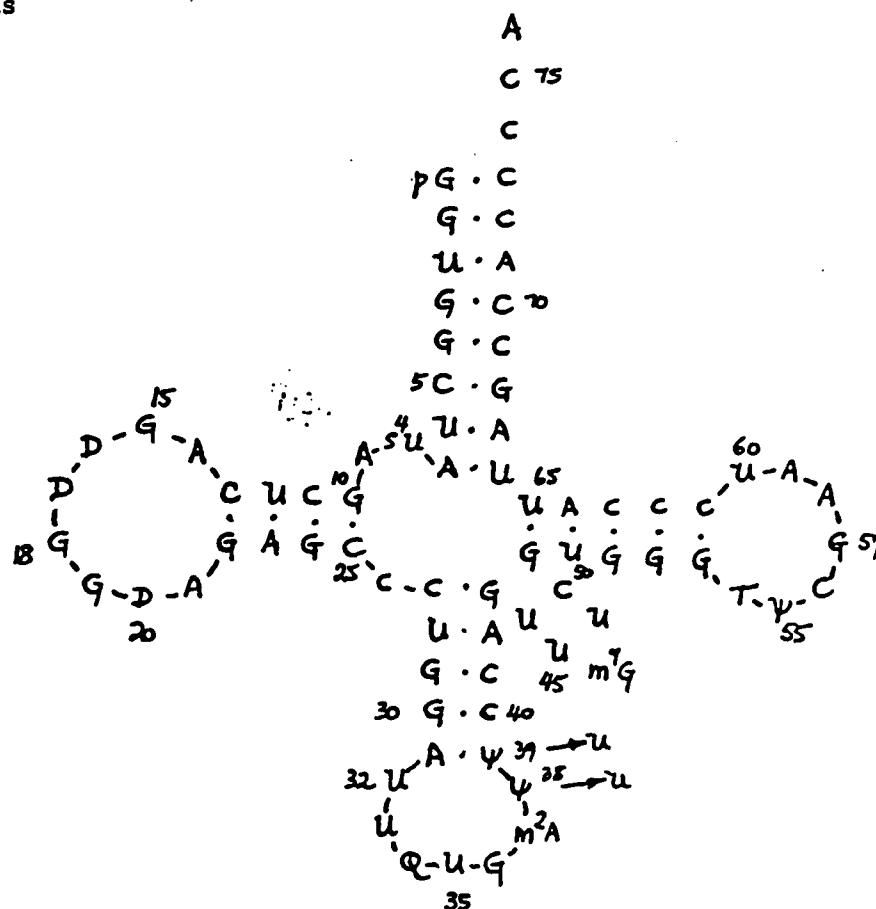
PROPOSED MECHANISM FOR tRNA ψ SYNTHASE IINHIBITION OF tRNA ψ SYNTHASE BY FU-tRNA

DIFFERENTIAL ACTION OF T₁ RIBONUCLEASE ON tRNA^{Phe}₂

Cleavage Sites	Mg ⁺⁺	% of Molecules Cleaved at Sites					
		0.1 u/ml		0.4 u/ml		1.0 u/ml	
		WT	hisT	WT	hisT	WT	hisT
3'-side of:							
G ₁₈ and G ₁₉	--	2.06	2.42	3.91	3.89	3.72	3.15
G ₂₂	--	1.17	1.20	2.72	2.82	3.94	3.67
G ₂₄	--	0.91	1.27	2.97	2.62	4.62	4.28
G ₃₄	--	2.80	3.57	6.59	9.67	11.43	17.97
	+	2.62	3.84	8.91	10.13	17.37	29.1
G ₄₄	+	---	---	1.09	2.43	1.0	2.67
G ₅₂ and G ₅₃	--	3.14	3.30	6.99	7.70	8.53	9.50
	+	---	---	1.52	1.40	2.66	3.42
G ₅₇	--	2.06	2.31	5.34	5.65	9.20	11.20
	+	---	---	1.49	1.53	3.28	5.91
G ₆₃	--	0.37	0.66	1.64	1.74	4.13	5.27
G ₆₅	--	0.22	0.24	1.05	0.96	2.31	2.95

DIFFERENTIAL ACTION OF U_2 RIBONUCLEASE ON $tRNA^{Phe}_2$

CLEAVAGE SITES	Mg ⁺⁺	% OF MOLECULES CLEAVED AT SITES SHOWN:					
		0.1 unit/ml		0.4 unit/ml		1.0 unit/ml	
		WT	hisT	WT	hisT	WT	hisT
3'-side of:							
A ₁₄	--	1.62	1.80	2.62	2.55	1.12	1.20
A ₂₁	--	0.83	0.82	1.49	1.09	0.90	1.0
A ₂₃	--	0.59	0.89	1.89	1.87	1.93	1.55
(G ₃₄), A _{35,36}	--	0.88	1.49	2.21	3.51	1.77	2.59
	+	0.68	0.93	1.37	1.96	1.50	2.14
A ₅₈	--	5.83	5.58	14.7	15.9	15.3	17.8
	+	0.70	0.86	1.76	2.00	2.26	3.06
A ₆₄	--	ND	ND	1.10	0.89	4.11	4.05



Action of SS Nucleases on 3'-End Labeled tRNA^{His}

Cleavage Sites		Mg++	% of Molecules Cleaved at Sites						
			10 u/ml		20 u/ml		40 u/ml		
			WT	hisT	WT	hisT	WT	hisT	
<u>S₁ Nuclease:</u>			--	2.12	2.73	2.78	4.16	6.11	7.37
5'-side of Residues 34-36			+	1.78	2.26	2.09	2.52	5.97	7.09
<u>Mung Bean Nuclease:</u>									
5'-side of G ₁₀			--	6.96	8.50	7.57	8.66	10.0	9.79
5'-side of residues 31-33			--	1.22	1.51	1.30	1.69	1.83	1.81
			+	1.04	1.43	1.42	1.63	1.34	2.05
5'-side of Q ₃₄ and U ₃₅			--	3.53	4.15	3.64	4.64	5.63	6.09
			+	4.97	6.29	6.93	11.59	9.58	15.61
5'-side of residues 37-39			--	0.91	1.91	0.91	2.08	1.25	3.29
5'-side of A ₄₂			--	2.40	4.49	2.58	5.19	4.50	7.37
5'-side of residues 44-46			--	1.58	1.38	1.62	1.73	2.65	2.30
5'-side of A ₆₇			--	1.19	1.40	ND	ND	4.20	5.08

DIFFERENTIAL EFFECT OF T_1 RIBONUCLEASE ON trNA^{His}

CLEAVAGE SITES:	Mg^{++}	% OF MOLECULES CLEAVED AT SITES SHOWN:			
		0.1 unit/ml		0.4 unit/ml	
3' side of:		WT	hisT	WT	hisT
G_{10}	--	1.12	0.93	1.21	1.20
G_{15}	--	2.51	2.28	3.58	2.88
$G_{18, 19}$	--	1.59	1.86	2.45	2.68
$G_{22, 24}$	--	0.66	0.33	0.72	0.71
$G_{29, 30}$	--	1.82	1.34	2.66	2.02
G_{36}	-- +	1.75 4.63	2.85 9.48	2.82 9.47	4.95 18.9
G_{43}	--	1.53	1.31	2.27	2.11
G_{51-53}	--	1.25	1.19	2.78	1.85
G_{57}	--	0.32	0.24	0.34	0.24
G_{68}	--	ND	ND	2.04	3.70

DIFFERENTIAL ACTION OF U_2 RIBONUCLEASE ON $tRNA^{His}$

CLEAVAGE SITES	Mg^{++}	% OF MOLECULES CLEAVED AT SITES SHOWN:			
		0.1 unit/ml		0.4 unit/ml	
		WT	hisT	WT	hisT
3'-side of:					
$A_{9, 14}$	--	2.68	4.21	4.91	5.66
	+	1.51	1.29	1.94	1.55
$(G_{30}), A_{31}$	--	5.35	10.0	9.12	13.1
	+	ND	ND	1.20	1.45
$A_{42}, (G_{43})$	--	4.10	7.09	10.7	14.5
$A_{58, 59}$	--	ND	ND	2.37	2.19
$A_{67}, (G_{68})$	--	ND	ND	3.74	4.80

END

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DTIC