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I am submitting herewith a thesis written by Robert Keith Owenby entitled "Characterization of a Tyrosine tRNA From a Suppressor Mutant of *Drosophila Melanogaster.*" I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

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We have read this thesis and
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CHARACTERIZATION OF A TYROSINE tRNA FROM A SUPPRESSOR MUTANT OF *DROSOPHILA MELANOGASTER*

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee

Robert Keith Owenby

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ABSTRACT

Transfer RNA, in particular, tyrosine tRNA, has been implicated in the mechanism of suppression mediated by the *sus* locus. *Drosophila melanogaster* $tRNA^{Tyr}$ can be separated into two major isoacceptors by RPC-5 chromatography. The early eluting isoacceptor, $tRNA_Q^{Tyr}$, has the anticodon QψA, while the late eluting isoacceptor, $tRNA_G^{Tyr}$, has the anticodon GψA. Previous evidence has indicated that both isoacceptors of *Drosophila melanogaster* $tRNA^{Tyr}$ from *v; bw* and a suppressor strain, *su(s)² v; bw*, differ in their biological activities. The chemical basis for this difference is explored. A procedure for purifying $tRNA^{Tyr}$ from *Drosophila melanogaster* without exposing the tRNA to enzymes or chemicals that might cause alterations is developed, and this procedure is used to purify *Drosophila melanogaster* $tRNA_G^{Tyr}$ from *v; bw* and *su(s)² v; bw*. The nucleoside compositions of the two tRNAs are then determined and compared. The *su(s)² v; bw* $tRNA_G^{Tyr}$ appears to contain one more N_2, N_2-dimethylguanosine and pseudoruidine than the *v; bw* $tRNA_G^{Tyr}$. Further confirmation that the two tRNAs are different is obtained by comparing the fingerprint patterns of the two tRNAs that were generated by digestion with T_1 RNase. One fragment in the *su(s)² v; bw* is identified that migrates faster than the corresponding fragment in the *v; bw* strain, indicating that the base composition of the two fragments and thus the two tRNAs are different. It is suggested that the *sus* locus is responsible for the difference in base composition of the *v; bw* and *su(s)² v; bw* tyrosine tRNAs, and that tyrosine tRNA may be involved in the mechanism of suppression mediated by the *sus* locus.

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CHAPTER I

INTRODUCTION

Historical Background

The fidelity of protein biosynthesis is achieved by a class of RNA molecules known as transfer-RNA. Transfer RNA acts as an adaptor molecule, carrying esterified, activated amino acids to the ribosomes, and decoding the genetic information carried in messenger RNA. The discovery of transfer RNA as the adaptor molecule solved the dilemma of how a sequence of nucleotides could specify a reproducible assembly of amino acids during protein biosynthesis.

The idea that a connection existed between nucleic acids and protein synthesis dates to the work of Caspersson[1,2] and Brachet.[3] They observed that secretory cells active in protein synthesis contained a high RNA content.

Prior to the establishment of the role of transfer RNA (tRNA), some people thought that DNA might act directly as a template for assembly of amino acids into proteins. Others believed that amino acids were held directly on RNA templates and linked enzymatically. Francis Crick rejected these concepts and proposed what is now known as the "adaptor hypothesis."[4] Crick reasoned that tRNA is primarily a sequence of sites at which hydrogen bonding can occur, and that any specific interaction between a molecule and RNA must involve hydrogen bonding. The twenty amino acids did not have enough hydrogen bonding diversity to permit them to specifically extract information from RNA. If the amino acids were to be properly sequenced on an RNA template, then they would have to be carried to the template by some sort of "adaptor" molecule, having a specific hydrogen-bonding surface which would combine specifically with the RNA template.

The first step in protein synthesis was solved when enzymes were found that could activate amino acids to an adenylated form.[5,6] Using a cell-free protein synthesizing system, the activated amino acids were then shown to become covalently bound to a type of soluble RNA.[7] Next, it was found that aminoacyl-sRNA could be substituted for free amino acid and that the transfer of amino acid from soluble RNA into protein depended on GTP.[8] It was soon established that soluble RNA, now termed transfer RNA or tRNA, was a mixture

of polynucleotides and that each tRNA was specific for a particular amino acid. The discovery of the role of tRNA and its specificity confirmed Crick's adaptor hypothesis.

Transfer RNA Structure

In the early 1900's Levene's work reached several conclusions concerning RNA structure. The pentose sugar in all nucleotides is D-ribose. The major heterocyclic bases are adenine, guanine, cytosine, and uracil. The furanose ring structure of the sugar moeity is attached to the 9 position of purine bases and to the 1 position of pyrimidines. Cohn[9] showed that after alkaline hydrolysis of RNA each nucleotide is a mixture of two isomers, and Brown[10] identified these as the 2′ and 3′ phosphates. Enzymatic degradation established that a phosphodiester linkage joining the 3′ position of one nucleotide with the 5′ position of the adjacent nucleotide was the internucleotide bond in RNA.[11,12,13]

Each tRNA molecule generally consist of a 75 to 90 unit polynucleotide chain with a 5′ phosphate at one end and a common sequence, CpCpA at the other, 3′ end. By biological macromolecular standards, tRNAs are small molecules with typical molecular weights of approximately 25,000 and a sedimentation constant of 4S. Each tRNA molecule is specific for a particular amino acid and each amino acid specific tRNA can exist in multiple forms known as isoaccepting tRNA species or tRNA isoacceptors.

The ability of different tRNAs to be discriminated against, as in aminoacylation, or to differentially discriminate as in decoding the message, can only depend on how the information stored in the nucleotide sequence is presented. Transfer RNA is a three dimensional molecule, having primary, secondary and tertiary structure. Therefore, tRNA can present information from all three structural levels. Since the secondary and tertiary structure of tRNA depends upon the primary structure, a study of tRNA structure should begin at the primary level.

The development of methods for the purification of different tRNA species was a significant accomplishment and was the first step which eventually led to the discovery of the primary structure of yeast alanine tRNA.[14] Other workers had previously shown that tRNAs have an ordered secondary structure composed of double stranded helical segments. From this information, Holley proposed several folded structures for alanine tRNA, one of

which is the now widely accepted clover-leaf structure. The primary structure of over 200 tRNA species are now known. Transfer RNAs appear to have a great deal of primary and secondary structure homology since all sequenced tRNAs to date can conform to the generalized clover-leaf structure.

Within the context of the clover-leaf structure, all tRNAs appear to have certain common features. Starting with the 5′ end of the molecule, all tRNAs possess a region of 7 base pairs termed the amino acid acceptor stem. Separated from the amino acid acceptor stem by two unpaired bases is the dihydrouridine or D stem. The D stem contains 3 or 4 base pairs, with the fourth base pair often being a non-Watson-Crick base pair. Following the D stem is a region of variable size known as the D loop. The variability in the D loop is confined to two variable regions α and β, with two constant guanine residues between them. The modified nucleoside, dihydrouridine, is commonly found in the variable region. A stem and a loop together is referred to as an arm, thus the D stem and the D loop make up the D arm.

The anticodon stem is a five base pair region separated from the D stem by one unpaired base. The anticodon loop consists of seven bases with the third, fourth and fifth comprising the anticodon.

On the 3′ side of the anticodon stem is a region of large size variability known as the variable loop or extra arm. The size of the variable loop allows tRNAs to be divided in two general classes,[15] one having an extra arm of 4 or 5 bases and the other 13 to 21 bases.

Following the extra arm is the TψC arm. The TψC loop consists of 7 bases while the stem contains 6 base pairs. This arm is one of the most highly conserved regions, and is believed to be important in the binding of tRNA to the ribosome.[16]

The final structural feature of tRNA is a 4 nucleotide sequence on the 3′ end, ending in a constant CpCpA. The 2′ or 3′ hydroxyl group of the terminal adenosine is the site of aminoacylation of all tRNAs.

There are several exceptions to this general clover-leaf structure.[17] However, many of these involve tRNAs with special roles such as initiator tRNAs and glycine tRNAs involved in cell wall metabolism.

While the clover-leaf structure is convenient for comparing different tRNAs, an understanding of the tertiary structure is necessary to explain the interaction of tRNA with aminoacyl-tRNA synthetases and ribosomes. Most of the information on the tertiary structure

of tRNAs has been provided by x-ray crystallographic studies. Difficulties in obtaining suitable crystals hampered progress until two groups independently obtained suitable crystals of yeast phenylalanine tRNA in 1971.[18,19] The three-dimensional structure of the monoclinic form of yeast $tRNA^{Phe}$ has now been solved to 2.5 Å.[20] Independent studies using the orthorhombic form[21] and the monoclinic form[22] have yielded essentially the same structure.

The molecule can be viewed as the letter T,[23] or when viewed from the side appears to have an inverted L shape.[15] The amino acid stem and TψC stem stack together forming one arm while the anticodon stem and D stem stack to form the other arm. The three functional regions of the molecule appear to be maximally separated. The 3′-CAA and the anticodon are at opposite ends of the molecule, 78 Å apart, while the TψC loop, which is believed to play a role in the binding to the ribosome,[16] is at the junction of the two arms. Nine tertiary hydrogen bonds hold the structure together and most of the bases involved in these hydrogen bonds are invariant or semi-invariant in all tRNAs.[24,25]

The chemical reactivity of yeast tRNA in solution with carbodiimide and methoxyamine[26] correlates well with the exposed bases that would be available to react with the reagents according to x-ray crystallographic studies.[15] In addition, physical chemical studies of tRNA in solution, such as NMR, can also be interpreted in terms of the same structure that exist in the crystal. These results indicate that the crystalline structure of yeast $tRNA^{Phe}$ is probably very similar to the solution structure.

Base Modifications and tRNA Function

One characteristic of mature tRNA is its high content of modified bases.[27,28] Modified bases usually arise by post-transcriptional modifications of the nucleotides initially assembled by RNA polymerase.

Over 50 different modified bases have been identified.[28,29] Most of these involve simple methylations, thiolations, or acetylations; however, some very complex nucleosides such as methylthio isopentyl adenosine and queuosine have been found. Generally, prokaryotic tRNAs have about 5% of their bases modified as opposed to 20% in eukaryotic tRNAs.[30]

Certain modifications, such as 1-methylguanosine at the junction of the acceptor and D stem, N^2-methylguanosine as the first paired base in the D stem, N^2, N^2-dimethylguanosine at the junction of the D and anticodon stem, 1-methyladenosine in the TψC loop and 5-methylcytosine in the variable loop are characteristic of eukaryotic tRNAs. Pseudouridine and 5-methyluracil are found in the TψC loop of nearly all tRNAs.

While it is difficult to generalize on the role of most modified bases in tRNA, their diversity and the appearance of specific modifications at characteristic positions suggest that they have an underlying function. Indeed, modifications located in the anticodon loop have been found to influence codon-anticodon interactions.[31,32] Anticodon bases 2 and 3 pair with the first two bases in the codon by standard Watson-Crick base pairing. Crick[33] proposed that the first base of the anticodon could also pair with the third base of the codon by non-standard "wobble" hydrogen bonding. This explained how a single tRNA could read different codons for one amino acid differing in the third base of the codon. Modified bases at the wobble position either expand or restrict the decoding capabilities of the tRNAs in which they are found. Inosine and uridine-5-oxyacetic acid (V) both pair with U, A and G.[34] Thiolated modifications of U restrict decoding capabilities. The hyper-modified base queuine appears to have decoding properties similar to G, except that it prefers U over C.[35]

Base modifications may also influence tRNA function in other ways. Some methylations have been shown to affect ribosome binding or aminoacylation efficiencies.[31,32] Also, pseudouridylations of tRNAs have been linked to transcriptional regulation of several amino acid biosynthetic operons.[36]

Transfer RNA and Suppression

Mutations that produce missense and stop codons or frameshift alterations result from a change in a component of the translational system so that the messenger RNA codon is misread. Mutations in tRNA that alter the tRNA decoding properties may result in suppressor tRNAs. Suppressor tRNAs that suppress nonsense codons are known as nonsense suppressors. Thus, while protein synthesis usually terminates when a nonsense or stop codon is reached, there are several examples of termination codons being suppressed so that an

amino acid is inserted into the polypeptide chain and protein synthesis continues. The three termination codons are UAG (amber), UAA (ochre) and UGA (opal).

Most suppressor tRNAs result from a mutation in a tRNA structural gene which alters the anticodon of the tRNA allowing misreading. The amber suppressor Su^+_3 from Escherichia coli results from a mutation in one of the two genes specifying $tRNA^{Tyr}_1$. The mutation changes the tRNA anticodon from QUA to CUA so that the tRNA now recognizes the nonsense codon UAG instead of the normal tyrosine codons UAU and UAC.[37] Thus, the tRNA now inserts tyrosine into the protein in response to the UAG termination codon. The E. coli amber suppressor Su^+_7 is a result of a mutation in the single gene for $tRNA^{Trp}$ that changes the anticodon from CCA to CUA.[38] The anticodon change alters the aminoacyl tRNA synthetase recognition so that this tRNA will preferentially accept glutamine over tryptophan.[39,40] All amber suppressors sequenced to date have the anticodon CUA. The ochre suppressors from E. coli $tRNA^{Tyr}_1$ [41,42] and $T_4\,tRNA^{Gln}$ [43] both have a modified uridine in the first position of the anticodon.

The only identified UGA suppressor arising from an anticodon base change is $T_4\,tRNA^{Arg}$ in which the $\overset{*}{U}CU$ anticodon is changed to $\overset{*}{U}CA$ ($\overset{*}{U}$ is a modified uridine residue).[44]

However, not all nonsense suppressors involve modifications of the tRNA anticodon. It has long been accepted that neither the stability or specificity of the aminoacyl-tRNA interaction with the ribosome-message complex could be explained simply by triplet-triplet nucleotide interactions.[45] One example that illustrates this is the UGA nonsense suppressor identified by Hirsh.[46] This E. coli $tRNA^{Trp}$ suppressor contains the normal anticodon, CCA. The structural alteration in this tRNA was found at position 24 in the D stem, where G is replaced by A.

Suppressors acting solely on UAG codons have been observed in both yeast and bacteria. Ochre suppressors acting solely on UAA codons have been observed only in yeast, while suppressors acting on both UAA and UAG codons are observed only in bacteria. Amber and ochre suppressors in *Saccharomyces cerevisiae* have all been found to insert one of three amino acids. The amino acids inserted during translational suppression of UAG or UAA codons are tyrosine, serine and leucine. Assuming that the suppressor tRNAs are not misacylated *in vivo*, a situation that does not always hold true in E. coli,[40] we can assume that

these suppressors probably arise by mutation of tyrosine, leucine, and serine-accepting tRNAs.

Nonsense mutations and their suppressors have played a major role in the study of gene expression in bacteria and yeast. Furthermore, much of our knowledge of tRNA structure-function relationships, biosynthesis and genetics has depended on the study of suppressor tRNAs.

While many examples of suppressor tRNAs have been identified in yeast and bacteria, much less is known about suppressor tRNAs in higher eukaryotes. The possibility of developing suppression systems in higher eukaryotes similar to those in yeast and bacteria has stimulated considerable research on nonsense mutants and suppressors in animal cells.

In mammalian cells and their viruses premature chain termination mutations have been identified in the Adenovirus-2-SV40 hybride virus,[47] Herpes thymidine kinase,[48] mouse immunoglobin mutants,[49] and Chinese hamster lung fibroblast mutants involving the genetic locus for hypoxanthine-guanine phosphoribosyl transferase.[50] In each case, the convincing evidence for premature chain termination mutants was provided by the *in vitro* suppression of the mRNA translation product by a yeast suppressor tRNA. However, no mammalian suppressor tRNA was actually demonstrated in any of these cases.

Recently, a $tRNA^{Trp}$ was purified from rabbit reticulocytes which suppresses the UGA termination codon of rabbit β-haemoglobin mRNA.[51] This was the first demonstration of naturally occurring readthrough in a nonviral system.

Question of Differences in Base Composition Between *v; bw* and *su(s)*^{2} *v; bw* Tyrosine tRNAs

Many suppressor mutants are known in *Drosophila melanogaster.* One suppressor locus that has been well studied in *Drosophila melanogaster* is the *su(s)*^{2} locus. Several lines of evidence indicate tRNA may be involved in the mechanism of suppression mediated by this locus.

The structural locus for tryptophan oxygenase in Drosophila is probably the vermilion locus.[52] When this enzyme activity is absent, no synthesis of xanthommatin, the brown eye pigment, occurs. Alleles of the vermilion locus may be assigned to two groups: v^u for

alleles in which the synthesis of brown eye pigment is not restored by a suppressor mutant, *su(s)*^{2}, and v^s for alleles that are suppressible.[53] It has been suggested that tRNA may be involved in the mechanism by which tryptophan oxygenase activity is restored by the *su(s)*^{2} mutant.[54]

RPC-5 chromatography of *Drosophila melanogaster* tRNA yields two major tyrosine tRNA isoacceptors. The late eluting isoacceptor, $tRNA_G^{Tyr}$, has the anticodon GψA while the early eluting isoacceptor, $tRNA_Q^{Tyr}$, has the anticodon QψA. Jacobson's group[55] found that $tRNA_G^{Tyr}$ from *su(s)*^{2} *v; bw* flies could not be charged by the tyrosyl-tRNA synthetase when the synthetase was prepared in one manner. This enzyme form could charge *su(s)*^{2} *v; bw* $tRNA_Q^{Tyr}$ and also the two tyrosine isoacceptors from wild-type Drosophila tRNA. If prepared in a different manner, the enzyme would charge $tRNA_G^{Tyr}$ from *su(s)*^{2} *v; bw* as well as the other three tRNAs. From this data, it was proposed that the *su(s)*$^+$ locus was responsible for an enzyme that modified a nucleotide that would therefore be found in $tRNA_G^{Tyr}$ from *v; bw* but not *su(s)*^{2} *v; bw*. It was suggested that both the "discriminating" form of the synthetase and the tryptophan oxygenase were unable to react with the $tRNA_G^{Tyr}$ from *su(s)*^{2} *v; bw* but that $tRNA_G^{Tyr}$ from *v; bw* could react with these enzymes due to the modified nucleoside produced either directly or indirectly by the product of the *su(s)*$^+$ locus. The $tRNA_G^{Tyr}$ from *v; bw* could therefore bind and inhibit tryptophan oxygenase.

If *v; bw* $tRNA_G^{Tyr}$ does inhibit tryptophan oxygenase and give rise to the lack of brown eye pigment seen in this fly, then the reduction of the relative amount of $tRNA_G^{Tyr}$ in *v; bw* would be accompanied by restoration of brown eye pigment synthesis. Indeed, it has been shown that brown eye pigment synthesis is restored in *v; bw* when $tRNA_G^{Tyr}$ levels are decreased by dietary methods.[56]

All the previous arguments are valid only if the second isoacceptor species of *v; bw* and *su(s)*^{2} *v; bw* are not identical. In the present study, these two tRNAs will be compared by base analysis and fingerprint patterns of the purified isoacceptors.

Recent evidence has indicated that $tRNA_Q^{Tyr}$ from *v; bw* and *su(s)*^{2} *v; bw* may also be different.[57] This study found that $tRNA_G^{Tyr}$ from wild-type *Drosophila melanogaster* could suppress the tobacco mosaic virus mRNA UAG stop codon, producing a read-through protein, while wild-type $tRNA_Q^{Tyr}$ could not. Both tyrosine tRNA isoacceptors from

su(s)^2 *v; bw* flies were found to produce a read-through protein. Thus, *su(s)*^{2} restores the ability of $tRNA_Q^{Tyr}$ to read the TMV-RNA stop codon and produce a read-through protein.

Thus, it would appear that the *su(s)*$^+$ and *su(s)*^{2} genotypes produce $tRNA^{Tyr}$, both isoacceptors of which are distinguishable from each other by their biological activities. The chemical basis for this difference is the goal of this study.

CHAPTER II

MATERIALS AND METHODS

Materials

The materials used in this research and the source of purchase are summarized in Table I.

Table I. Sources of Materials

Compound	Company
Miracloth	Chicopee Mills, Inc.
[^{3}H] tyrosine and [^{14}C] tyrosine	Schwarz-Mann
ATP (disodium salt)	P. L. Biochemicals, Inc.
Benzoylated DEAE-cellulose	Schwarz-Mann
3 MM filter paper disc	Whatman, Ltd.
Acrylamide, methylene bisacrylamide, urea, ammonium persulfate	BioRad Laboratories
3-dimethylaminopropionitrile (DMAPN)	Eastman Chemical Co.
Adogen 464	Ashland Chemical Co.
Millipore filters (0.45 μm, type HA)	Millipore
Triton X-100	Rohm and Haas
BBOT (2.5-bis-[2-(5-tertbutyl-benzoxazolyl)] thiophene)	Packard Inst. Co.
Kodak X-omat RP Film (XRP-1)	Eastman Kodak
Cellogel strips (2.5 cm X 55 cm)	Kalex, Inc.
Cellulose thin-layer plates (glass back)	E. Merck
Nuclease T_1	Calbiochem
Nuclease P_1, T_4 Polynucleotide Kinase, Yeast Hexokinase	Boehringer
Adenosine 5'[γ-^{32}P] triphosphate	Amersham
Bacterial Alkaline Phosphatase	Worthington
Potassium periodate and potassium borohydride	Sigma
EN [^{3}H] ANCE	New England Nuclear
Whatman DEAE-cellulose paper (DE-81)	Reeve Angel and Co.
Yeast $tRNA^{Phe}$	Boehringer

Growth of Drosophila

The diet for rearing *Drosophila melanogaster* consists of 8% cornmeal, 2.9% sucrose, 2.9% dried brewer's yeast, 5.8% glucose and 0.6% agar. Flies were raised at 25°C in 230-ml bottles containing 50 ml of medium or in large growth boxes containing 4–6 L of medium. All flies were harvested at less than 2 days after eclosion and stored at −80°C for not more than 6 months.

Preparation of Aminoacyl-tRNA Synthetase

Crude synthetases were prepared from wild-type adult flies by a modification of the method of Twardzik.[58] Frozen *Drosophila melanogaster* (25 grams) were homogenized in 100 ml of buffer A (10 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 10 mM β-mercaptoethanol and 10% glycerol) in a Waring blender by alternating the speed between high and low every 30 seconds for 2 minutes. The homogenate was centrifuged at 9,000 to 10,000 g for 10 minutes and then filtered through miracloth. The supernatant was centrifuged at 76,000 g for 90 minutes in a Spinco preparative ultracentrifuge, collected and frozen overnight at −85°C. The supernatant was thawed and added to a DEAE-cellulose (DE-23) column (3.8 cm × 30.0 cm) that had been equilibrated overnight with 1000 ml of buffer A. The column was washed with buffer A until the A_{280} fell below 0.10 A_{280}/ml and the enzyme was then eluted with buffer A containing 0.30 *M* NaCl. The tyrosyl-tRNA synthetase was located by aminoacylation, pooled and stored at −20°C. The crude synthetase is generally stable for over a year when prepared in this way.

Aminoacylation of tRNA

Column fractions were aminoacylated to locate the tyrosine tRNA in a final reaction volume of 50 μl. Each reaction contained 50 mM Tris-HCl (pH 8.0), 10 mM magnesium acetate, 10 mM β-mercaptoethanol, 10 mM potassium chloride, 6 μM ^{3}H-tyrosine, 10 mM ATP, a 1:5 dilution of the column fraction, and a 1:4 dilution of the crude aminoacyl-tRNA synthetase. The reaction time was 15 minutes since this time was shown to be adequate to

completely charge the tRNA. The aminoacylation reaction was followed by pipetting 40 μl samples onto 3MM filter paper discs and removing acid soluble radioactive amino acids.[59]

Conditions for aminoacylation of each pooled tRNA fraction to determine purity was as previously described except ^{14}C-tyrosine at 9 μM was used instead of ^{3}H-tyrosine at 6 μM, and the tRNA concentration should be present at 0.3 A_{260}/ml or greater to obtain adequate charging. The tyrosine concentration was increased from 6.0 to 9.0 μM since this concentration was shown to increase the charging level (Table II). Also ribosomal RNA was included in some reactions to help control possible nuclease contamination, however, in most cases, the ribosomal RNA did not increase the extent of charging.

Table II. Effect of tyrosine concentration on extent of charging

Transfer RNA	[^{3}H] tyrosine concentration (mM)	Aminoacid acceptance (picomole/A_{260})
Crude *v; bw* $tRNA^{Tyr}$	3	7.6
	6	9.7
	9	10.1
	12	10.1
Partially purified *v; bw* $tRNA_G^{Tyr}$	3	112.
	6	198.
	9	242.
	12	227.

tRNA Isolation

Transfer RNA was prepared by a modification of the method described by Twardzik et al.[58] Homogenization was performed at 0–4°C in equal volumes of 88% phenol and buffer T (50 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 5 mM β-mercaptoethanol and 50 mM NaCl), with 5 ml of each per gram of flies. The homogenate was centrifuged at 10,000 xg for 15 min, and the aqueous layer removed. The phenol layer was re-extracted

with one-half volume of buffer T and centrifuged as before. The aqueous layers, containing the nucleic acids, were combined and precipitated by the addition of two and one-half volumes of 95% ethanol at −20°C. After overnight storage at −20°C, the precipitate was recovered by centrifugation and resuspended in buffer T. The solution was loaded onto a DEAE-cellulose (DE-23) column previously equilibrated with buffer T. The column was washed with buffer T containing 0.25 *M* NaCl until the A_{260} observed was less than 0.100. The tRNA was eluted with buffer T containing 1.0 *M* NaCl, pooled and precipitated by the addition of two and one-half volumes of 95% ethanol at −20°C. The tRNA was collected by centrifugation and dissolved in buffer B (10 mM sodium acetate, pH 4.6, 10 mM magnesium acetate, 5 mM β-mercaptoethanol, and 300 mM NaCl). At each step of the tRNA purification small aliquots of the tRNA may be saved to determine its specific acceptance in an aminoacylation reaction.

Benzoylated DEAE-Cellulose Chromatography

Whereas the capacity of BD-cellulose is approximately 100 A_{260} units per ml, I did not exceed two-thirds this concentration. The length to diameter ratio of the column was at least 8:1 in all cases and ideally should be even greater. The BD-cellulose column was poured and stored in 0.05 *M* sodium acetate, pH 4.6, 2 *M* NaCl, and 0.02% sodium azide. Prior to sample loading, the column was washed with 15 volumes of buffer B overnight at 4°C. The tRNA, dissolved in buffer B, was diluted to a concentration of 20 A_{260} units per ml and loaded onto the BD-cellulose column. The sides of the column were rinsed with buffer B, and the tRNA eluted with a linear, 3-L gradient, varying the NaCl concentration from 0.3 *M* to 1.0 *M*, with buffer B present throughout. The flow rate for loading and eluting the tRNA was approximately one column bed volume per hour and 15 ml fractions were collected. An A_{260} profile was taken and the tyrosine tRNA located by aminoacylation. The tRNA was brought to a NaCl concentration of 1.0 *M* and precipitated with two and one-half volumes of 95% ethanol at −20°C. This method of location and precipitation of the tRNA was followed in all subsequent steps.

The ethanol precipitated tRNA of the BD-column was collected by centrifugation and dissolved in 10 mM magnesium acetate. From this point forward, all solutions that came in

contact with the tRNA were treated with diethylpyrocarbonate prior to contact with the tRNA.

RPC-5 Chromatography

RPC-5 chromatography was as described by Jacobson et al.,[55] with several modifications. The columns were precision-bore, high-pressure, jacketed glass columns from Glenco Scientific, Inc. The packing consisted of Plaskon beads coated with Adogen 464, and was prepared as described by Pearson et al.,[60] according to method C. The standard temperature was 37°C. A high-speed piston pump rated at 1000 lb. per in.^{2} from Milton-Roy was used. Dry packing was prepared for use by suspending it in the desired buffer containing at least 0.3 *M* NaCl, and blending in a Waring blender for two minutes at low speed. The RPC-5 slurry was then poured into the column and packed under pressure. As the column packed, the buffer above the forming bed was removed and additional RPC-5 slurry added and packed until the bed filled the column. Before using a freshly packed RPC-5 column, one to three milligrams of crude tRNA were applied and eluted to condition the column and create a final stable bed.

Two sizes of columns were used. The large diameter column for the initial separation was 2 cm × 20 cm and the smaller diameter column used for all subsequent separations was 0.6 cm × 58 cm. Although the capacity of an RPC-5 column probably approaches 50 A_{260} units per ml of bed volume for a crude mixture of tRNAs, the capacity of a column for a partially purified tRNA sample that is enriched for a particular species may be much less. The resolution on the larger diameter column was much lower and the peaks broader, but the smaller diameter column did not have the needed capacity. A longer column with a smaller diameter would have been preferable for the initial separation.

Thirteen A_{260} units per ml of bed volume was applied to the 2 cm × 20 cm column in buffer A (10 mM sodium acetate, pH 4.6, 10 mM magnesium acetate, 1.0 mM EDTA) containing 0.3 *M* NaCl. A linear gradient was used that varied the NaCl concentration from 0.48 *M* to 0.58 *M*. The total gradient volume was one L and buffer A, containing freshly added 5 mM β-mercaptoethanol, was present throughout. The flow rate was held between 1.5 and 2.0 ml per minute and 8 ml fractions were collected. Annealed tubes were used to

collect all fractions off the RPC-5 columns. Peaks I and II of the tyrosine tRNA were located, pooled separately and precipitated as previously described.

The tRNA was pelleted by centrifugation; unpelleted tRNA in the ethanol supernatant was then filtered onto a 0.45 μm type HA Millipore filter that was previously washed with 0.5 *N* NaOH followed by three washes with double-distilled water.

The pelleted tRNA and tRNA on the Millipore filter were then dissolved in the appropriate buffer, in this case 10 mM sodium acetate, pH 4.6. This method was used for the collection of ethanol precipitates after all RPC-5 columns.

The tRNA was then loaded onto a 15 ml bed volume DEAE-cellulose (DE-23) column that had been previously equilibrated with buffer I (10 mM sodium acetate, pH 4.6 and 1.0 mM EDTA) containing 0.1 *M* NaCl. The column was washed with 20 volumes of buffer I containing 0.25 *M* NaCl and then eluted with buffer I containing 1.0 *M* NaCl. This step was to assure that as much Mg^{+2} as possible was removed from the tRNA since the next column run was in the absence of Mg^{+2}.

A series of column runs were then made on a 0.6 cm X 58 cm RPC-5 column. In each case the tRNA was loaded onto the column in the appropriate buffer containing 0.30 *M* NaCl. Linear 600 ml gradients using the appropriate buffers and varying NaCl concentrations were run. In all column runs 5 mM freshly added β-mercaptoethanol was present and 3 ml fractions were collected. A maximum of 100 A_{260} units of tyrosine tRNA peak II was loaded onto the first 0.6 cm X 58 cm column and a 0.50 *M* to 0.60 *M* NaCl gradient was run with buffer A minus magnesium acetate present throughout. The tyrosine tRNA was located and collected as previously described. The gradient for the next column varied the NaCl concentration from 0.50 *M* to 0.58 *M* and buffer A was present throughout. The tRNA was located and collected as before and loaded onto a 0.6 cm X 58 cm RPC-5 column that varied the NaCl concentration from 0.48 *M* to 0.56 *M*. The sodium acetate in buffer A was replaced by 10 mM sodium formate at pH 3.0 for this column run. The level of incorporation of ^{14}C-tyrosine should be at least 700–900 pmoles per A_{260} unit of tRNA upon aminoacylating the ethanol precipitated pool of this tRNA.

Before each run the RPC-5 column was washed with 1.5 *M* NaCl containing the appropriate buffer and then equilibrated with the buffer containing the lowest NaCl concentration

present in the gradient. It was found that adding one drop of Adogen 464 per 500 ml of buffer used on the RPC-5 column greatly increased the life of the column.

Purification of $tRNA_Q^{Tyr}$ on RPC-5 required only that the upper and lower limits of the NaCl concentration on each gradient be lowered 0.02 *M*. It is impossible to predict what steps would need to be taken to purify other tRNA species on RPC-5. Any conditions that would have an effect on the secondary or tertiary structure of the tRNA might be considered. Thus, variations in pH, magnesium concentration, and temperature are often employed.

Two-Dimensional Polyacrylamide Gel Electrophoresis

The methods used for the final purification of the tRNA have been discussed by Farmerie et al.[61] The method involves two-dimensional polyacrylamide gel electrophoresis in the presence of 4 *M* urea. Many tRNAs show differential denaturability in the presence of 4 *M* urea and can thus be separated by polyacrylamide gel electrophoresis on this basis.

The composition of the gel for the first dimension was 10% acrylamide (w/v), 0.4% bisacrylamide, 0.08 *M* Tris-HCl, pH 8.3, 0.09 *M* boric acid, 0.0025 *M* EDTA, and 4 *M* urea. Before the gel solution was brought to its final volume, the pH was checked and adjusted if necessary. The solution was filtered through a 0.45 μm Millipore filter and degassed. Because the gels were poured and run at 4°C, the gel solution was chilled before adding the initiator and catalyst. The gel plates were 40 cm long, and 0.5 mm thick. The gel was polymerized by adding 0.36% DMAPN (3-dimethlaminopropionitrite) and 0.09% ammonium persulfate. The gel was poured between the plates up to a line 5 to 6 cm below the expected level of the loading slots. Before the gel polymerized, the surface was covered with running buffer (0.09 *M* Tris-HCl, pH 8.3, 0.09 *M* boric acid, 0.0025 *M* disodium EDTA and 4 *M* urea).

After allowing the gel to polymerize for two hours and washing the top of the gel with 0.05 *M* Tris-HCl at pH 6.7, a stacking gel was poured on top of the 10% gel. The composition of the stacking gel was 5% acrylamide, 0.25% bisacrylamide and 4 *M* urea buffered to pH 6.7 with 0.05 *M* Tris-HCl. The gel solution was handled as previously described and polymerized by adding 0.40% BMAPN and 0.08% ammonium persulfate. The samples, dissolved in 10 μl of a loading solution composed of 20% RNAse-free sucrose, 4 *M* urea, 0.1 *M* sodium acetate,

pH 4.5, 0.01% xylene-cyanole-ff marker, and 0.01% bromophenol blue, were loaded into the interior lanes of the gel. The top of the gel was connected to the upper buffer chamber by Miracloth which had been sandwiched between parafilm sheets to retard evaporation. The samples were run through the stacking gel at 300 volts and the voltage then increased to 700 volts for the remainder of the run.

The gel was run until the slowly migrating xylene-cyanole-ff, blue dye marker, was within 5 to 10 cm of the bottom of the gel. If one or more A_{260} units were loaded onto the column the tRNA bands could be visualized by the UV shadowing technique.[62] The region to be transferred was selected and cut out by using a clean scapel.

The gel for the second dimension must be only slightly wider than the section transferred from the first dimension. The length of the gel was 48 cm and the thickness was again 0.5 mm. The gel composition was 20% acrylamide, 0.8% bisacrylamide, 0.08 *M* Tris-HCl, pH 8.3, 0.08 *M* boric acid, 0.0025 *M* EDTA, and 4 *M* urea. The gel was polymerized by adding 0.36% DMAPN and 0.08% ammonium persulfate. The transferred gel strip was turned 90° for the second dimension. The gel solutions were again chilled prior to being polymerized. The gel solution was poured to within 1 cm of the bottom of the transferred gel strip and carefully topped with running buffer containing 4 *M* urea.

After allowing the gel two hours to polymerize, a layer of 10% gel (identical to that in the first dimension) was added to surround and just cover the transfer from the first dimension. The second dimension was run at 300 volts until the xylene-cyanole-ff migrated into the 20% gel and the voltage then increased to 700–1000 volts. The xylene-cyanole-ff dye marker was run through the gel and a second xylene-cyanole-ff marker added and ran two-thirds to three-fourths the length of the gel. The second dimension required three to four days. The tRNA was identified by the UV shadowing technique but if necessary the tRNA may be identified by staining the gel.

The tRNA band was cut out of the gel, placed in an Eppendorf tube and eluted for 24 h with water or 2 *M* NaCl, the latter being preferred. The tRNA band was then re-extracted a second time in the same manner.

Base Analysis

Hydrolysis of tRNA. The base composition of yeast $tRNA^{Phe}$ and of *v; bw*, and *su(s)*[2]

v; bw $tRNA_G^{Tyr}$ was determined by a modification of Randerath's tritium derivative method.[63] Thirty nanograms of nuclease P1 was incubated with 0.02 A_{260} units (approximately one microgram) of tRNA in 10 μl of water. After 12 to 18 h incubation, ammonium bicarbonate, pH 8.0 and bacterial alkaline phosphatase were added to final concentrations of 10 mM and 2.0×10^{-4} units/μg tRNA respectively. Incubation was at 37°C for 6 h in a final reaction volume of 12 μl. The sample was then lyophilized 3 or 4 times to remove the ammonium bicarbonate.

Periodate Oxidation. One microgram of digested RNA, equal to approximately 3 nanomoles of nucleoside, was dissolved in 15 μl of water. In a well-ventilated fume hood, 4.5 μl of a 2 mM potassium periodate solution was then added to the nucleoside solution. The reaction was allowed to proceed for 2 h at room temperature in the dark.

Potassium Borohydride Reduction. Potassium borohydride (^3H) was obtained as a dried powder from Amersham. The $KB(^3H)_4$ was dissolved in carbon dioxide-free 0.1 *N* potassium hydroxide so that the final borohydride concentration was 0.1 *M*. Unlabelled 0.1 *M* KBH_4 in 0.1 *N* KOH was then added to adjust the specific activity 4.0 Ci/mmole. The KBH_4 may then be stored for up to 6 months at −80°C until needed.

Following periodate oxidation, the solution was cooled on ice and 1 *M* potassium phosphate buffer, pH 6.8, was added to a final concentration of 15 mM. One microliter, 100 nanomoles, of the KBH_4 was added, the solution was mixed thoroughly and incubated for 2 h in the dark, in a hood.

Decomposition of Excess (^3H) Potassium Borohydride and Preparation for Chromatography. Following borohydride reduction, a 500-fold molar excess of acetic acid to borohydride was added and the solution was incubated in an unstoppered tube for 30 min in the hood. Caution should be taken when the acetic acid is added because the excess borohydride will now be released as tritium gas. After the 30 min incubation, the solution was evaporated to dryness in a stream of filtered air and the residue dissolved in 30 μl of water.

Chromatography. For purposes of chromatography, 1.5 nanomoles of nucleoside (15 μl) was lyophilized dry and resuspended in 2 to 3 μl of 0.1 *N* formic acid. The solution was

applied at an origin 2.5 cm from the bottom, left-hand edge of a 20 X 20 cm glass-backed cellulose thin-layer plate. The solution was applied slowly, with drying between applications, to keep the sample area small.

Development in the first dimension was with acetonitrile/ethyl acetate/n-butanol/ isopropanol/6 *N* ammonia (7/2/1/1/2.7, by volume) to 17 cm from the origin. The chromatogram was thoroughly dried in a fume hood and the thin-layer plate was etched at 1.5 cm from the top and bottom edges of the plate.

The solvent for the second dimension was t-amyl alcohol/methyl ethyl ketone/ acetonitrile/ethyl acetate/water/88% formic acid (4/2/1.5/2/1.5/0.28). A 6 cm Whatman 1 wick was attached to the top of the plate (the first dimensional right-hand side) and the chromatogram developed to 5 cm on the Whatman 1 wick. In both dimensions, the chromatography was started immediately after adding the solvent to the tank.

After the plates were thoroughly dried, the Whatman 1 wicks were removed and the cellulose plates were each sprayed with 2 to 3 light coats of EN(^{3}H) ANCE. The plates were then marked with S^{35}-ink and fluorographed at −80°C for 7 to 10 days.

Elution of Compounds and Counting. With the aid of the S^{35}-ink marks, the film and thin-layer plate were aligned and the location of the labelled nucleoside trialcohols were marked on the back side of the plate. Each cellulose area corresponding to a nucleoside trialcohol was then collected by scraping the area with a disposable glass rod and vacuuming the cellulose into an Eppendorf disposable pipet tip which was plugged at its small end with glass wool. The tritiated nucleoside trialcohols were then eluted from the cellulose with 2 *N* ammonia by placing the plugged end of the pipet tip in a 400 μl Eppendorf test tube, adding 50 μl of 2 *N* ammonia, and spinning it in an Eppendorf microcentrifuge. This procedure was repeated several times (usually 4 to 5 times) until no more counts eluted from the cellulose. Fifty microliters of sample were added to 950 microliters of water and the solution was counted in 10 ml of a counting scintillant composed of 1 part Triton X-100 and 2 parts 0.4% BBOT in toluene.

The percent of the total counts in each spot is determined and then expressed as the number of residues in a chain length of 78. This number was chosen because it represents an average tRNA chain length and because yeast $tRNA^{Tyr}$ contains 78 residues.

Fingerprinting of $tRNA_G^{Tyr}$

Complete T_1 Digestion and Dephosphorylation. The $tRNA_G^{Tyr}$ was denatured by heating in boiling water for one 1 min and then quick-cooling on dry ice. The digestion reaction contained 0.5–5 μg (normally 2.5 μg) of tRNA, 0.25 units of T_1 RNase per microgram of tRNA, and 20 mM Tris-HCl, pH 8.0. The nuclease digestion was for 3 h at 37°C in a reaction volume of 10 μl. The tRNA fragments were dephosphorylated by adding 1×10^{-3} units of bacterial aklaline phosphatase per microgram tRNA and incubating at 37°C for 2 h. The phosphatase reaction was terminated by cooling the reaction solution to room temperature, adding 0.1 volume of 50 mM nitrilotriacetic acid (NTA), and incubating for 20 min at room temperature. The mixture was then heated in boiling water for 90 s and quick-cooled on dry ice.

The *in vitro* phosphorylation reaction contained 0.25–0.5 μg of the digested tRNA, 1.7 nanomoles of (γ-^{32}P) ATP at 100–200 Ci/mmole per 0.25 μg tRNA, 20 to 40 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 10 mM dithiothreotol and 5 units of T_4 polynucleotide kinase per 0.25 μg of digested tRNA. Incubation was at 37°C for 30 min in a final reaction volume of 18 μl. After 30 min, 25 nanomoles of glucose and 0.002 units of yeast hexokinase per 0.25 μg digested tRNA were added and incubated for 10 min at 37°C. The mixture was cooled to room temperature and 2.5 nanomoles of unlabelled ATP per 0.25 μg of digested tRNA were added. After incubation for 10 min, another equal amount of unlabelled ATP was added and incubated an additional 10 min. The mixture was then heated in boiling water for one min and quick-cooled on dry ice.

These reactions were designed to promote quantitative labelling of oligonucleotides. However, some oligonucleotides, especially those with modified bases at the 5′ terminus, may not be labelled as efficiently.

Two-Dimensional Separation of Oligonucleotides. The high voltage paper electrophoresis equipment was from Savant Instruments, Inc. The two dimensional separation procedure was essentially Sanger's procedure,[64] as modified by Silberkland et al.[65] For a detailed description, see Barrell.[66]

The labelled oligonucleotide solution was lyophilized dry and resuspended in 1.5 μl of water. The lower portion of the electrophoresis tank was filled with a pH 3.5 buffer

consisting of 0.5% pyridine and 5% acetic acid. The remainder of the reservoir was filled with Varsol.

The Cellogel strips (2.5 cm X 55 cm) were stored in 50% methanol at 4°C. Prior to use, the Cellogel strip was soaked in a pH 3.5 buffer containing 5% acetic acid, 0.5% pyridine, 7 *M* urea and 1 mM EDTA. Excess buffer was blotted in the area of sample application and the sample was applied as a spot in the middle of the Cellogel strip at an origin 6 cm from the cathode end. Approximately one microliter of a tracking dye consisting of 1% xylene-cyanole-ff, 1% methyl orange and 1% acid fuchsin in water was applied to each side of the sample. To keep the strip from drying out during sample application, all but the sample area was covered with Saran-Wrap. After the sample area was dry, excess buffer was blotted and the strip was dipped in Varsol. The strip was placed on a rack and lowered into the tank so that the sample area was above the level of the pH 3.5 buffer and both ends of the strip were in the pH 3.5 buffer. Electrophoresis was from cathode to anode at 45 v/cm until the xylene-cyanole-ff (blue dye marker) had migrated a total of 18 cm.

The procedure for the transfer of oligonucleotides from Cellogel to DEAE-paper (46 cm X 57 cm) was as described by Barrell,[66] and modified by Silberkland et al.[65] Three Whatman 3 MM strips (46 cm X 3 cm) were placed under the DEAE paper at the 6 cm origin. The excess Varsol was allowed to drip off the Cellogel strip and the strip was then placed, oligonucleotide surface down, on the DEAE-paper between the yellow and pink spots on the Cellogel.

Three more Whatman strips identical to the others were then soaked with water and placed on top of the Cellogel strip. A glass plate was then put on top and a lead brick was placed on top of the glass plate for added weight. After 15 min, the glass plate was lifted and the Whatman 3 MM papers on top of the DE-81 paper were wetted again. The glass plate and brick were then put back on top, and the transfer allowed to continue for an additional 15 min.

Following the transfer, the DEAE-paper was placed on a rack and the wet area was soaked with 95% ethanol. After drying, the paper was placed on a glass surface with a glass rod under the paper at the 6 cm origin. Glass rods were then placed on both sides of the 6 cm origin on top of the paper. The paper was wetted along the outside edges of the glass rods

with 7% formic acid in such a way that the liquid from each side reached the 6 cm origin at the same time. The rest of the cathode half of the DEAE-paper was wetted with 7% formic acid, leaving about 1 cm of dry area at the top and the DEAE-paper was then placed back on the rack. It was useful to prefold the paper before wetting with formic acid to facilitate the placement of the paper on the rack. The rest of the paper was wetted with 7% formic acid and lowered into the electrophoresis tank containing 7% formic acid. Electrophoresis was at 700 volts until the xylene-cyanole-ff dye had migrated a total of 25 to 30 cm. The DEAE-paper was dried, marked with S^{35}-ink and autoradiographed to locate the oligonucleotides.

CHAPTER III

RESULTS

Aminoacylation of tRNA

Initially one-half volume of the aminoacylation reaction consisted of the column fraction containing the tRNA, since it was felt that increasing the tRNA concentration would increase the extent of charging and facilitate identification of $tRNA^{Tyr}$. However, it was found that under these conditions the extent of charging was much lower than expected and in some instances undetectable. Examination of aminoacylation conditions revealed high sodium chloride concentrations inhibited the aminoacylation reaction (Fig. 1). Since the major source of sodium chloride was the column fraction, the salt concentration was being increased proportional to the increase in the tRNA concentration. Thus it was necessary to either remove the sodium chloride from each fraction to be assayed, or find a dilution that would remove or greatly reduce the inhibitory effect of the sodium chloride and still supply enough tRNA to obtain adequate levels of charging. It was found that a 1:5 dilution of the column fraction in the aminoacylation reaction, which lowered the sodium chloride concentration to approximately 0.11 M, produced better results than the 1:2 or the normal 1:4 dilution. This dilution was used in all the aminoacylation reactions to scan the columns.

Isolation and Purification of tRNA

Phenol extraction and DEAE-cellulose chromatography of *v; bw* and *su(s)*^{2} *v; bw* flies produced 25.0 and 24.3 A_{260} units per gram of flies respectively. This was within the generally accepted range of 20 to 25 A_{260} units per gram of flies normally obtained using this method.

Purification of the tRNAs was accomplished by benzoylated DEAE-cellulose chromatography followed by a series of RPC-5 columns and finally two-dimensional gel electro-

Fig. 1. Effect of NaCl on the extent of aminoacylation of *v; bw* tyrosyl-tRNA. [^{3}H] tyrosine acceptance of partially purified $tRNA_Q^{Tyr}$ (●) and crude $tRNA^{Tyr}$ (○) from *v; bw*.

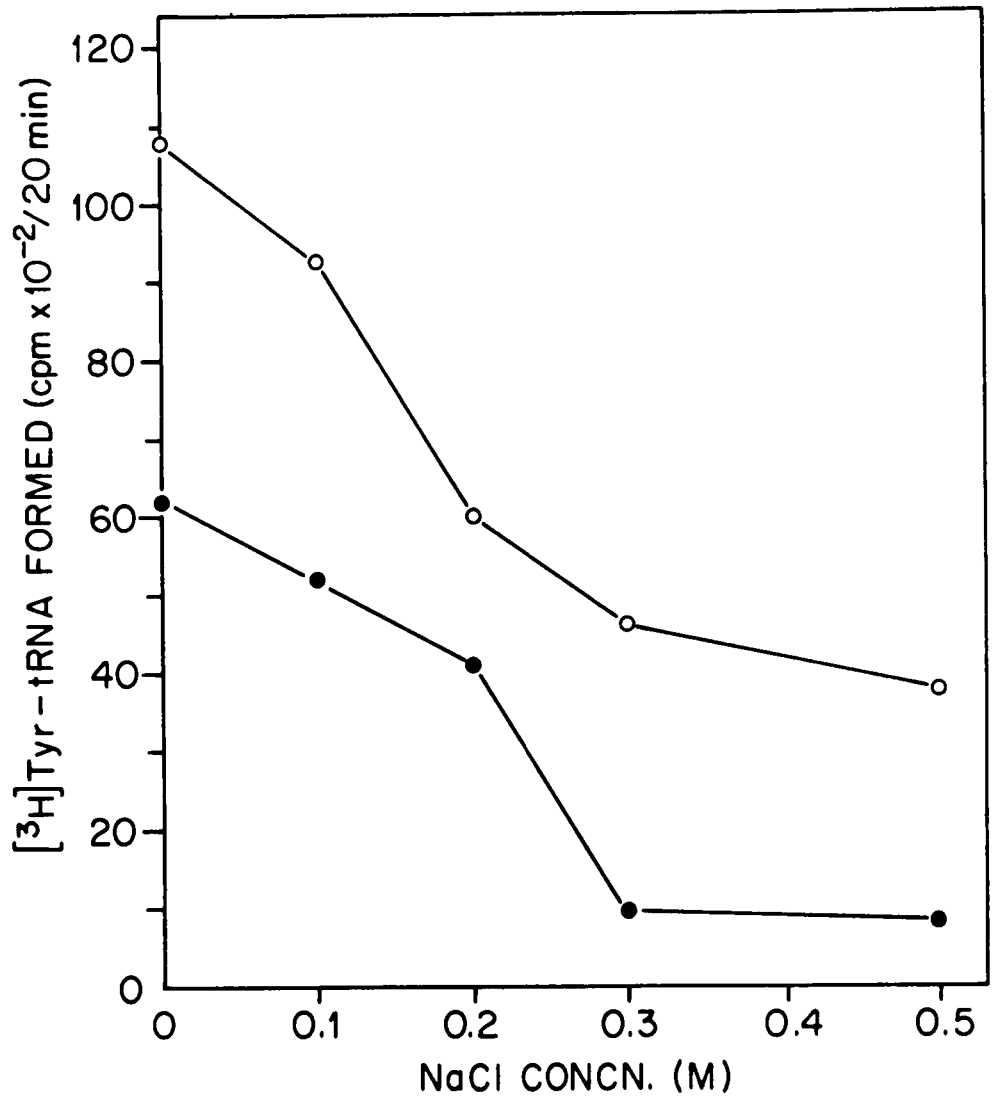
120
100
80
60
40
20
0
[3H]Tyr-tRNA FORMED (cpm x10-2/20 min)
0
0.1
0.2
0.3
0.4
0.5
NaCl CONCN. (M)

Figure 1

phoresis. Chromatography on BD-cellulose (Fig. 2) resulted in approximately a two-fold increase in the purity of the tRNA (Table III) and was desirable because of the high capacity of BD-cellulose.

The major proportion of the tRNA purification was accomplished by a series of RPC-5 column runs during which the magnesium concentration and pH were varied (Figs. 3 and 4). After the final RPC-5 chromatography, the tRNA A_{260} peak and the amino acid accepting peak coincided (Fig. 4). At this stage the tRNA accepted 700–900 picomoles tyrosine/A_{260} unit, representing at least a 70-fold increase over crude tRNA (Table III). The recovery of $tRNA_G^{Tyr}$ after all RPC-5 chromatography was greater than 20% as determined by tyrosine acceptance of the A_{260} material (Table III).

When an end group determination of tRNA was performed, more than one nucleotide was found. To purify the tRNA further it was subjected to two-dimensional polyacrylamide gel electrophoresis, which separated the A_{260} absorbing material into 3 bands. The major band was shown to be $tRNA_G^{Tyr}$ by aminoacylation, while the two minor bands, equivalent to 20 to 30% of the total A_{260} material did not accept tyrosine. Thus, the gel resulted in at least an additional 20% purification of the tRNA.

Two-dimensional gel electrophoresis followed by dialysis of the extracted sample against water resulted in approximately one-half of the total applied being recovered as $tRNA_G^{Tyr}$. The loss of A_{260} material occurring in the two-dimensional gel was due to tRNA being lost in the extraction process, in dialysis and in the process of purification.

Base Analysis

One microgram of tRNA was analyzed for base composition by the method of Randerath et al.[64] Base analysis was performed on $tRNA_G^{Tyr}$ from *v; bw* and *su(s)² v; bw* and also on yeast $tRNA^{Phe}$ as a control. After two-dimensional thin layer chromatography, the positions of the labelled nucleoside trialcohols were identified by fluorography (Fig. 5). Base analysis of yeast $tRNA^{Phe}$ helped to establish that the procedure was quantitative under the conditions employed (Table IV). A comparison of the base composition of $tRNA_G^{Tyr}$ from *v; bw* and *su(s)² v; bw* showed that, as expected, the tRNAs were very similar (Table V). However, $tRNA_G^{Tyr}$ from *su(s)² v; bw* appeared to have an additional m_2^2G and ψ.

Fig. 2. Benzoylated DEAE-cellulose chromatography of *v; bw* tRNA. The sample ($2501A_{260}$) was applied to a 1.6 cm × 17.5 cm column and 16 ml fractions collected. The tyrosine tRNA was located by aminoacylation with [^{3}H] tyrosine.

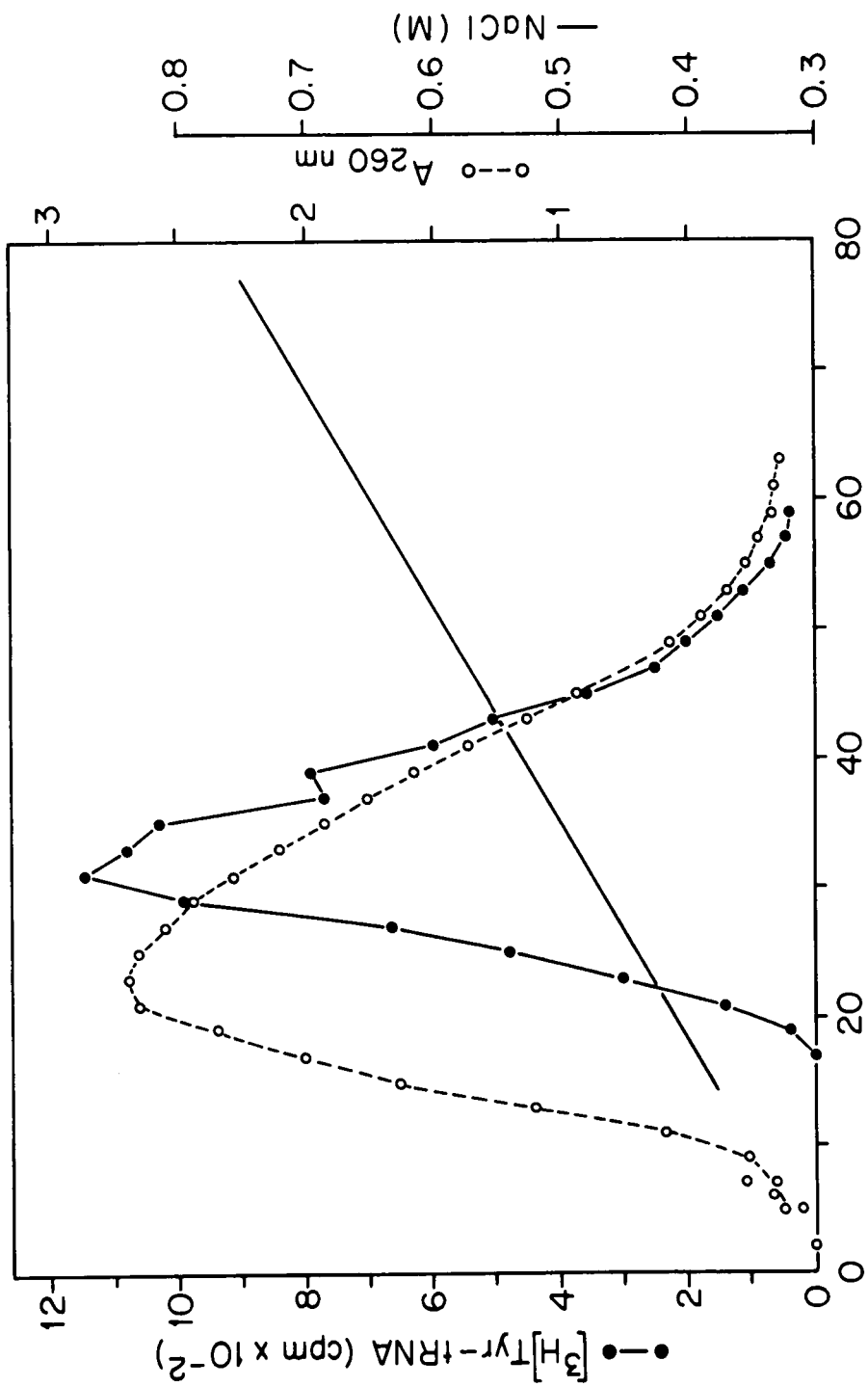
●—● [³H]Tyr-tRNA (cpm x 10⁻²)
o---o A260 nm
— NaCl (M)
FRACTION NO.
0
2
4
6
8
10
12
0
1
2
3
0.8
0.7
0.6
0.5
0.4
0.3
20
40
60
80

Figure 2

Table III. Chromatography stages in the purification of $tRNA_G^{Tyr}$

Genotype	Stage	A_{260} units	% initial/ A_{260} units	Specific acceptance picomoles Tyr A_{260} unit	Total picomoles $tRNA^{Tyr}$	$tRNA^{Tyr}$ total $tRNA^{Tyr}$	Total picomoles $tRNA_G^{Tyr}$	% initial picomoles $tRNA_G^{Tyr}$
su(s)^2 *v; bw*	DE-23 (crude tRNA)	2384.0	100.0	10.1	24078.4	0.35	8427.4	100.0
	BD-cellulose	795.0	33.3	21.8	17331.0	0.35	6065.8	72.0
	RPC-5 (2 × 20 cm)	96.0	4.0	48.2	4627.2	–	4627.2	54.9
	1st RPC-5 (0.6 × 54 cm)	12.0	0.50	255.	3060.0		3060.0	36.3
	2nd RPC-5 (0.6 × 54 cm)	5.04	0.21	487.	2454.5	–	2454.5	29.1
	3rd RPC-5 (0.6 × 54 cm)	2.45	0.10	851.	2085.0		2085.0	24.7
v; bw	DE-23 (crude tRNA)	2501.	100.0	10.0	25010.	0.35[a]	8753.5	100.0
	BD-cellulose	970.	38.8	19.9	19303.	0.35	6756.	77.2
	RPC-5 (2 × 20 cm)	78.4	3.1	59.8	4688.	–	4688.	53.6
	1st RPC-5 (0.6 × 54 cm)	50.	2.0	–	–	–	–	–
	2nd RPC-5 (0.6 × 54 cm)	9.48	0.38	294.	2787	–	–	–
	3rd RPC-5 (0.6 × 54 cm)	2.9	0.12	702.	2036.		2036.	23.3

[a] Fraction of *v; bw* $tRNA^{Tyr}$ present in the G form was assumed to be 0.35 based on previous results with *v; bw* $tRNA^{Tyr}$ and on the value obtained for *su(s)*^{2} *v; bw* $tRNA_G^{Tyr}$.

Fig. 3. First RPC-5 chromatography of *v; bw* tRNA. The sample was applied to a 2 cm × 20 cm column and a linear 1-liter gradient varying the NaCl concentration from 0.48 *M* to 0.58 *M* was run with buffer A present throughout. The fraction size was 8 ml and the tRNA was located by aminoacylation with [^{3}H] tyrosine.

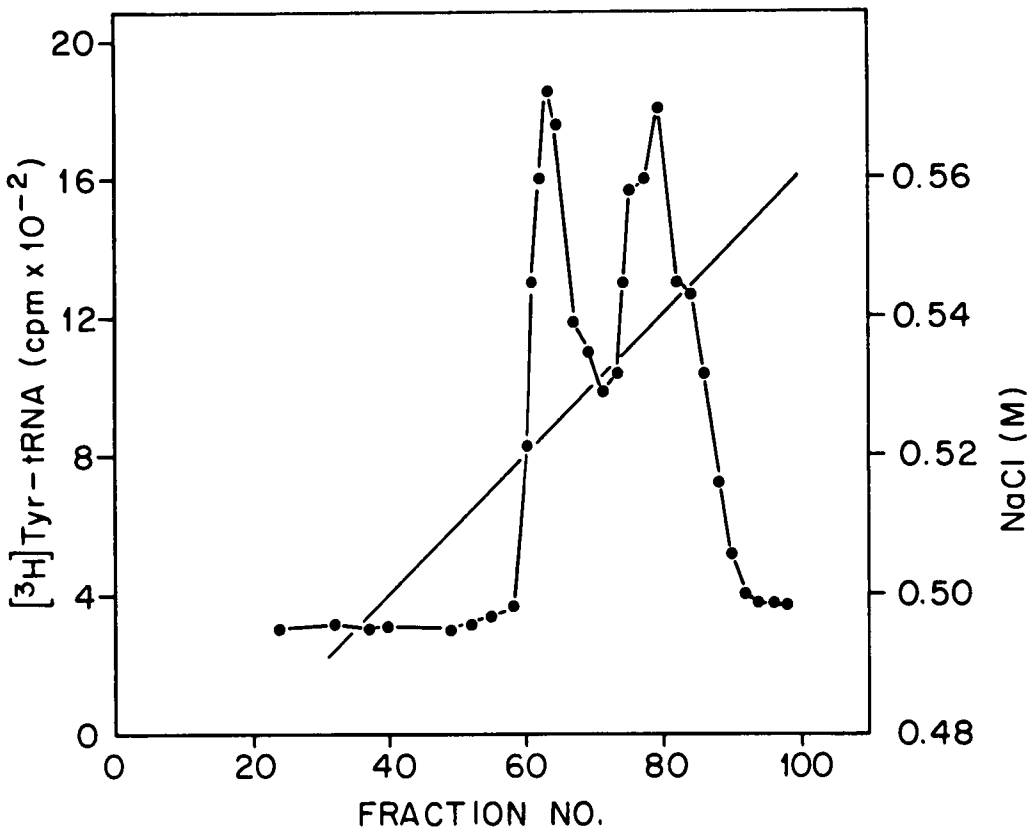
20
16
12
8
4
0
[3H]Tyr-tRNA (cpm x 10-2)
0
20
40
60
80
100
FRACTION NO.
0.56
0.54
0.52
0.50
0.48
NaCl (M)

Figure 3

Fig. 4. Subsequent RPC-5 chromatography of *v; bw* **$tRNA_G^{Tyr}$.** Column size was 0.6 cm × 58 cm and 4.0 ml fractions were collected. The gradient volume was 600 ml and elution of the tRNA was monitored at 260 nm (○). The $tRNA^{Tyr}$ was located by amino-acylation with [^{3}H] tyrosine (●).

(a) Chromatography of the ethanol precipitated pool of $tRNA_G^{Tyr}$ (fractions 72–90) from the 2 cm × 20 cm RPC-5 column. The gradient varied the NaCl concentration from 0.50 *M* to 0.60 *M* and buffer A minus magnesium was present throughout.

(b) Chromatography of the ethanol precipitated pool (fractions 75–93) from (a). The gradient varied the NaCl concentration from 0.50 *M* to 0.58 *M* and buffer A was present throughout.

(c) Chromatography of the ethanol precipitated pool (fractions 38–50) from (b). The gradient varied the NaCl concentration from 0.48 *M* to 0.56 *M*. The sodium acetate in buffer A was replaced by 10mM sodium formate at pH 3.0.

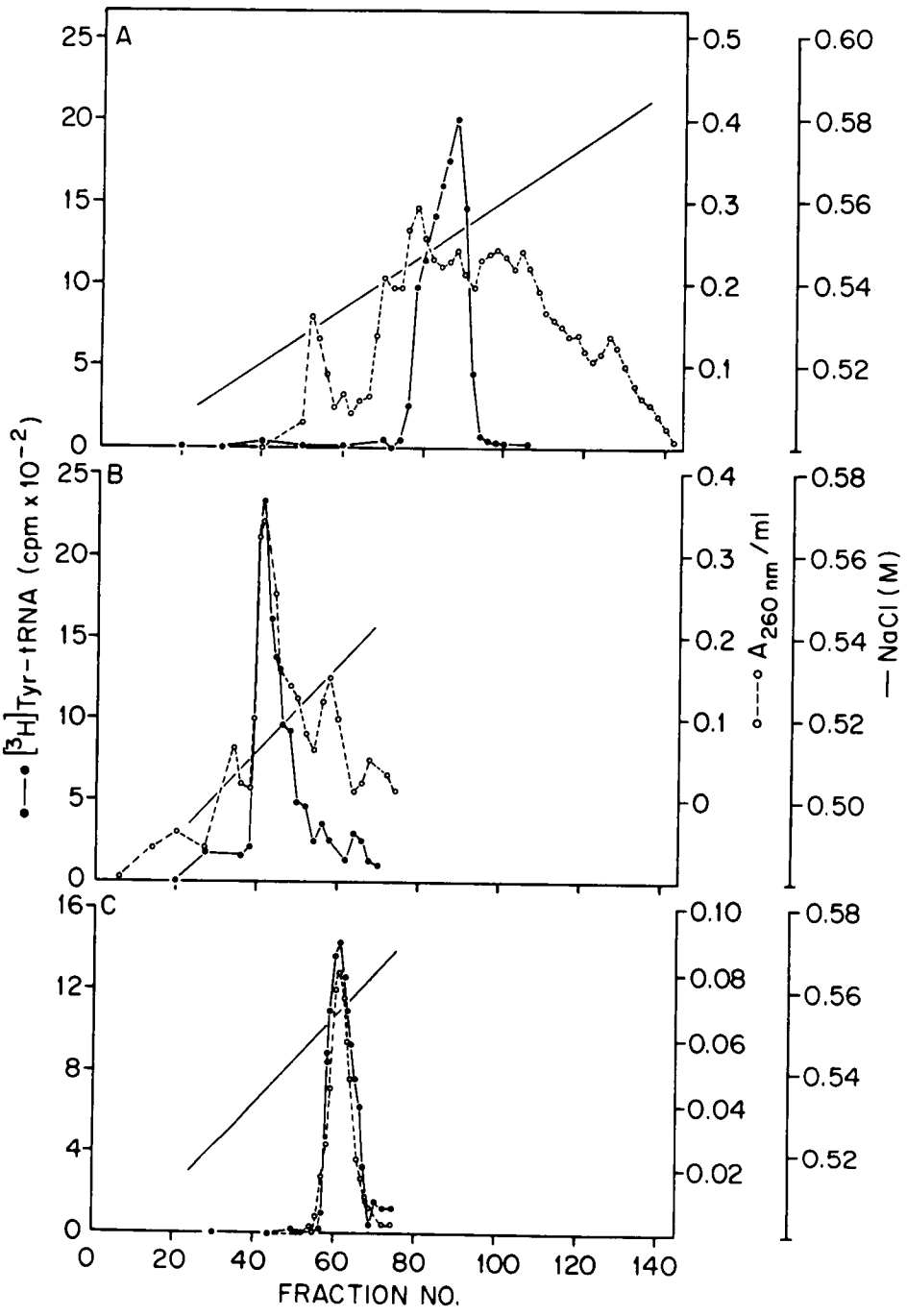
A
B
C
•—• [3H]Tyr-tRNA (cpm x 10−2)
o---o A260 nm/ml
— NaCl (M)
FRACTION NO.
0
20
40
60
80
100
120
140

Figure 4

Fig. 5. Tritium labelled nucleoside trialcohol derivative of *Drosophila melanogaster* (a) $tRNA_G^{Tyr}$ from *v; bw* and (b) $tRNA_G^{Tyr}$ from *su(s)*^{2} *v; bw*. First dimension is from bottom to top and second dimension is from left to right. ψ-m is a breakdown product of ψ and X is believed to be a 3-(3-amino-3-carboxypropyl) uridine.[68] Background spots are labelled b. The exposure time was 7 days.

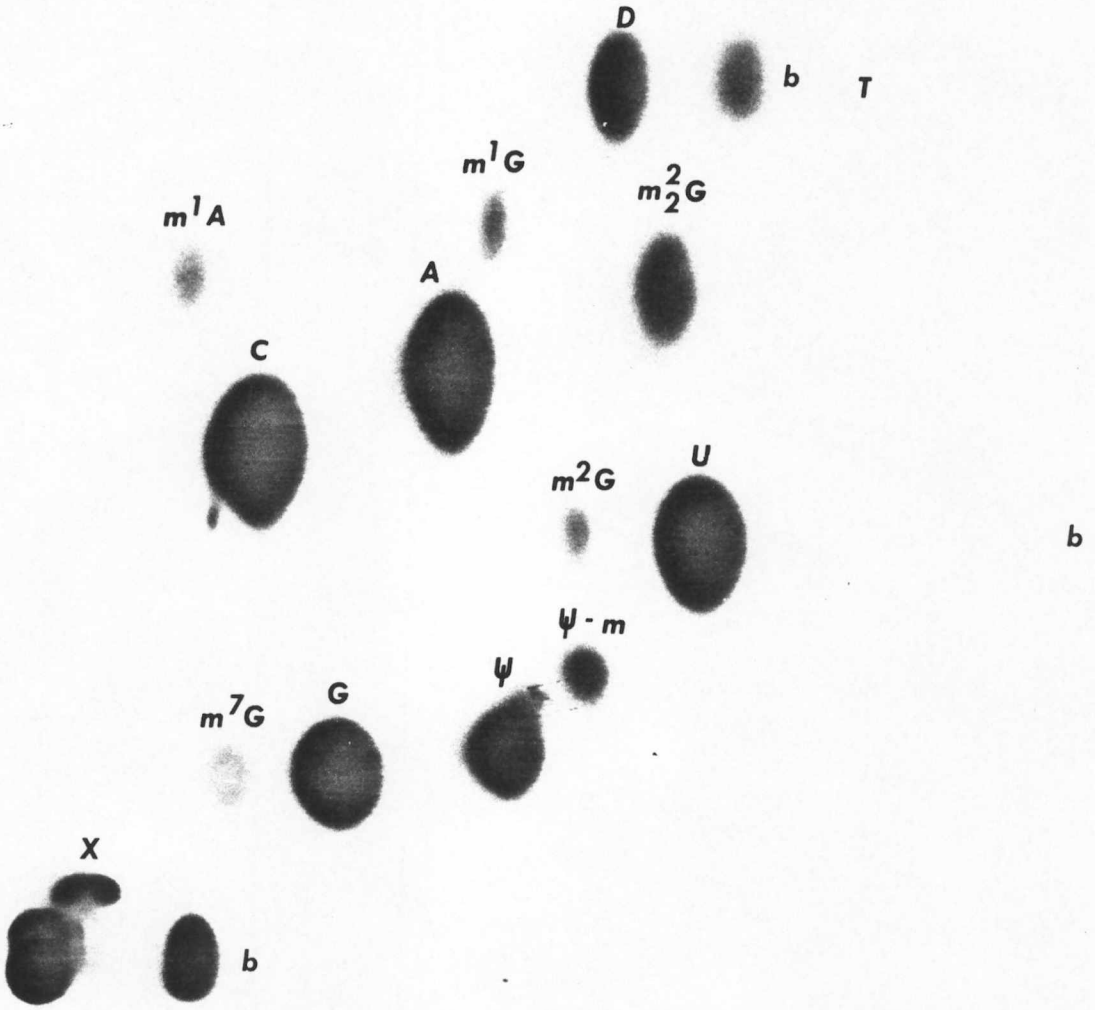

Figure 5(a)

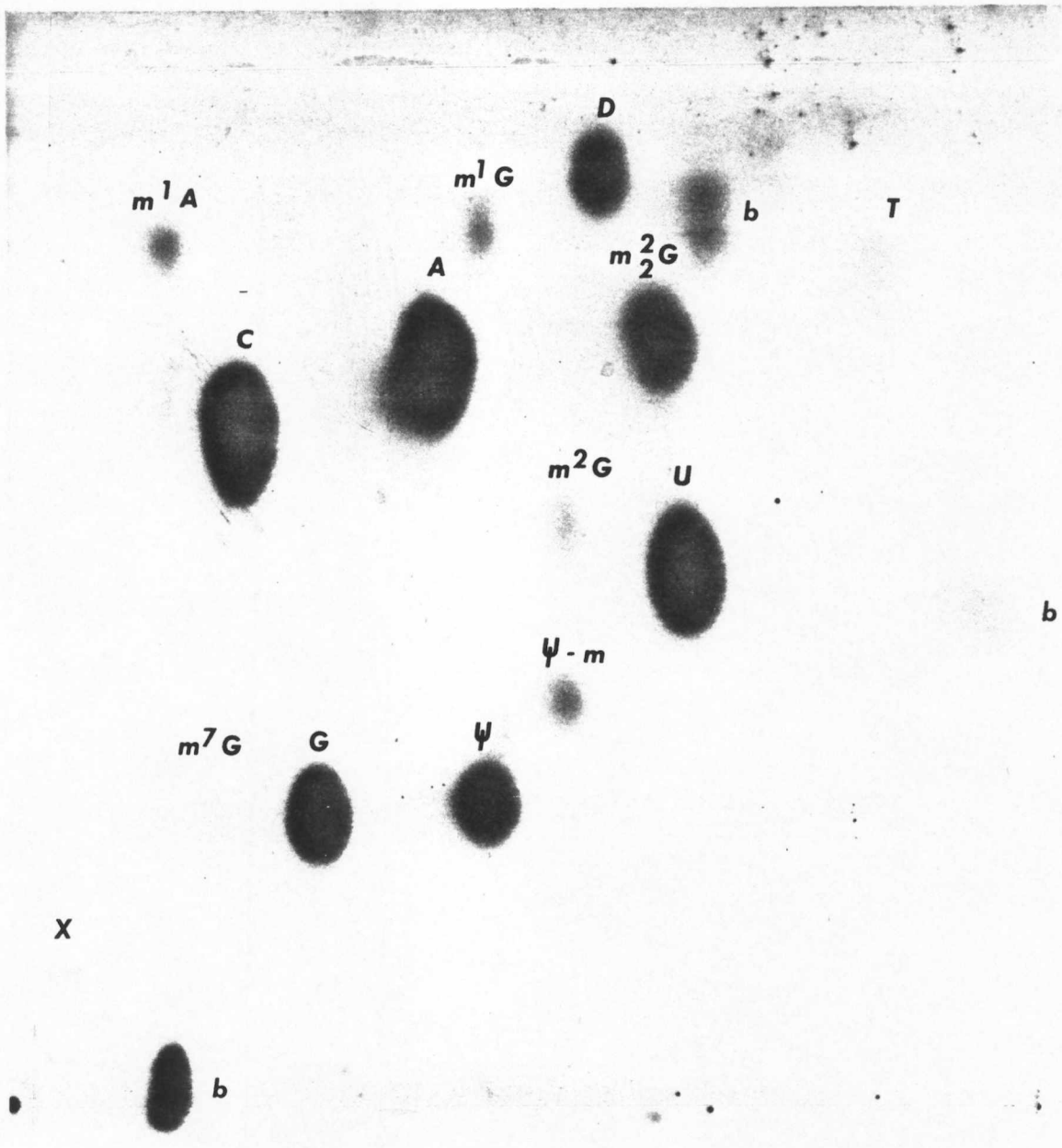

Figure 5(b)

Table IV. Nucleoside composition of yeast $tRNA^{Phe}$

Spot No.	Nucleoside	Calculated[a]	Known
1	m^7G	0.80	1
2	G	18.3	18
3	$\psi + \psi$–m	1.96	2
4	U	11.7	12
5	m^2G	0.94	1
6	A	16.9	17
7	C	15.7	15
8	m^5C	1.81	2
9	m^1A	1.08	1
10	m_2^2G	1.07	1
11	D	2.26	2
12	T	0.99	1

[a] Expressed as the number of residues in a chain length of 73. This number was chosen because 73 was the total number of residues in yeast $tRNA^{Phe}$ capable of being labelled using this procedure.

Table V. Comparison of nucleoside composition of *v; bw* and *su(s)² v; bw* $tRNA_G^{Tyr}$ from *Drosphila Melanogaster.*

Spot No.	Nucleoside	$tRNA_G^{Tyr}$ [a]	
		su(s)² v; bw	*v; bw*
1	x	0.95 ± .012	0.90 ± .060
2	m^7G	1.00 ± .038	0.93 ± .078
3	G	13.98 ± .217	15.37 ± .510
4–5	$\psi + \psi$–m	6.91 ± .343	5.85 ± .076
6	U	12.06 ± .200	12.73 ± .276
7	m^2G	0.68 ± .067	0.77 ± .036
8	C	19.00 ± .357	19.15 ± .765
9	m^1A	0.76 ± .010	1.03 ± .095
10	A	14.90 ± .409	14.48 ± .389
11	m^1G	0.65 ± .055	0.82 ± .036
12	m_2^2G	4.07 ± .035	2.72 ± .250
13	D	2.67 ± .025	2.97 ± .118
14	T	0.39 ± .010	0.31 ± .029

[a] Expressed as the number of residues in a chain length of 78. This number was chosen because it represents an average tRNA chain length and because yeast $tRNA^{Tyr}$ contains 78 residues.

Fingerprint Analysis

Fingerprints of $tRNA_G^{Tyr}$ from *v; bw* and *su(s)²v; bw* were obtained by RNase T_1 digestion of the intact tRNA, 5′-^{32}P end labelling of the generated oligonucleotides with T_4 polynucleotide kinase and two-dimensional separation of the oligonucleotides (Fig. 6). The circled xc in each indicates the position of the xylene-cyanole-ff blue dye marker.

The oligonucleotide patterns were very similar. Fragment 3 from *su(s)²v; bw* migrates slightly faster in the second dimension than the corresponding fragment from *v; bw*. This was shown by the fact that fragment 3 from *v; bw* migrates slightly behind the blue dye marker while fragment 3 from *su(s)²v; bw* migrates slightly ahead of the blue dye marker. The distance fragment 3 migrates in the second dimension can also be compared to the migration of other fragments. Results of this comparison are shown in Table VI. The distance migrated was determined by measuring from the origin to the midpoint of the spot. In all cases fragment 3 from *su(s)²v; bw* migrates comparatively faster. It can also be seen that in *su(s)²v; bw*, fragment 3 migrates faster than fragment 4 while in *v; bw*, the reverse is true. The distances migrated in the second dimension cannot be compared directly because electrophoresis on the two sets of tRNA fragments had to be run separately.

Table VI. Comparison of $tRNA_G^{Tyr}$ fragment 3 from *v; bw* and *su(s)²v; bw*

Fragment	Distance migrated (cm)		Mobility of fragment 3 relative to listed fragment	
	v; bw $tRNA_G^{Tyr}$	*su(s)²v; bw* $tRNA_G^{Tyr}$	*v; bw*	*su(s)²v; bw*
16	5.2	4.6	4.75	5.04
13	15.6	13.2	1.58	1.76
4	25.6	22.6	0.965	1.03
3	24.7	23.2	1.00	1.00
2	30.9	27.0	0.80	0.86
1	35.4	30.8	0.70	0.75

Fig. 6. Two-dimensional fingerprint of ribonuclease T_1 digest of *Drosophila melanogaster* **$tRNA_G^{Tyr}$ from (a)** *v; bw* **and (b)** *su(s)*^{2} *v; bw*. XC is the position of the blue dye marker (xylene-cyanole-ff). Background spots are labelled (b). First dimension is from left to right; second dimension is from top to bottom.

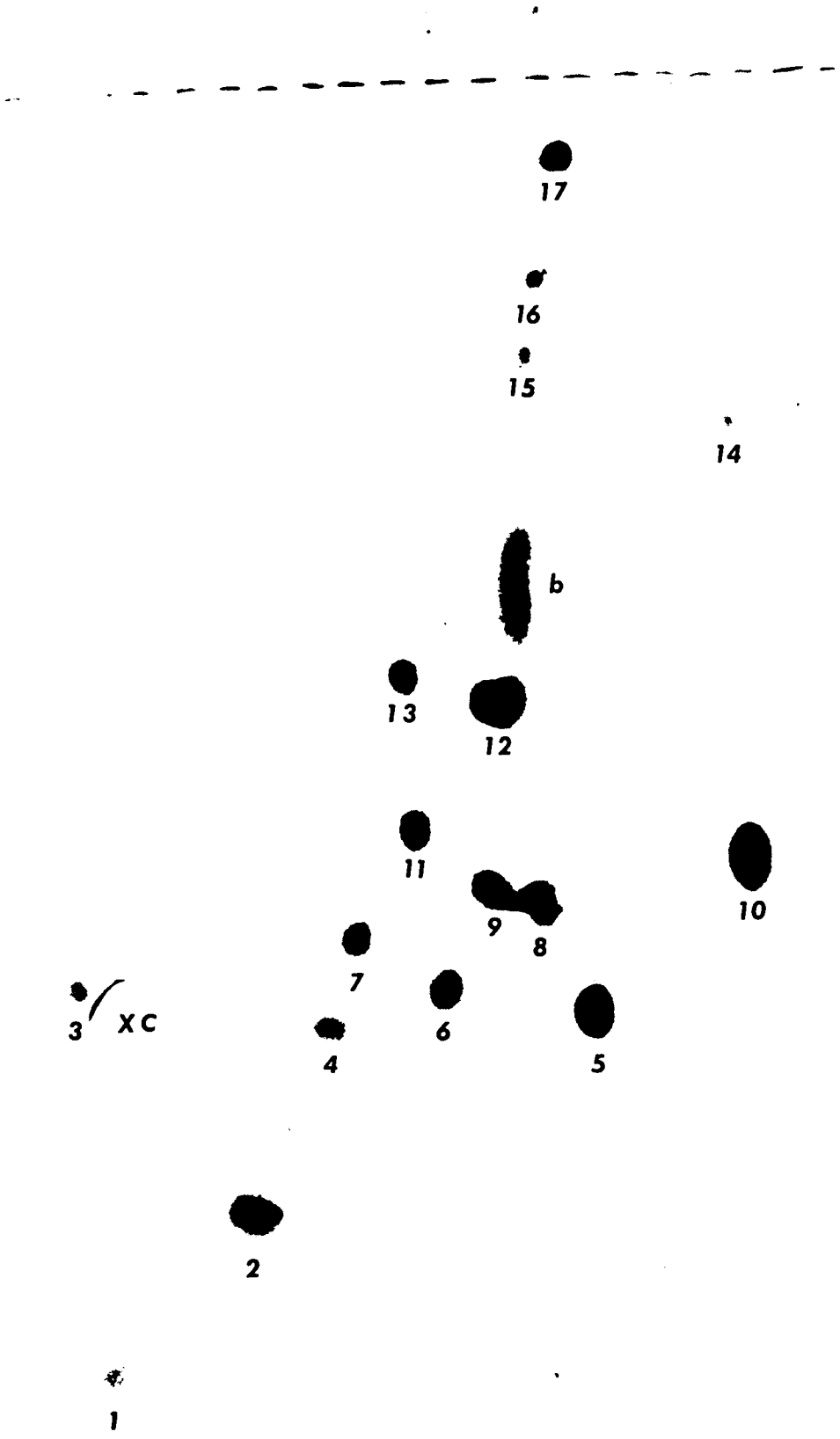

Figure 6(a)

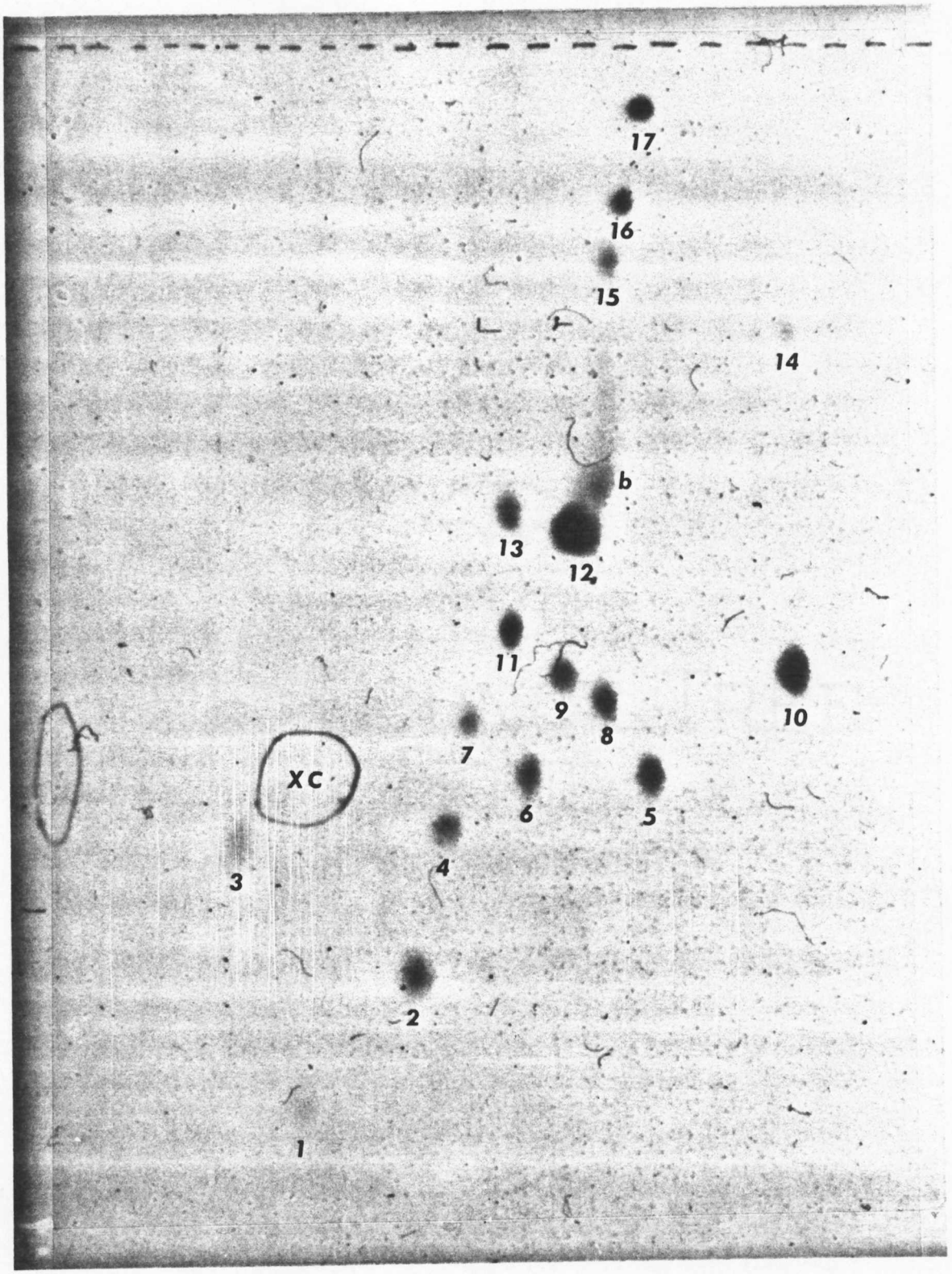

Figure 6(b)

CHAPTER IV

DISCUSSION

The method employed for the purification of the tRNA was devised so that the bulk of the tRNA was never exposed to enzymes or chemicals that might cause alterations. Up to 100–200 μg of $tRNA^{Tyr}$ can be purified using this procedure and the absence of minor fragments in the T_1 RNase fingerprint analysis indicate that the tRNA is very pure. Under suitable conditions, the method should be applicable to the purification of virtually any tRNA species. Since three tRNAs were separated on the two-dimensional gel, it seems likely that a mixture of several tRNAs could be purified using the gel. Indeed, this system has been used to separate the individual tRNA species from crude tRNA mixtures.[67,68] However, because of capacity considerations, less than one microgram of an individual species could be purified from a crude mixture of tRNAs.

Using an *in vivo* system and injecting TMV-mRNA and tRNA into Xenopus oocytes, Kubli found that $tRNA_G^{Tyr}$ from both the *v; bw* and *su(s)*^{2} *v; bw* genotypes read through the TMV-RNA stop codon while of the wild type and 21 mutants tested only $tRNA_Q^{Tyr}$ from *su(s)*^{2} *v; bw* produced a read-through protein.[57] It is thought that the G to Q change at the first position of the anticodon is the only difference in the G and Q tyrosine isoacceptors. One laboratory has reported that $tRNA_Q^{Tyr}$ also contains an m^5C not found in $tRNA_G^{Tyr}$.[69] If G-Q is the only difference in the two isoacceptors, then it appears that the modification at the first position of the anticodon prevents the $tRNA^{Tyr}$ from reading the TMV-RNA stop codon. Because $tRNA_Q^{Tyr}$ from *su(s)*^{2} *v; bw* does cause read-through, it appears that the *su(s)*^{2} locus, or the absence of the *su(s)*$^+$ locus, is responsible for a base difference that restores the ability of $tRNA_Q^{Tyr}$ to read-through the TMV-RNA stop codon.

Jacobson et al.[55] showed that a "discriminating" form of tyrosyl-tRNA synthetase will charge *v; bw* $tRNA_G^{Tyr}$ but not *su(s)*^{2} *v; bw* $tRNA_G^{Tyr}$.[55] He has also indicated that $tRNA_G^{Tyr}$ may be involved in the mechanism by which tryptophan oxygenase activity is restored by the *su(s)*^{2} mutant.[54,55] Based on Jacobson's and Kubli's results, it appears that both tyrosine

isoacceptors of the *v; bw* and *su(s)*^{2} *v; bw* tRNAs are biochemically different and it is probable that the *su(s)*^{2} locus affects both tyrosine isoacceptors in the same manner.

Geller and Rich[51] have proposed that the read-through phenomenon may represent a control of gene expression at the level of termination. They believe that certain read-through proteins occur in certain developmental stages of an organism, or after transformation. This concept is of interest since significant changes can be observed in the ratio of queuine to guanine containing $tRNA^{Tyr}$ isolated from different developmental stages in Drosophila.[70] Also, in transformed mammalian cells, the queuine containing tRNAs disappear almost completely.[71] The *su(s)*^{2} locus appears to destroy the ability of $tRNA_Q^{Tyr}$ to respond to the TMV-RNA stop codon and cause termination. The *su(s)*^{2} locus therefore restores suppressor activity to the $tRNA_Q^{Tyr}$. Thus the *su(s)*^{2} locus may be destroying a regulatory role for one or more of the four queuine containing tRNAs in *Drosophila melanogaster*. The *su(s)*^{2} locus does appear to have an adverse effect on the flies. The *su(s)*^{2} *v; bw* genotype appears to have a shorter life span than the *v; bw* genotype and the *su(s)*^{2} *v; bw* strain is more sensitive to toxic metals such as cadmium.[72]

The base analysis indicated that the *su(s)*^{2} *v; bw* $tRNA_G^{Tyr}$ contained one more m_2^2G and ψ than the *v; bw* $tRNA_G^{Tyr}$. The *su(s)*^{2} *v; bw* $tRNA_G^{Tyr}$ also contained less G and U residues, providing further evidence for the presence of the additional modified bases in *su(s)*^{2} *v; bw* $tRNA_G^{Tyr}$. It should be emphasized that the numbers in Table III are relative numbers, since the exact number of bases in $tRNA_G^{Tyr}$ from *Drosophila melanogaster* are not known and 2'-0-methylated nucleosides are not susceptible to periodate oxidation.

It is assumed that the difference in the fingerprints of the two tRNAs is due to the presence of the additional m_2^2G and ψ in the *su(s)*^{2} *v; bw* $tRNA_G^{Tyr}$. The fast migrating fragment 3 in the *su(s)*^{2} *v; bw* $tRNA_G^{Tyr}$ may be due to the presence of m_2^2G in the *su(s)*^{2} *v; bw* $tRNA_G^{Tyr}$, since it is known that methylations generally cause a slight increase in the rate of migration in the second dimension.[66]

Since only one clear difference exists in the fingerprint patterns, the position of the other modified base is unknown. Both additional modified bases in the *su(s)*^{2} *v; bw* $tRNA_G^{Tyr}$ could be in the same oligonucleotide. Alternatively, because a single base alteration in a large fragment would not be expected to alter the migration of the fragment to a detectable extent,[66] any of the larger fragments are good candidates to contain the other modified base.

Ideally, the sequence of the fragments in the fingerprints should be determined to identify all differences. However, attempts at sequencing the fragments have only been partially successful and differences in the fragments of the two genotypes have not been established. The key point, however, is that the fingerprint patterns of the two tRNAs are different, which indicates that the sequences of the tRNAs are not identical.

It is not clear how the *su(s)*^{2} locus gives rise to the altered tRNA. One interpretation is that the *su(s)*^{2} locus codes for a mutant tRNA which is a true amber suppressor. However, by in situ hybridization, no $tRNA^{Tyr}$ genes have been found to map at the *su(s)*^{2} locus. Thus Kubli, Jacobson and others believe that the *su(s)*^{2} locus codes for a modification enzyme and the modification restores the ability of $tRNA_G^{Tyr}$ to read the TMV-RNA stop codon.

Kubli has sequenced the anticodon regions of wild-type $tRNA_G^{Tyr}$ and both $tRNA^{Tyr}$ isoacceptors from *su(s)*^{2} and found that the anticodons are all GψA or QψA.[73] Thus the difference in the two $tRNA_G^{Tyr}$ isoacceptors is somewhere other than the anticodon.

As observed earlier, modified bases at the first position of the anticodon have been shown to play a role in suppression. Recently, an ochre suppressor $tRNA^{Ser}$ from *Saccharomycces cerevisiae* has been found that lacks the modified uridine found in the wobble position of the wild-type tRNA but contains instead another modification near or in the anticodon.[74] This suppressor tRNA also has a G→U substitution in the middle position of the anticodon. It is not clear if one or both changes are necessary for suppression. Single base modifications at positions other than the anticodon have also been shown to cause suppression of termination codons.[46]

One or both base modifications in the *su(s)*^{2} *v; bw* $tRNA_G^{Tyr}$ may be necessary for suppression. Presumably only one base modification is dependent on the *su(s)*^{2} locus. The presence of the additional modification may be due to the fact that the two strains of *Drosophila melanogaster* are not isogenic.

In order to test this prediction, strains of *su(s)*^{2} and *su(s)*$^+$ *Drosophila melanogaster* were constructed that differed only in the tip of the X chromosome. This was accomplished by a series of crosses utilizing male flies with an attached X that were *y*^{2} *cv v f* and *su(s)*^{2} *cv v f*. The end result was two strains that were *y*^{2} *cv v f* and *su(s)*^{2} *cv v f*, both in a Sammarkand background. Thus, these strains were isogenic except for the tip of the X

chromosome which was y^2 *su(s)*$^+$ in one strain and y^+*su(s)*^{2} in the other. Both $tRNA^{Tyr}$ isoacceptors of these tRNAs have been purified and base analysis will be performed on all four tRNAs. If the only base that is different between the comparable isoacceptors is an m_2^2G or a ψ, then we will have identified the base modification for which *su(s)*^{2} is responsible. In addition, if the *su(s)*^{2} *cv v f* $tRNA_Q^{Tyr}$ still functions as a suppressor in Kubli's system while the y^2 *cv v f* $tRNA_Q^{Tyr}$ does not, then we will have identified the base modification responsible for the suppressor activity.

It would then be of considerable interest to sequence the corresponding $tRNA^{Tyr}$ isoacceptors to locate the exact position of the modified base in the *su(s)*^{2} strain. Physical-chemical studies such as chemical modification of exposed bases followed by fingerprinting and NMR studies could then be performed to determine if the modified base caused any change in the tertiary structure of the molecule. This information would hopefully help us to gain additional insight into tRNA interactions with synthetases and with the ribosome-message complex.

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