A STUDY OF tRNA BIOSYNTHESIS IN *Escherichia coli*

by

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ABSTRACT

Escherichia coli was grown in the presence of amino acid analogues or in the absence of required amino acids. The tRNAs were isolated and characterized. Numerous changes were observed in the total tRNA acceptance for particular amino acids although in no instance did these changes occur for amino acids corresponding to the adverse growth condition. The isoacceptor patterns for particular labelled aminoacyl-tRNAs were determined on the anion exchanger RPC-5. Novel isoacceptor tRNAs were observed under several growth conditions. Significant changes in tRNA isoacceptor distributions were noted. In certain instances it appeared that changes in total amino acid acceptance could be explained in terms of the increased or decreased synthesis of particular tRNA isoacceptors while for other tRNAs it seemed that changes occurred in the synthesis of all isoacceptors for a particular amino acid such that the relative amounts of isoacceptors remained constant even when total amino acid acceptance changed considerably.

E. coli was grown over a wide temperature range, 17°C to 44°C, and the tRNA isolated and characterized. Novel tRNA isoacceptors were observed at both high and low growth temperatures for most but not all tRNAs. It was shown that the same isoacceptors could be formed at both extremes of temperature. Preliminary results suggest that the novel isoacceptors are formed as the result of a temperature aggravation of a nutritional problem at extremes of growth temperature.

One of the novel tRNA isoacceptors formed under a variety of adverse growth conditions, $tRNA_3^{Val}$, was purified and partially characterized. The results are consistent with $tRNA_3^{Val}$ being an undermodified precursor of the major isoacceptor $tRNA_1^{Val}$.

E. coli str^D was grown and the tRNA isolated and characterized. Major differences in the amino acid acceptances for several tRNAs were observed. These changes were accomplished without any significant changes in the relative isoacceptor distributions as determined by RPC-5 chromatography.

Gel electrophoretic analysis was performed on tRNA from cells grown at extremes of growth temperature. Significant differences were observed in the 5S region; there was an accumulation of material in cells grown at low temperature and a decrease of material in cells grown at high temperature.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

Page

Acknowledgements

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DEDICATION

to

June,

Carrie

and

David

ABBREVIATIONS USED

The abbreviations summarized below are those suggested by the IUPAC-IUB Combined Commission on Biochemical Nomenclature [Revised Tentative Rules, 1965 (261) and Recommendations, 1970 (262)]. Recommendations (1970) (262) replace Section 5 of the Revised Tentative Rules (1965) (261).

A, C, G, U: -	the ribonucleosides of the four bases; adenine, cytosine, guanine, and uracil.
Ap, Cp, Gp, and Up: -	the 3'-ribonucleoside monophosphates
GDP: -	the 5'-ribonucleoside diphosphate of guanosine
GTP: -	the 5'-ribonucleoside triphosphate of guanosine
ATP: -	the 5'-ribonucleoside triphosphate of adenosine
pppGp: -	the ribonucleoside-5'-triphosphate-3'-monophosphate of guanosine
ppGpp: -	the ribonucleoside-5'-diphosphate-3'-diphosphate of guanosine
pppGpp: -	the ribonucleoside-5'-triphosphate 3'-diphosphate of guanosine
poly(C, G,A): -	polyribonucleotide with a random sequence containing C, G and A
DNA: -	deoxyribonucleic acid
RNA: -	ribonucleic acid
rRNA: =	ribosomal ribonucleic acid
mRNA: -	messenger ribonucleic acid
tRNA: -	transfer ribonucleic acid
$tRNA^{Val}$: -	nonacylated valine tRNA

Val-$tRNA^{Val}$: -	aminoacylated valine tRNA
$tRNA_1^{Val}$: -	one of the isoaccepting species of $tRNA^{Val}$
Val, Ser, Leu, Thr: -	amino acids, valine, serine, leucine, threonine
SDS: -	sodium dodecyl sulfate
EDTA: -	ethylenediaminetetraacetate
RPC: -	reverse phase chromatography
DEAE-cellulose: -	O-(diethylaminoethyl) cellulose
BD-cellulose: -	benzoylated DEAE-cellulose
A_{260}: -	absorbance at 260 nm
A_{260} unit: -	the amount of material giving an absorbance of 1.0 in 1.0 ml of solution at neutral pH in a 1 cm light path at 260 nm.
RNase: -	ribonuclease
TuTs: -	transfer factors in protein synthesis
G-factor: -	translocation factor
str^D: -	a mutant form that is dependent upon streptomycin for growth
d.p.h.: -	growth rate expressed in terms of doublings of turbidity per hour at 420 nm

INTRODUCTION

The manner in which tRNA is involved in the complex process of protein synthesis has been the subject of numerous reviews (1-8). Physical and chemical characterization of tRNA (4, 9-13) has likewise been reviewed in great detail. The introduction to this thesis will therefore not attempt to cover all aspects of present day knowledge of tRNA structure and function but will attempt to bring together and summarize the important information on tRNA transcription, processing of the primary transcriptional product and finally the maturation of the tRNA particularly with respect to modified nucleoside composition. Emphasis will be placed on known and postulated control mechanisms for the synthesis and maturation of tRNA. A number of review articles are available which deal with various aspects of these topics (14-25, 137).

tRNA Genes in E. coli

Hybridization experiments have suggested 40 to 80 loci for tRNA genes in Escherichia coli (20) and primarily from studies of missense and nonsense suppressor tRNA mutations, it has been shown that these tRNA genes are widely distributed around the E. coli chromosome (20,23). Evidence has been put forward, however, that suggests that tRNA genes are usually found in clusters. Squires et al. (26) showed that gly T su^+ 36 (a mutant form of $tRNA_2^{Gly}$), $tRNA_2^{Tyr}$ and $tRNA_3^{Thr}$ genes are in very close proximity. Squires and Carbon (27) earlier presented evidence for the existence of several closely linked identical

copies of the genes for $tRNA_3^{Gly}$. Ohlsson et al. (28) suggest that the ochre suppressor su_B and the amber suppressor su II are 50 to 150 base pairs from each other. $tRNA_1^{Tyr}$ has been shown to exist as two identical tandem genes (29). Orias et al. (30) demonstrated that gly T su^+_{36} and the ochre suppressor supl5B (supM) are closely linked. This is probably the same result obtained by Squires et al. (26) in that supl5B inserts tyrosine. Unpublished work (31) suggests that in vivo E. coli tRNA transcripts may often be up to five tRNAs long since in one instance they observed a transcript containing five covalently linked $tRNA^{Leu}$ molecules. Transcripts having single copies of several different tRNA genes (similar to that described by Squires et al. (31)) were also observed. Several authors (32,33) in the course of isolating tRNA genes have likewise suggested that tRNA cistrons may be clustered in groups of two or three.

tRNA Transcription

In E. coli, tRNA genes are transcribed by an RNA polymerase complex in the presence of the required nucleoside triphosphates and appropriate co-factors. The initial transcription product is larger than mature tRNA and constitutes a precursor which is matured by selective nuclease action and minor base modification. The size of the primary transcriptional product has been the subject of considerable research.

Kinetic studies by Vickers and Midgley (34) suggest that the average tRNA precursor in E. coli might be 170 ± 40 nucleotides long. Individual precursor tRNAs or pre-tRNA molecules

have been studied. In particular, Altman and co-workers (35, 36) have used a Ø 80 phage, carrying mutations in $tRNA^{Tyr}_{Su+++}$, to cause the accumulation of precursor $tRNA^{Tyr}_{Su+++}$. Presumably processing is slower because the maturation enzymes do not have the same affinity for the mutant substrate. The total nucleotide sequence of the precursor was determined and found to have a 5'-terminal pppGp, suggesting natural initiation, followed by an additional 40 nucleotides and then the nucleotide sequence which is eventually converted into $tRNA^{Tyr}_{Su+++}$. Ribosome-associated RNase P was shown to remove selectively the extra 41 nucleotides at the 5'end (22,37). The 3'OH end of the precursor was three nucleotides longer than the mature molecule and had the completed pCpCpA end characteristic of mature tRNA. Primakoff and Schedl (31) have shown in unpublished work that the 3'OH end of the Altman precursor is shorter than the true precursor. Loewen et al. (305) have recently determined the sequence of 23 nucleotides beyond the CCA end. Primakoff and Schedl confirm Altman's results regarding the extra nucleotides at the 5'end and report that four specific endonucleases are normally involved in the maturation of the precursor. Two specific cleavages occur at each end. They postulate that cleavage by RNase P, liberating the 41 nucleotide fragment, is prerequisite for the other three cleavages. The 41 nucleotide fragment is cleaved specifically by a second nuclease. One might ask why the cleaved 41 nucleotide fragment undergoes a second highly specific cleavage and is not just simply degraded by a general exonuclease.

Dijk and Singhal (38) have recently reported the accumulation of pre-tRNA in E. coli either starved for methionine or inhibited by chloramphenicol. By Sephadex chromatography they distinguished a group of precursors which they call α's, which are about 200 nucleotides long, and a second group called β's, about 120 nucleotides long. Both groups are very heterogeneous when chromatographed on RPC-5. Both groups can be cleaved to 4S material by crude cell extracts and have sequence homology with tRNA as determined by competitive hybridization. Similarly, bands C, D and F' observed by gel electrophoresis by Griffin and Baillie (39) are tRNA precursors transiently accumulated upon the relief of growth restricting conditions. Unpublished work of Primakoff and Schedl (referred to earlier) suggests that E. coli tRNAs may also be transcribed in units of three to five genes. The α-group (38) could certainly be transcripts of tandem genes. Recent work by Ghysen and Celis (40) suggests that the two tandem genes for $tRNA_1^{Tyr}$ of Ø 80 psup 3 (Kyoto) are transcribed as a single precursor in vivo.

Phage T4 codes for at least eight tRNAs (41). Three precursor tRNAs can be obtained by acrylamide gel electrophoresis and each has been shown to contain two tRNAs. In one instance it appears that a band conaining two tRNAs is not the primary transcription product (42). Since it is known that all eight tRNA genes are closely clustered (43-45) it is postulated that all eight are transcribed as a single transcript.

Some work has been done with higher organisms. Mammalian precursor tRNA is known to be slightly larger than mature tRNA

(see Burden review (18)). Its conversion to 4S RNA by crude mammalian extracts has been demonstrated. Kinetic studies confirm a precursor product relationship. Similar results have been obtained for insect tRNA (46,47,303).

The nature of the RNA polymerase complex involved in tRNA transcription in E. coli has been the subject of considerable interest. In addition to the core enzyme (24) several other protein factors appear to be involved. In vitro studies on tRNA transcription suggest that sigma factor (48) is necessary for correct initiation of transcription and that rho factor (49) is necessary for correct termination. In this regard tRNA transcription is like mRNA transcription. An additional factor, psi (50), has been implicated in tRNA and rRNA synthesis. Psi factor has been shown to stimulate stable RNA synthesis up to 100 fold. An interesting observation, the implications of which will be discussed later, is that psi factor is in fact the elongation factor complex TuTs (51). Multiple RNA polymerase activities with different template specificities have been observed in crude E. coli extracts (52). A protein complex sedimenting at approximately 16S is most active in the synthesis of stable RNA. The molecular weight of this complex is consistent with the presence of TuTs and an affinity for Tu has been demonstrated. In other work (53) it has been shown that DNA must be activated before the efficient transcription of stable RNA can begin. This activation is thought to involve a change in the structure of the promoter region. It has been suggested that psi factor interacts with RNA polymerase so as to allow

the polymerase complex to recognize the activated promoter (54). Recent unpublished data (55) suggest that the protein H, a DNA unwinding protein, is involved in stable RNA promoter activation.

Results of Haseltine (56) and others have tended to argue against a role for psi in stable RNA synthesis. They observe extensive synthesis of stable RNA without the addition of psi and little or no stimulation when psi is added. These results can be reconciled with those of Travers et al. in that Haseltine worked with a system which was already maximally derepressed for stable RNA synthesis. The DNA had been disrupted so that stable RNA synthesis was no longer under natural control. Any RNA polymerase complex and not just the stable RNA polymerase complex (52) was active in stable RNA transcription.

The 3'OH Ends of tRNA

The 3'OH ends of all functional tRNA molecules terminate in the trinucleotide sequence pCpCpA (14). The absence of the terminal adenylate residue renders the tRNA incapable of accepting its cognate amino acid in aminoacylation reactions. The presence or absence of one or more of the terminal nucleotides constitutes a potential control mechanism to regulate the functional tRNA levels of the cell. Although in one instance it has been suggested that the pCpCpA end is a primary transcription product (35,36), there is strong evidence that it is not in several other instances (14,42,306). The enzymes responsible for the addition of the three terminal nucleotides are called tRNA nucleotidyltransferases. They are primarily biosynthetic enzymes and have little or no nuclease activity

in vivo (57). Other enzymes are implicated in the removal of the terminal sequence (58,59) since it has been demonstrated that the 3'OH terminal nucleotides undergo rapid exchange. In normally growing E. coli (60-62) and yeast (60) virtually all tRNA molecules are intact with regards to the pCpCpA sequence. In non-lactating mammary gland (63) slow growing yeast (60), Bacillus subtilis spores (64,65) and unfertilized sea urchin eggs (66) the tRNAs are, to a considerable degree, missing this sequence. Sporulation (64) and sea urchin egg fertilization (67,68) result in immediate repair of incomplete termini. In the case of Bacillus subtilis sporulation it has been conclusively proven that the addition of the terminal pCpCpA sequence is in no manner rate limiting in overall protein synthesis (64). In E. coli it has been shown (69) that the amount of nucleotidyltransferase that is present in the cell is in vast excess of the amount needed for normal synthesis and repair. Though not rate limiting, 3'OH termini repair is related to protein synthesis in that turnover of this sequence in E. coli increases with increased growth rate (62) and virtually ceases in amino acid starved E. coli or chloramphenicol treated E. coli (61). Although theories have been proposed (70) there is at this time no evidence for a cell directed control mechanism to limit or alter specifically protein synthesis via the readdition or removal of pCpCpA. Potential for such control does however exist.

The Stringent Response[1]

In bacteria there is a strong correlation between nucleic acid synthesis and protein synthesis. Early work (71) showed that when bacteria are deprived of an essential amino acid, protein synthesis virtually ceases and a drastic decrease in RNA synthesis is observed showing that RNA synthesis is coupled to protein synthesis. Mutations were found at a single locus, the rel locus, which relieved the dependency of RNA synthesis on protein synthesis. The wild type is said to exhibit a stringent response to amino acid deprivation and the mutant is considered relaxed with regard to this control mechanism. Stringency acts as an overriding control mechanism whenever there is a deficiency of aminoacyl-tRNA for any codon in the process of being translated. When RNA synthesis is restricted, it is the stable RNAs (tRNA and rRNA) which are most dramatically affected. Functional mRNA continues to be made for repressible, inducible and constitutive proteins. Cashel and Gallant (72,86) noted that stringent but not relaxed cells when deprived of an essential amino acid accumulate two unusual nucleotides MSI and MSII. MSI and MSII were subsequently identified as ppGpp and pppGpp respectively (307). It had been earlier shown that antibiotics which function as ribosome inhibitors produced the relaxed phenotype in genetically stringent cells. These

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[1] It is important to this thesis that this phenomenon be discussed at some length but because of the enormous volume of literature on the subject, a minimum number of original references are used. The reader can find most of the original references in a comprehensive review article by Ryan and Borek (19).

same antibiotics likewise prevent MSI and MSII accumulation. Cashel and Gallant proposed that the MS nucleotides were synthesized in an idling reaction in protein synthesis and that the MS nucleotides must in some manner preferentially limit stable RNA synthesis. Studies by several workers established that the stringent response was not limited to amino acid deprivation but that the MS nucleotides were in fact part of a control mechanism that is operative during stepdown transition (73-75) and NaCl inhibition (76) etc. (77-79) i.e. a general control mechanism for the synthesis of stable RNA. Relaxed mutants accumulate far less MS nucleotide when starved for amino acids but respond to stepdown transition in basically the same manner as do stringent cells. In a series of elegant experiments in vitro Haseltine and co-workers (80) showed that (a) MS nucleotides are synthesized on the ribosome; (b) this synthesis requires the G translocation factor and a protein factor which can be washed off ribosomes by high salt concentration; (c) synthesis of MS nucleotides is suppressed by inhibitors of G factor; (d) GDP is the precursor for MSI and GTP for MSII with ATP supplying the additional 3'OH phosphates; (e) the high salt wash factor appears functionally absent from the ribosomes of genetically relaxed cells. Since all rel^- are leaky (81) mutants, some of this protein is present. This may explain why rel^- synthesize MS nucleotides during stepdown transition. Perhaps the protein is functional with respect to step down transition but not with respect to amino acid deprivation. Non-ribosomal synthesis of MS nucleotides in vitro has recently been achieved (82).

Kinetic data suggest that the MS nucleotides bring about the preferential shut off of stable RNA synthesis. Although MS nucleotides are potent inhibitors of many cell processes it is very important to determine precisely how they can differentiate between mRNA transcription and stable RNA transcription so as to inhibit one but not the other. This distinction is possible because stable RNA transcription uniquely requires the protein factor psi. Psi factor has been shown to be strongly inhibited by ppGpp (50). The 16S RNA polymerase complex referred to earlier is likewise strongly inhibited while RNA polymerase complexes not implicated in stable RNA synthesis are not (52). Since psi is in fact the elongation complex TuTs as previously mentioned the direct coupling of protein synthesis and stable RNA synthesis is explained. Some controversy has arisen in the literature (83-85) because ppGpp was not inhibitory of stable RNA transcription in vitro. Under the experimental conditions used, the DNA is transcribed in a nonphysiological manner in that the authors attempted to make as much transcript as possible and in doing so they used DNA which was in a conformation where all natural control is lost. The ability of the cell to regulate the synthesis of specific RNAs via the heterogeneity of the RNA polymerase complexes becomes meaningless in that all polymerase complexes recognize all promoters because the DNA is in an unnatural state.

A final point to be mentioned is that the rel gene function should be considered an overriding control mechanism. Other

possible control mechanisms for tRNA biosynthesis could for survival reasons be overridden by it. tRNA synthesis is not simply related to gene dosage but must involve highly specific cytoplasmic control (87,88).

Specific Control of tRNA Synthesis

The previous discussion shows that control of tRNA biosynthesis on an overall level has been well established. Because of the central role of tRNA in protein synthesis it is important to determine if individual tRNAs are subject to specific control mechanisms. Possibly all tRNAs are made in quantities dictated by the needs of the cell. Alternatively, some tRNAs may be controlled in a passive, essentially constitutive manner, while the synthesis of selected key tRNAs is carefully regulated. Lastly it is possible that all tRNAs are synthesized in a constitutive manner as a function of polymerase levels etc.

In many studies done to date the acceptance of tRNA for a particular amino acid has been measured in order to correlate any changes in levels observed with the state of the system. As there are usually several tRNA isoacceptors for any particular amino acid most studies have included a chromatographic analysis of the relative amounts or distribution of individual isoacceptors. Often new chromatographic peaks are observed. It should be remembered that such peaks are of several possible origins. They may reflect the transcription of tRNA genes not previously transcribed. They may represent undermodified forms of the normally observed isoacceptor and lastly they may rep-

resent normal isoacceptors in an altered physical state. The most thorough studies attempt to differentiate between these possibilities. In order to attempt an understanding of any possible control mechanism for tRNA synthesis it is necessary to know the identity and hence origin of all the individual tRNA isoacceptors.

Functional Adaptation

A term that Garel (89) has coined which represents one form of tRNA control is "functional adaptation". If a cell is able to regulate the amount of tRNA for a particular amino acid, it is to the advantage of the cell to synthesize tRNAs in proportion to the amino acid composition of the proteins that it is making at a particular time. This concept has been tested in several systems.

The silk gland of Bombyx mori has a posterior part, the secreteur, which becomes specialized for the synthesis of the protein fibroin, and a middle portion, the reservoir, which becomes specialized for the synthesis of the protein sericin. During development there is a ten fold increase in the tRNA of the secreteur and a five fold tRNA increase in the reservoir. The synthesis of tRNA is such that those tRNAs capable of accepting the four amino acids predominant in fibroin (glycine, alanine, serine and tyrosine) constitute 2/3 of the total tRNA population at the 8th day of development (90,91). A linear correlation exists between these predominant amino acids of fibroin and the corresponding tRNAs during the secretion phase

(92). The work of Suzuki and Brown (93) on the oligonucleotide distribution of the isolated fibroin mRNA suggests that the messenger RNA mainly contains the codon GCU for alanine, the codons GGA and GGU for glycine and the codon UCA for serine. Based upon the coding responses of the fractionated isoacceptors for alanine and glycine, Garel et al. (94) and Chen and Siddiqui (95) suggest that the isoacceptor distribution for these amino acids is qualitatively adapted to their translational function for fibroin mRNA. In the reservoir, tRNAs for serine, glycine, aspartate, glutamate and alanine, the five major amino acids of sericin, likewise constitute 2/3 of the post ribosomal supernatant tRNA pool by the 7th day. While that group of tRNAs most important to both fibroin and sericin synthesis reach peak concentrations at 7 to 8 days a second group of tRNAs can be distinguished. This second group reaches maximum pool concentrations at 4 to 5 days where upon which their synthesis apparently ceases. These tRNAs would appear to be necessary for the protein synthesis which precedes fibroin and sericin production. A mechanism therefore appears to select for the continued synthesis of certain tRNAs and the decreased synthesis of others. The significance of such results is that they show that a cell is not genetically programmed to have a specific nonvariable tRNA population. tRNA populations can change in a highly specific manner.

Functional adaptation has been suggested in several other systems. It has been reported (96-98,302) that tRNA of rabbit

reticulocytes is specialized for hemoglobin synthesis. Garel et al. (99) compared the tRNA from the relatively specialized internal cortical zone cells of bovine lens tissue with those from the less specialized external cortical zone. They observed that for 10 of 12 tRNA species examined, the level of tRNA was functionally adapted for the crystallin synthesis characteristic of the internal cortical zone. Ortwerth (100) noted major differences in amino acid acceptance levels and tRNA isoacceptor distribution on RPC-2 between mammalian lens and muscle tissue. He postulated tissue specialization of tRNA populations as a function of the proteins being synthesized.

Yang (101) reported a correlation of seven representative tRNAs with the amino acid composition for IgF myeloma proteins in MOPC31C plasma cell tumor. Lactating mammary gland is specialized for the synthesis of casein, a glutamate-rich glycine-poor protein. Elska et al. (102) observed that at lactation there is a 78% increase in glutamate acceptance and a 62% decrease in glycine acceptance compared to virginal mammary tRNA. They also noted markedly increased in vivo aminoacylation levels at the onset of lactation. Rat granulation tissue is specialized for the synthesis of large amounts of proline- and glycine-rich collagen. As the granulated tissue developed, 30% increases in glycine and proline acceptances were observed (103). Chromatographically one of three glycine isoacceptors was shown to undergo a major increase in size relative to the two others. Estrogen-induced synthesis of serine-rich phosvitin in rooster livers results in a 25% increase in total serine

acceptance (104) due to an increase in the content of the minor isoacceptor $tRNA^{Ser}_{II}$ and the major isoacceptor $tRNA^{Ser}_{III}$ (105-107). These changes were observed to be reversed upon the termination of phosvitin synthesis (105).

In systems where a wide variety of proteins is made at possibly very different rates a control mechanism such as functional adaptation is more difficult to recognize. However, Yamane (108), studying four different bacteria, observed that the levels of five aminoacyl-tRNA roughly correlated with the amino acid composition of total protein for each bacterium. Since it has been shown that the tRNAs of growing bacteria are nearly completely aminoacylated (109,110) this is equivalent to saying that the tRNA population of bacteria is functionally adapted to the amino acid composition of the bulk of the proteins being synthesized.

tRNA During Differentiation and Development

Differentiation and development are potentially tRNA directed and controlled processes. By regulation of the size and nature of the tRNA pool, cells and tissues could alter the rate of translation of certain mRNAs or even terminate that translation. Numerous studies have been done on these topics and certainly in a few instances altered translational ability or efficiency is implicated. The synthesis and degree of modification of specific tRNAs is the instrument which the cell uses to achieve these ends.

In plants, comparing eleven tRNAs of wheat embryos and seedlings, Vold and Sypherd (111) found minor differences for

acceptors of lysine, proline and serine. Also working with wheat Shugart (112) noted chromatographic differences in $tRNA^{Phe}$ obtained from growing and nongrowing tissue (apical end vs. basal end of the same leaf). Minimal differences in chromatographic patterns (113) of cytoplasmic tRNAs have been found for four different developmental stages during cotyledon embryogenesis and germination in cotton. Soybean cotyledons during senescense show a three fold increase in $tRNA^{Leu}_{5 \text{ and } 6}$ (114) relative to the other leucine isoacceptors. As total leucine acceptance decreases markedly it would appear that the synthesis of $tRNA^{Leu}_{1-4}$ is preferentially restricted while that of the 5th and 6th isoacceptors is not.

In animals total tRNA acceptance was shown to decrease with age in mouse brain (115). Rat brain likewise showed an overall decrease in tRNA with age. However, certain tRNAs were enriched relative to others (116). Quantitative but no qualitative changes were observed in aging rat spleen (117). Mouse placental tRNA was shown to double the number of peaks coding for histidine at the 14th day of pregnancy (118).

Different methionine and arginine tRNA profiles were obtained for tRNA from larval bull frog (Rana catesbeiana) erythrocytes and adult erythrocytes (119). Minor differences were noted between Xenopus embryo and adult with respect to codon response for isoleucine, glutamate and arginine (120). Comparing the encysted gastrula and nauplius larval stages of brine shrimp, Bagshaw et al. (121) showed chromatographically quantitative differences in acceptors for nine amino acids and

no differences at all for nine others. Comparing five stages of sea urchin development with respect to four different amino acids, small changes were observed in $tRNA^{Lys}$ and a new $tRNA^{Ser}$ was observed at gastrulation (122). An extra serine isoacceptor is seen in trophozoites of Aconthamoeba castellanii in addition to the two found in the precyst (123).

It is not sufficient simply to observe changes in tRNA populations. It is necessary to determine how the system brought such tRNA changes about. Recent work by White et al. (124,125) on the development of Drosophila does that. While a great many quantitative chromatographic differences were observed between 1st instar, 3rd instar and adult, a definite pattern emerged for those tRNAs which accept histidine, tyrosine, aspartate and asparagine. Each group of isoacceptors could be divided into δ and γ forms. These forms were shown in two instances to be homogeneic i.e. derived from the same gene but differing in the degree of modification of minor nucleosides; in this instance Q. During development, the total acceptance of these four amino acids by tRNA remains relatively constant but the amounts of the δ forms decrease from the egg to the third instar and thereafter increase until two weeks after eclosion. The amounts of the γ forms vary inversely. The δ form contains Q. Since the presence of Q changes the coding characteristics of the tRNA this type of base modification is potentially an important translational control mechanism.

Work by Ilan (126,127) has suggested that in Tenebrio molitor a new leucine acceptor activity is found in the 7th

day pupae but not in 1st day or juvenile hormone-treated pupae. The mRNA for adult cuticle is present at an earlier time but is not translated until the appearance of this new $tRNA^{Leu}$. The implication is that a new $tRNA^{Leu}$ is required to translate a limiting leucine codon in the adult cuticle mRNA.

Hormonal Effects on tRNA

Hormone-mediated changes in tRNA have been observed in several systems. Estrogen induced phosvitin synthesis and the accompanying changes in the $tRNA^{Ser}$ profile (104) have already been discussed in terms of tRNA functional adaptation. Possible changes in $tRNA^{Leu}$ in the meal worm Tenebrio molitor (126) associated with juvenile hormone have been discussed in terms of development and differentiation. There are many other instances where hormones influence tRNA populations but where the relationship is less obvious.

Busby and Hele (128) observed estrogen-induced changes in the $tRNA^{Lys}$ of chicken liver. The first isoacceptor off a methylated albumin kieselgur column, $tRNA_{1}^{Lys}$, was substantially increased in amount relative to $tRNA_{II}^{Lys}$. Several changes in total amino acid acceptance by tRNA for other amino acids were also noted. A new $tRNA^{Leu}$ species has been detected during differentiation of bovine mammary gland (129) and as noted earlier (102) large changes in amino acid acceptance are observed during lactation. Changes in $tRNA^{Leu}$ have likewise been observed during bull frog tadpole metamorphosis induced by triiodothyronine (130).

Altman and co-workers (131) noted that administration of hydrocortisone to female rats gave rise to a new $tRNA^{Leu}$ which was transient and subsequently disappeared.

Removing hormone secreting organs often affects the tRNA population of target tissue. Thyroidectomy gave rise to altered profiles for rat liver $tRNA^{Lys}$ and $tRNA^{Phe}$ (132). Ovariectomy changed the $tRNA^{Ser}$ profile of pig uterus but not pig liver (133). Hypophysectomized rats show up to 30% decreases in specific amino acid acceptor activities (134) although only minor chromatographic differences were observed. When growth hormone was administered to hypophysectomized rats, increases in amino acid acceptances were observed (135) and two new $tRNA^{Asp}$ isoacceptors were detected (136). These new isoacceptors were shown, however, to be characteristic of growing liver and not a specific hormonal response.

Organ and Tissue tRNA Differences

Quantitative and qualitative differences in tRNA populations of different organs, tissues and organelles have often been observed. It is tempting to speculate that such differences represent functional adaptation in some manner to the proteins being synthesized in each instance. These differences, unless already previously discussed, will not be elaborated upon any further here. This thesis will attempt to concentrate on those tRNA control mechanisms and tRNA differences which are best understood and on those which are most related to the experimental work to be described. Earlier references are contained in a

review by Sueoka and Kano Sueoka (15). The review by Littauer and Inouye (137) is also helpful. Additional references to tRNA composition of organelles can be obtained from the paper by Ritter and Busch (138).

tRNA and Phage Infection

Phage infection of E. coli has been shown to bring about changes in the observed tRNA population. Phage T5 is known to have as a part of its genome at least 14 tRNA genes (139) and phage T4 is thought to code for at least 8 tRNAs (41). The T4 tRNA genes are transcribed from the light strand of T4 DNA (44) and are closely clustered (140).

T-even phages are unique in that they code for a highly specific nuclease which inactivates $tRNA_1^{Leu}$ of the host (141-145). Approximately 60% of the host $tRNA_1^{Leu}$ is rendered inactive by a single cleavage which gives two specific fragments; one, 48 nucleotides long from the 5'-end and the other 39 nucleotides long arising from the 3'-end (146). $tRNA_1^{Leu}$ recognizes CUG, a triplet which is rare in T-even phage mRNA (141). It has therefore been postulated that the inactivation of $tRNA_1^{Leu}$ limits the ability of the host cell to translate its own mRNA and is one of the mechanisms for shutting off host protein synthesis (141,147).

An interesting explanation for the presence of tRNA genes in the T4 genome has recently been put forward by Wilson (45). Numerous T4 strains are available carrying characterized deletions in the T4 tRNA genes. Wilson (45) demonstrated that

burst size of the tRNA deficient strains is inversely proportional to the size of the deletion. No single tRNA was more important than any other and strains deleted in all tRNA genes are still viable but have a 40% reduction in burst size. Using SDS polyacrylamide gel electrophoresis he was able to correlate the amounts of two tail fiber proteins, P34 and P37, with the size of the tRNA deletion. It would seem that phage proteins, particularly P34 and P37, contain codons for which the host has a limiting amount of tRNA and thus the T4 tRNAs ensure optimal rates of protein synthesis by supplementing the tRNA content of the host. Scherberg and Weiss (148) have shown in triplet binding studies that the T4 arginyl-, glycyl-, isoleucyl- and leucyl- tRNAs recognize those codons for which the host E. coli has the least amount of tRNA. It is also possible that laboratory E. coli strains are not the natural host for T4 and that T4 tRNA is absolutely required in another host. There is some experimental evidence for this (45). Infection of E. coli by the RNA phage Qβ is also interesting in that after infection $tRNA^{Pro}$ responds to a poly C message two to three times less efficiently than the $tRNA^{Pro}$ from the uninfected cells (149). Considering the limited coding capacity of the Qβ genome it is hard to visualize how Qβ infection brings about this response.

tRNA and Cancer

A great deal of study has been undertaken to characterize cancerous and potentially cancerous systems in biochemical terms. In such tissues major changes in protein synthesis, both quantitative and qualitative, are usually observed. Since tRNA has

been postulated as a potential mediator of translational control, aberrant tRNA synthesis is potentially a causative agent of cancer (308,150). Much work has been done to test this theory cf. Sueoka (15) and Littauer (137). Despite an enormous wealth of literature on the subject the exact relationships between cancer and tRNA are still poorly defined. Major quantitative and qualitative tRNA changes have been observed in some systems (151,152). Other systems show minimal tRNA differences in the neoplastic state (153,154). Altered tRNA methylase activity is common and probably accounts for many of the qualitative differences observed. The turning on or shutting off of specific tRNA genes is nevertheless a credible possibility. The potential for such control mechanisms is discussed in detail in several other parts of this thesis.

tRNA and Growth Conditions

The composition of tRNA of lower organisms is dependent upon the growth condition or growth stage. Differences in isoacceptor distribution for $tRNA^{Phe}$ and $tRNA^{Trp}$ have been observed for Rhodopseudomonas spheroides grown aerobically as compared to anaerobically (155). Protein synthesis is known to differ in the two states. Neurospora crassa which was grown without shaking had a $tRNA^{Arg}$ population which readily bound to poly (C,G,A) but not poly (A,G) in ribosome binding assays. However, shaken cultures had $tRNA^{Arg}$ which strongly bound poly (A,G) and bound poly (C,G,A) to a lesser extent (156).

tRNA profiles of Bacillus have been shown to change under selected circumstances. An additional $tRNA^{Lys}$ is present in vegetative or sporulating cells which is present in very low concentrations in spores (157,158). Lazzarini (159) proposed that the extra peak was growth medium dependent. Others disagree (158). Changes in $tRNA^{Val}$ during sporulation have been reported (161-163). Similarly different tyrosine tRNAs predominate in exponential (form I) and stationary (form II) states. A recent paper by Vold (164) comparing tRNAs from spores and exponentially growing cells, by the improved chromatographic technique RPC-5, shows no tRNA differences for tRNAs accepting phenylalanine, valine, alanine, aspartate, isoleucine, proline, methionine and histidine; changing ratios for tRNAs accepting glycine, tyrosine, leucine, serine, threonine and asparagine and qualitative differences for tRNAs accepting lysine, glutamate and tryptophan.

Skjold et al. (165) found that in E. coli the tRNA to DNA ratio increased substantially with increasing growth rate but the percent of the total acceptance of individual amino acids remained essentially constant. They observed no chromatographic differences for tRNAs for seven amino acids when examined by BD-cellulose chromatography. Bartz et al. (166) report no chromatographic differences in E. coli tRNA from early, mid and late log cells. They did observe a new $tRNA^{Phe}$ isoacceptor in stationary cells. Ms^2i^6A and i^6A content was shown to be highest in early log cells and much lower in later stages of growth. Some differences in amino acid acceptance between

various stages were observed. A much lower level of $tRNA_1^{Leu}$ was observed in stationary cells than in exponentially growing cells (167). Gross and Raab (168) reported major differences in $tRNA^{Tyr}$ profiles between early log and late log E. coli cells. In early log the two isoacceptors are present in approximately equal amounts but in late log $tRNA_2^{Tyr}$ is predominant. It has been reported (169) that anaerobically grown E. coli have an altered $tRNA^{Ile}$ profile. E. coli (170) grown on less than 10^{-7} M iron have one or two additional $tRNA^{Phe}$ isoacceptors. Likewise E. coli grown on low phosphate media have an additional $tRNA^{Phe}$, the amount of which is dependent upon the growth rate, slower growing cells having more (171).

Bacteria have been grown under adverse conditions and the tRNAs characterized. Such growth conditions disrupt the normal synthesis and maturation of tRNA.

Early work by Waters (172) showed that chloramphenicol treated E. coli had altered chromatographic profiles for $tRNA^{Leu}$, $tRNA^{Phe}$ and $tRNA^{Tyr}$. Waters et al. (173) confirmed the early results and showed the formation of new peaks to be a general phenomenon. In addition, they showed that tRNA made during chloramphenicol treatment differs from normal tRNA in that it has 60 - 70% less of the minor nucleotides 4-thiouridine and dihydrouridine. Most methylated minor nucleotides were essentially normal. The tRNA synthesized during chloramphenicol treatment was able to function normally in an in vitro hemoglobin synthesizing system. The dihydrouridine and 4-thiouridine changes have been independently demonstrated by other

workers (174). All data are consistent with the new tRNA isoacceptors being undermodified forms of normally observed tRNAs. Treatment of E. coli with chloramphenicol causes the accumulation of novel isoacceptors $tRNA_I^{Phe}$ and $tRNA_{II}^{Phe}$. Mann and Huang (175) have presented kinetic evidence that during recovery from chloramphenicol treatment $tRNA_I^{Phe}$ is converted to $tRNA_{II}^{Phe}$ which in turn is converted to the normal $tRNA^{Phe}$.

If a prophage carrying a tRNA gene is induced abnormal tRNA profiles are obtained for those tRNAs carried. The new tRNA peaks are undermodified tRNAs which accumulate because the phage-carried tRNA gene is present after induction in very large quantities and therefore abnormally large quantities of this tRNA are transcribed. Hence the modification enzymes are rate limiting in the formation of mature tRNA. This technique has been used to obtain novel tRNA isoacceptors in many instances (26,176,177). The physiological significance of the extra peaks is discussed in this thesis in the section dealing with minor base function.

New isoacceptors are also observed if E. coli are starved of a required amino e.g. ref. (173,178-188). This is particularly apparent in the case of methionine limitation. It has been established in these instances that the new tRNA isoacceptors are deficient in modified bases. Starvation of a mevalonic acid auxotroph for mevalonic acid gives rise to undermodified tRNAs (189). This is observed because mevalonic acid can act as a precursor for portions of certain modified nucleosides. From the literature it appears that many adverse growth

conditions are capable of giving rise to novel tRNA isoacceptors and that similar novel isoacceptors can be obtained from several growth conditions.

In other work with E. coli, starvation of a leucine or tryptophan auxotroph was shown to have little or no effect on the total acceptance for the amino acid (190) absent from the medium. Repression of the arginyl biosynthetic pathway did not significantly alter the arginyl-tRNA profile (191).

Minor Nucleosides in tRNA

In addition to the four common RNA nucleosides tRNA has a wide variety of minor nucleosides characterized by the presence of unusual bases (hyper-modified forms of the common bases). In many instances these minor bases have been shown to play an important role in tRNA functions. The minor bases are classified into three distinct groups depending upon their location in the tRNA molecule. One group consists of those found in the first position of the anticodon. A second group includes those located next to the 3'-OH end of the anticodon. The third group consists of all minor bases not included in the first two. Nearly forty modified nucleosides are known. For the purposes of this thesis only those found in lower organisms and particularly E. coli will be discussed at length.

The minor bases of the first grouping have received considerable attention because of their ability to alter codon-anticodon interactions. The minor nucleoside uridine-5-oxyacetic acid occupies the first position of the anticodon of E. coli $tRNA_1^{Val}$ (192-194), E. coli $tRNA_1^{Ser}$ (195) and its presence has

been suggested at that position in E. coli $tRNA_2^{Ala}$ (196). Uridine-5-oxyacetic acid has unique characteristics in that it pairs with U in addition to A and G (195,197,198). In triplet-ribosome binding studies E. coli $tRNA_1^{Val}$ recognized GUU with 20% of the efficiency with which it recognized GUA and GUG (197). Similarly E. coli $tRNA_1^{Ser}$ recognized UCU with 20-35% of the efficiency with which it recognized UCA and UCG (195,198). In an in vitro protein synthesizing system E. coli $tRNA_1^{Ser}$ was shown to recognize the UCU codon of the f2 coat protein (199).

Studies on E. coli $tRNA^{Arg}$ have suggested that inosine is located in the first position of the anticodon (200,201). In other systems inosine has been shown to base pair with U, C and A of the third position of the codon (202,203). One species of E. coli $tRNA^{Arg}$ recognizes the triplets CGU, CGC and CGA.

Uridine-5-oxyacetic acid and inosine have in common the ability to pair with a base with which the unmodified forms (U and A respectively) cannot pair. tRNAs deficient in these odd bases have a more restricted translational capacity.

The minor nucleoside Q is found in the first position of the anticodon in E. coli tRNAs that accept tyrosine (309) histidine, asparagine and aspartic acid (204,205). All E. coli tRNAs which recognize U and C in the third position and A in the second position of the codon contain Q (205). Q is a highly modified G and is unique in that it has a much greater affinity for U than C.

A number of minor nucleosides occupying the first position of the anticodon have been shown to be 2-thiouridine derivatives.

5-Methyl-aminomethyl-2-thiouridine has been found to occupy that position in E. coli $tRNA_2^{Glu}$ (206,207) and $tRNA^{Gln}$ (208). Similar derivatives have been found in yeast $tRNA_3^{Glu}$ (209) and rat liver $tRNA_3^{Glu}$ and $tRNA_2^{Lys}$ (210). 2-Thiouridine derivatives are unique in that they show strict base pairing with A and never pair with G. This characteristic prevents miscoding in several instances (206).

Those minor nucleosides located immediately adjacent to the 3'-OH end of the anticodon are likewise very interesting. Two such nucleosides are 2-methylthio-N^6-(Δ^2-isopentenyl) adenosine (ms^2i^6A) and N^6-(Δ^2-isopentenyl) adenosine (i^6A). It has been shown that E. coli tRNAs which respond to a codon starting with U contain one of these bases at that position (211-213). These nucleosides have been identified in a number of tRNAs of other systems. They have been implicated in ribosomal binding of tRNA (176,214) in that when tRNAs which normally contain these nucleosides are lacking them, the tRNA can no longer bind to ribosomes efficiently. tRNAs responding to codons beginning with U but lacking these modified nucleosides, can in some systems bind to ribosomes with normal efficiency (215,216). Nucleosides such as ms^2i^6A and i^6A have been shown to have strong cytokinin activity (25,217). This is however not a property dependent upon their position or presence in tRNA (218-220). Considerable work has been done to determine the mechanism of biosynthesis for these nucleosides (221-223).

A number of $tRNAs^{Phe}$ of eukaryotic systems have at this position the unusual nucleotide Y or a related derivative

(224-227) the structures of which have been determined (227-232). These nucleosides appear necessary to maintain a particular conformation of the anticodon loop (233-234). The removal of Y from $tRNA^{Phe}_{yeast}$ results in the loss of ability of this tRNA to participate in protein synthesis (235).

A group of highly modified nucleosides including N-[9-(β-D-ribofuranosyl)purin-6-yl-N-methyl carbamoyl] threonine (mt^6A) and N-[9-(β-D-ribofuranosyl)purin-6-yl-carbamoyl] threonine (t^6A) was found to occupy the position immediately adjacent to the 3'-OH end of the anticodon in tRNAs of E. coli which recognize codons starting with A (236-238). Little is known about the function of these nucleosides although it is postulated that they play a role in ribosome binding similar to that described for the other minor nucleosides at that position. The mode of biosynthesis of these nucleosides has been investigated (239-241). A number of other modified nucleosides have been identified at this position. N^6-Methyl A is uniquely found adjacent to the 3'-OH end of the anticodon in E. coli $tRNA^{Val}_1$ (192,193). 1-Methyl G has been found at that position of E. coli $tRNA^{Leu}$ (CU series) (242,243). 2-Methyl A has been identified at that position in the E. coli $tRNA^{Glu}_2$, $tRNA^{Asp}_1$, $tRNA^{His}_1$ and $tRNA^{Arg}$ (205,206,244). The frequency of such modifications at this position in tRNA suggests that the modified bases play a definite, perhaps critical role, in tRNA functioning.

The third group of minor nucleosides is very diverse. Only selected examples will be discussed here. The reader is referred to several good sources of additional information (16,21,25).

Much of the early work done on the third group of minor nucleosides consisted of a cataloging of the kinds of modified nucleosides present and their location in tRNA. More recent work has been concerned with the assignment of particular functions or properties to these nucleosides and with the understanding of how such minor nucleosides are involved in overall tRNA function. A good example of such work is that described by Roe et al. (245). They have clearly demonstrated that the N^2-methyl G in $tRNA^{Phe}$ at position 10 from the 5' end dramatically affects the rate of aminoacylation by the cognate synthetase, increasing V max ten fold. As they point out, where minor nucleosides are shown to have such dramatic effects on an important cellular process, the presence or absence of such minor nucleosides constitutes a potential control mechanism over cell function and development. In other instances undermodified tRNA has been shown to have different charging characteristics (246,247). This aspect of minor nucleoside function will be considered again at length in the Discussion portion of this thesis.

Another possible function for methylated nucleosides has been suggested. Igo-Kemenes and Zachau (248) postulated that it is the function of the positively charged minor nucleosides such as 1-methyl A and 7-methyl G to stabilize certain regions of the tRNA structure by interaction with the negatively charged phosphates of adjacent regions.

Another minor nucleoside of this group which has been the subject of interesting research is pseudouridine. In Salmonella, $tRNA^{His}$ of histidine T mutants was shown to have an altered

chromatographic profile. When the nucleoside composition of $tRNA^{His}$ was examined it was found that the tRNA was lacking two pseudouridines in the anticodon arm. Functionally, the under-modified tRNA was unable to participate, as the fully modified form does, in the repression of the histidine biosynthetic pathway (310). Other tRNAs normally having pseudouridine in the anticodon arm also had altered chromatographic profiles (311) and were unable to participate in enzyme repression (250). Those pseudouridines found in the common sequence TΨCG were present suggesting that several pseudouridine forming enzymes for tRNA exist in the cell. Conversion of the uridine to pseudouridine in tRNA has been achieved *in vitro* (249). A key role in the control of cellular metabolism can therefore be tentatively assigned to the pseudouridines of the anticodon arm.

The results of most of the tRNA studies to date suggest that tRNA populations are not just random mixtures of a variety of tRNAs but are specific populations of particular isoacceptors. This implies that sensitive control mechanisms are operative which can measure the amounts of individual tRNAs present and which can bring about specific quantitative and qualitative changes in the cellular tRNA population.

In *E. coli* the *rel* gene control of total tRNA biosynthesis has been studied extensively. We hypothesize that additional more selective control mechanisms are operative to regulate individual tRNA biosynthesis. In this thesis, an attempt has been made to demonstrate specific tRNA biosynthesis in *E. coli*

in response to adverse growth conditions. As a starting point we have sought to test the hypothesis that individual tRNAs are involved in regulation of tRNA biosynthesis acting as feedback repressors at the transcriptional level. This hypothesis has not been adequately tested in the literature. There is considerable evidence (315-319) that conformational changes in tRNA accompany aminoacylation. We hypothesize that aminoacylation thus converts an inactive tRNA repressor to the active form. Individual aminoacylated tRNAs could then specifically feedback inhibit transcription of those particular tRNA genes. This hypothesis does not require that all tRNA biosynthesis would be controlled in this manner. Several possibilities exist and will be discussed. The first experiments involved the alteration of specific aminoacyl-tRNA levels by amino acid analogues or amino acid depletion and the analysis of the tRNA populations of the E. coli under these growth conditions. The hypothesis presented predicts that an increased transcription of tRNA will be observed for those tRNAs which are feedback inhibited by a particular aminoacyl-tRNA.

Materials

The following amino acid analogues were purchased from Calbiochem: DL-2-amino-n-butyrate, DL-p-fluoro-phenylalanine, and 1-amino-2-(4-hydroxyphenyl) ethyl phosphonate. The analogue O-methyl-DL-threonine was purchased from Cyclo Chemical Co. The materials for RPC-5 columns, Plaskon CTFE 2300 powder and Adogen 464, were obtained from Allied Chemical Corp. and Ashland Chemical Co. respectively. RPC-5 was prepared as described in the literature (124,251).

Sephadex G-25 was obtained from Pharmacia. Pancreatic RNase, RNase T_2, RNase T_1, snake venom phosphodiesterase and E. coli alkaline phosphatase were purchased from Worthington Biochemicals.

Cellulose thin layer chromatographic sheets were purchased from Eastman Kodak Co. These were without fluorescent indicator (no. 13255).

Purified $tRNA_1^{Val}$, (1500 picomoles of valine acceptance per A_{260} unit) was a kind gift from Dr. Mildred Cohn. Crude commercial tRNA was obtained from Schwarz-Mann Co.

All common chemicals, unlabelled amino acids etc. were obtained commercially and were of reagent grade.

All radioactive amino acids were purchased from New England Nuclear Corp. Potassium borohydride-[^{3}H] 7.6 Ci/mmole was purchased from Amersham/Searle. The counting cocktail, Aquasol, was purchased from New England Nuclear Corp.

E. coli NF162 (Arg^- Met^- rel^-) and E. coli NF161 (Arg^- Met^- rel^+) were gifts from Dr. J. Gallant. E. coli B was obtained

from Dr. R.A.J. Warren. *E. coli* B str^D, an additional *E. coli* B strain and *E. coli* W were obtained from Dr. W.J. Polglase. Frozen commercial *E. coli* (mid log) was purchased from Grain Processing Co.

Methods

Growth of E. coli

The various E. coli strains were grown on 0.4% glucose plus minimal salts medium (252). The medium for NF161 and NF162 was supplemented with 50 µg per ml of each of the required amino acids. E. coli B str^D was grown in the presence of 1 mg per ml of dihydrostreptomycin sulfate. Turbidity was monitored at 420 nm on the absorbance scale using a Bausch and Lomb Spectronic 20, from an initial turbidity of 0.05 to a final value of 0.95. Logarithmically growing cells were used as inoculum to minimize growth lag in the experimental culture. Growth rate was expressed in terms of the number of doublings of turbidity per hour.

In large scale preparations the bacteria were grown at 37°, unless otherwise noted, in 20 l. batches in an American Sterilizer Biogen under aerobic conditions (15 p.s.i. air pressure, 142 rpm agitator speed and setting 2 on the Biogen flow meter). Because of technical problems the growth medium was not sterilized but contamination was monitored and if more than 4% of the cells were contaminants the run was discarded (see Discussion). Cells were harvested using a Sharples continuous flow centrifuge.

In small preparations, cells were grown as previously described in 250 ml volumes in 1 l. erlenmeyer flasks with shaking in a New Brunswick Scientific Co. water bath shaker. These cells were then harvested in a Sorvall RC-2B centrifuge.

The identities of NF161 and NF162 cells were easily confirmed by the yield of tRNA and by their inability to grow on unsupplemented media. In experiments in which E. coli B were grown at extreme temperatures the cells at the time of harvesting were plated out to check for contamination. Selected plated colonies were also checked for sensitivity to phage T7.

The strains were maintained in appropriate media in 50 ml volumes in 125 ml erlenmeyer flasks in a water bath shaker at 37° or on 1.5% agar plates containing the medium required for growth of that strain.

In experiments where E. coli NF162 was starved for arginine, 13.4 µg/ml of arginine and 50 µg/ml of methionine were added. In those experiments where E. coli NF162 was starved for methionine 2.1 µg/ml of methionine and 50 µg/ml of arginine were added. In each instance the chosen amino acid became growth limiting such that after three hours of starvation the turbidity at 420 nm was 0.95.

All growth inhibitors were added at the time of inoculation except α-amino-n-butyrate which was added when the turbidity at 420 nm reached 0.50.

Preparation of Aminoacyl-tRNA Synthetases

Crude E. coli aminoacyl-tRNA synthetases were prepared from commercially grown cells (mid log) essentially by the method of Muench and Berg (253) except that the cells were disrupted using a Polytron (Kinematica GMBH, Switzerland) and after the DEAE-cellulose chromatography step, the synthetase preparation was dialyzed against Tris-HCl (pH 7.4) 20 mM; 2-mercaptoethanol

10 mM; 50% glycerol. The product was stored at -20°C. The glycerol was removed before the aminoacylation reactions by chromatography on Sephadex G-25.

Preparation of tRNA

Crude E. coli tRNA was prepared by the Kirby phenol method (254) followed by DEAE-cellulose chromatography. E. coli was homogenized in 2 volumes of 88% phenol and buffer A. Buffer A contained 10 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride and 1 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 X g for 15 min and the aqueous layer removed. The phenol layer was re-extracted with 1 volume of buffer A and the homogenate was centrifuged as before. The combined aqueous fractions were mixed with 0.1 volume of 2.0 M potassium acetate (pH 4.5), then with 2.5 volumes of 95% ethanol and left overnight at -20°C. The nucleic acid precipitate was collected by centrifugation, resuspended in buffer A and applied to a DEAE-cellulose column previously equilibrated with buffer A. For small preparations the nucleic acid precipitates were collected on Millipore filters and then handled subsequently as described below. The column was washed with buffer A containing 0.3 M NaCl and the crude tRNA eluted with 1.0 M NaCl in buffer A. The tRNA was precipitated with ethanol and collected by centrifugation after being stored overnight at -20°C. It was then dissolved in distilled water, dialyzed against distilled water and freeze-dried.

Aminoacylation of tRNA. Acceptance Levels

Except where noted in particular experiments, aminoacyla-

tions were carried out at 21°C in reaction volumes of 0.20 ml. Each reaction contained per ml, Tris HCl (pH 7.4), 50 μmoles; 2-mercaptoethanol, 5 μmoles; ^{14}C-labelled amino acid, 50 nmoles (20 μCi/μmole); $MgCl_2$, 10 μmoles; ATP, 10 μmoles, 3 to 5 A_{260} units of crude E. coli tRNA and sufficient crude aminoacyl-tRNA synthetase to achieve complete charging within 5 min. To follow the progress of the reaction, 50 μl aliquots were pipetted onto filter paper discs and the trichloroacetic acid-insoluble radioactive amino acids determined. Amino acid acceptance values were expressed relative to an internal standard, the acceptance of threonine. Measurements were considered valid if amino acid acceptance was directly proportional to tRNA added and if amino acid acceptance at 5, 10 and 20 minutes of incubation were identical, thus indicating rapid saturation of the acceptor.

Aminoacylation of tRNA for RPC-5 Chromatography

Aminoacyl-tRNA for chromatography on RPC-5 was prepared essentially as described to obtain acceptance levels except that the labelled amino acids were of higher specific activity and were present at concentrations of 10-30 nmoles per ml and 0.4 ml reaction volumes were used. The amounts of synthetase and tRNA were scaled up proportionately. The labelled aminoacyl-tRNAs thus prepared were then chromatographed on DEAE-cellulose as described by Yang and Novelli (255) to obtain an aminoacyl-tRNA fraction free of protein and undesirable small molecules. Aminoacyl-tRNAs were stored in the elution buffer at -20°C.

RPC-5 Chromatography

Radioactively labelled aminoacyl-tRNAs were chromatographed on the RPC-5 system developed by Pearson et al. (251). RPC-5 columns were prepared and run as described by White et al. (124). Elution was with linear NaCl gradients in a buffer containing 5 mM sodium acetate (pH 4.5), 10 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. Analytical columns were 12 to 15 cm long and jacketed (37°C). As many as 18 A_{260} units of aminoacyl-tRNA were chromatographed each time. Columns were normally replaced after 20 such runs or as soon as they showed signs of deterioration (abnormal peak width). These columns were eluted at 15 ml/hr with a 100 ml gradient. The 0.5 ml fractions were shaken with 7 volumes of Aquasol and radioactivity was determined in a scintillation counter.

To obtain labelled aminoacyl-tRNA isoacceptors for 3'OH end analysis, as many as 40 A_{260} units of labelled aminoacyl-tRNA were chromatographed on a 60 cm X 0.9 cm column. These columns were eluted at 40 ml/hr with a 200 ml gradient. Radioactivity was determined in 50 to 100 µl of the 1 ml fractions and the desired fractions were pooled.

RPC-5 was also used in the purification of $tRNA_3^{Val}$. The details are included below.

Purification of $tRNA_3^{Val}$

Several batches of E. coli NF162 were grown under conditions of limiting arginine as previously described. The tRNA was isolated and pooled. The resulting 1.90 g of crude tRNA was used

for the isolation of purified $tRNA_3^{Val}$. The purification of $tRNA_3^{Val}$ involved standardized methods and thus will only be discussed briefly. The final purification step is described in detail so that the reader will be aware of the purity of the material used in subsequent experiments.

For convenience, the tRNA was divided into approximately equal portions. Each portion was aminoacylated and naphthoxyacetylated by standard procedures (263,264). The derivatized material was chromatographed on BD-cellulose and the fraction eluted with 1.0 M NaCl plus 20% ethanol, but not by 1.0 M NaCl plus 5% ethanol, was isolated. The salt fractions were reaminoacylated, derivatized, run on BD-cellulose and the 1.0 M NaCl plus 20% ethanol fraction was pooled with that from the first isolation. The naphthoxylacetylated Val-$tRNA^{Val}$ was hydrolysed in 1.8 M Tris buffer (pH 8.0) and the $tRNA^{Val}$ isolated in the salt fraction on BD-cellulose. The $tRNA^{Val}$ was then run on a 2.4 x 4.5 cm RPC-5 column (50 mesh sieve size) and eluted with standard RPC-5 buffers (pH 4.5) within a linear NaCl gradient from 0.50 to 0.65 M. [^{14}C]Valine acceptance was determined by charging using filter paper discs on a plastic support (266). The appropriate fractions were pooled and rerun on the same column under the same conditions. Again the [^{14}C]valine acceptance was determined and the appropriate fractions pooled. This material (47.8 A_{260} units) was then applied to the RPC-5 column described and eluted with a linear gradient (2 l.) of NaCl from 0.50 to 0.65 M in 10 mM Tris-HCl pH 7.0, 1 mM 2-mercaptoethanol.

The fraction size was 10 ml and the column flow rate 180 ml/hr. This column was the final step in the purification and the results are described in Fig. 40.

Polyacrylamide Gel Electrophoresis

To analyze RNA size distribution by polyacrylamide gel electrophoresis the following procedures were used.

Stock TEB buffer (256) contained 108 g Tris base, 9.3 g EDTA, disodium salt and 55 g boric acid per litre. Analytical gel slabs 3 mm thick were cast in a cell (257) purchased from the E-C Apparatus Co., St. Petersburg, Florida. The gels were made from 14.7% acrylamide and 0.3% methylenebisacrylamide in 6 M urea conaining 2% of stock TEB buffer. The stacking gel, in which the sample slots were cast, was 4.8% acrylamide, 0.2% methylenebisacrylamide in 2% stock TEB buffer (without urea). The lower reservoir was filled with 2% stock TEB buffer in 6 M urea and the upper with 2.0% stock TEB buffer without urea. The gel was prerun for 30-60 minutes and electrode compartments refilled with fresh buffer. Samples of RNA containing about 0.5 A_{260} units in 10 to 20 µl of water were mixed with 0.5 to 1 volume of 50% sucrose solution (ribonuclease free) and 1 µl of 0.001% bromophenol blue solution and transferred to the 1 cm wide sample slots. Electrophoresis was performed in the cold room (4°) with water (6°) circulating through the cooling jacket. At 15 V/cm it required 6 hr for the tracking dye to migrate to the bottom of the gel. The developed gel was removed, stained in Stains-All (258) overnight at 37° and destained in water by

bleaching with light. RNA markers were obtained from commercial tRNA of E. coli B (Schwarz/Mann, Orangeburg, New York) by electrophoresis in a 12% gel (11.65% acrylamide and 0.35% methylene-bisacrylamide) in 2% stock TEB buffer (no urea). Ten A_{260} units of crude RNA were loaded into the single slot 10 cm wide. The developed gel was lightly stained and the RNA samples recovered as described by Chia et al. (259). These procedures were carried out, as indicated in the acknowledgements, by Dr. I.C. Gillam.

Nucleoside Analysis of Purified tRNAs

Total nucleoside analysis was performed using the Randerath tritium derivative method (260) with the modifications that KBH_4 in KOH was used and the thin layer chromatography was performed using solvent systems F and G (260). A qualitative and quantitative comparison of the nucleoside composition of purified $tRNA_1^{Val}$ and $tRNA_3^{Val}$ was made.

The purified tRNA samples were digested in a total reaction volume of 50 µl using 1.0 A_{260} unit of purified RNA per digestion. The [^{3}H]potassium borohydride used had a specific activity of 7.6 Ci/mmole when purchased. The chromatograms were spotted with approximately 0.06 A_{260} of the labelled nucleosides.

The areas of the labelled nucleoside triols as deduced from the fluorograms were cut out and eluted in 4 ml of 2 M ammonium hydroxide for approximately 8 hrs. This length of time was necessary for complete elution of the triol from the chromatography plate rather than the 2 hours elution time suggested in the literature. From the radioactivity of the eluate the nucleoside composition was calculated assuming a tRNA length of 76 nucleoside residues.

Qualitative Nucleotide Analysis of $tRNA_3^{Val}$

Three A_{260} units of purified $tRNA_3^{Val}$ was digested overnight at 37°C in a total volume of 50 µl containing 10 µl of RNase T_2 and 10 µl of 0.1 M potassium acetate pH 4.3. The digestion products were spotted on cellulose thin layer chromatography plates and developed as described by Nishimura (16). Nucleotide positions were determined by UV absorption and by fluorescence after exposure to HCl vapors.

[^{3}H]Val-Oligonucleotides on DEAE-Cellulose

The procedure is basically that of Twardzic et al. (265). Individual [^{3}H]Val-$tRNA^{Val}$ isoacceptors were prepared as previously described, suspended in 0.10 M potassium acetate (pH 4.5), 2 mM disodium EDTA and incubated with RNase T_1. The total digest was applied to a DEAE-cellulose column (1.2 cm x 15 cm) which had been pre-equilibrated with 10 mM ammonium formate (pH 4.5). The column was eluted with a linear ammonium formate gradient, total volume 360 ml, from 10 mM to 0.25 M ammonium formate (pH 4.5). Undigested Val-$tRNA^{Val}$ was then eluted with 1.0 M ammonium formate (pH 4.5). The column flow rate was 30 ml/hr. and the fraction size was 2.0 ml. An aliquot of 0.4 ml from every second tube was counted.

Results and Discussion

The theory proposed in the introduction as a starting point for the investigation of the control of tRNA biosynthesis in E. coli would predict that the presence of amino acid analogues which limit aminoacylation or the absence of a required amino acid, would bring about specific derepression of the synthesis of tRNA for which there was a decreased level of aminoacyl-tRNA. This should result in increased amino acid acceptance of the isolated tRNA for that tRNA having the altered aminoacylation level. Although aminoacyl-tRNA levels were not determined in vivo, it is well established (267,289) that amino acid analogues and amino acid starvation do lead to correspondingly decreased aminoacyl-tRNA levels and it is assumed that such was the case in the experiments described here and that this resulted in the reduced growth rates observed.

Amino Acid Analogues

Initially 26 amino acid analogues were examined to determine or confirm their ability to inhibit E. coli growth and aminoacylation in vitro. Based upon the growth inhibition results obtained, results published in the literature, the availability of the analogues etc. it was decided that the analogues listed in Table 1 were the most suitable analogues for the subsequent experiments.

The isoleucine analogue, O-methyl-DL-threonine, was shown to be a potent growth inhibitor of E. coli under the growth conditions described. In subsequent growth experiments in the

Table 1

Effect of Amino Acid Analogues on *E. coli* Growth

Amino Acid Analogues	Growth Rate d.p.h.	Maximum Concentration used	Comments
Control-B	1.0	-	-
O-methyl-DL threonine	0.6 to no growth	10 to 300 µg/ml	isoleucine analogue
1-amino-2-(4-hydroxyphenyl) ethyl phosphonate	0.8	100 µg/ml	tyrosine analogue
DL-p-fluoro phenylalanine	0.55	15 µg/ml	phenylalanine analogue
DL-α-amino-n butyrate	0.50	200 µg/ml	valine analogue

E. coli were grown in 50 ml volumes in 125 ml flasks with vigorous shaking in a 37°C water bath, as described in Methods, in the presence of individual amino acid analogues. Growth was monitored by turbidity measurements at 420 nm.

Biogen it was far less growth inhibitory. The ability of an amino acid analogue to be an inhibitor of E. coli growth depends very much on the growth conditions used as can be seen by comparing growth rates in the presence of the analogues in Tables 1 and 2. O-Methyl-DL-threonine has been shown by other workers (312,313) to inhibit E. coli growth, to inhibit isoleucine aminoacylation, to be incorporated into protein and to competitively inhibit threonine deaminase feedback inhibition (313).

A large number of phosphonic acids were shown to inhibit E. coli growth at suitable concentrations. Only the tyrosine phosphonate, 1-amino-2-(4-hydroxyphenyl)ethyl phosphonate was capable of inhibiting aminoacylation in vitro. By use of a Dixon plot the tyrosine phosphonate was shown to be a competitive inhibitor of [^{14}C]Tyr-tRNA^{Tyr} formation having a Ki of approximately 10^{-4} M under the acylation conditions used and described in Methods. Several amino acid phosphonate analogues have been shown to inhibit aminoacylation in other organisms (314,276).

The phenylalanine analogue DL-p-fluorophenylalanine was shown to inhibit E. coli growth at relatively low concentrations. It has been demonstrated by many other workers that p-fluorophenylalanine is a potent E. coli growth inhibitor (277-283) that it is incorporated into protein (278-284) inhibits aminoacylation by phenylalanine (285,286) of tRNA^{Phe} and is an inhibitor of phenylalanine biosynthetic enzymes (287).

The valine analogue, DL-α-amino-n-butyrate was shown to be growth inhibitory atvery high analogue concentrations. This analogue has been shown by others to inhibit E. coli growth

(288) and acylation of $tRNA^{Val}$ by valine but not to be acylated itself (289). Of the four analogues in Table 1, two are incorporated under certain conditions into protein (p-fluorophenylalanine and O-methyl-threonine) and two are not (α-amino-n-butyrate and the tyrosine phosphonate).

tRNA of E. coli Treated with Amino Acid Analogues and of E. coli Depleted of Essential Amino Acids

The tRNA isolation procedures used lead to the isolation of tRNA which is nearly completely deacylated (G.M. Tener - personal communication). Thus the [^{14}C]amino acid acceptance (as determined by the procedure described in methods) for each tRNA is a relatively accurate measure of the individual tRNA content in the crude tRNA fraction. However, it is necessary to express the acceptance for each amino acid relative to an internal standard (the acceptance for threonine) because amino acid acceptance per A_{260} of different batches of crude tRNA was variable. This problem has also been encountered by other workers (304). In most growth experiments, the ratios of the acceptances of most amino acids to the acceptance of threonine were constant. For this reason threonine was chosen as an internal standard. To confirm the reproducibility of the method crude tRNA was isolated from E. coli B grown in five batches under carefully regulated conditions (37°, no inhibitor). It was found that the ratios of acceptance of valine to acceptance of threonine all fell within the range 1.26 to 1.32.

Table 2 shows the results of amino acid acceptance measurement of crude tRNA from E. coli grown in the presence of amino acid analogues. The tRNA from E. coli B grown in the presence of 100 μg/ml of the tyrosine analogue, tyrosine phosphonate, had a tyrosine to threonine acceptance ratio of 0.65 which is identical to that of control untreated cells. Growth in the presence of 15 μg/ml of the phenylalanine analogue, p-fluorophenylalanine, resulted in a crude tRNA preparation having a phenylalanine to threonine acceptance ratio of 0.73. This value is, within experimental error, the same ratio as that of the control. E. coli NF162 cells grown in the presence of the valine analogue, α-amino-n-butyrate (400 μg/ml) yielded tRNA with a valine to threonine acceptance ratio of 1.22. The same value was obtained from the tRNA of untreated NF162 cells. Table 3 shows the results of amino acid acceptance measurements of crude tRNA of E. coli NF162 cells depleted of an essential amino acid. As can be seen from the table, tRNA from cells depleted of arginine had an arginine to threonine acceptance ratio of 1.94 compared to a value of 2.02 from nonstarved cells. This slight difference falls within experimental error. When cells were depleted of methionine, the tRNA had a methionine to threonine acceptance ratio of 1.30. An identical value is obtained with nonstarved cells. Based upon the yield of tRNA per gram of cells isolated, it is calculated that approximately 3/4 of the total tRNA of the amino acid starved cells was synthesized after the onset of the starvation conditions. Thus in five clearly defined experiments which give specific decreased levels of

Table 2. Amino acid acceptance of crude tRNA from E. coli grown in the presence of amino acid analogues

E. coli strain	Addition	Growth Rate dph	Acceptance $\frac{[^{14}C]Val}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Tyr}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Phe}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Pro}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Ser}{[^{14}C]Thr}$	Picomoles $[^{14}C]Thr$ Acceptance per A_{260} unit
B	Control	1.0	1.28	0.65	0.70	1.56	1.19	65
B	Tyrosine Phosphonate	0.58	1.42	0.65	0.68	1.71	1.33	52
B	p-fluorophenyl-alanine	0.58	1.26	0.63	0.73	1.70	1.18	65
NF162	Control	1.09	1.22	0.80	0.69	1.66	1.21	55
NF162	O-methyl-DL-threonine	0.92	1.07	0.60	0.65	1.49	1.03	76
NF162	α-amino-n-butyrate	0.18	1.22	0.68	0.72	1.50	1.23	69

Table 3. Amino acid acceptance of crude tRNA of *E. coli* NF162 cells depleted of an essential amino acid

Growth Condition	Growth Rate dph	Acceptance $\frac{[^{14}C]Arg}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Met}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Phe}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Tyr}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Val}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Ser}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Pro}{[^{14}C]Thr}$	Picomoles $[^{14}C]Thr$ Acceptance per A_{260} unit
Control 162	1.09	2.02	1.30	0.69	0.80	1.22	1.21	1.68	55
-Arg	0.19	1.94		0.72	0.61	1.48	1.05	1.38	61
-Met	0.29	2.25	1.30		0.65	1.15		1.51	38

aminoacyl-tRNAs no effect was observed on the amino acid acceptance and hence tRNA level of those specific tRNAs. These results strongly argue against any role for aminoacyl-tRNA levels in the control of tRNA biosynthesis.

Another experiment in which specific changes in total amino acid acceptance might be observed, but for which data have not been presented, is that in which E. coli NF162 cells were grown in the presence of the isoleucine analogue O-methyl-DL-threonine (75 µg/ml). Rapid saturation of the isoleucine tRNA with [^{14}C] isoleucine was not achieved. However, in all the experiments performed, the isoleucine to threonine acceptance ratio was less with tRNA from the analogue treated cells than in the control. It is possible that undermodified isoleucine tRNAs from the analogue treated cells were incompletely detected because they are only slowly aminoacylated (246).

Although specific tRNA changes were not observed, that is changes in tRNA species corresponding to the analogue or starvation condition used, non-specific changes in amino acid acceptance ratios were seen (tables 2 and 3). An 11% increase in the valine to threonine acceptance ratio, a 12% increase in the serine to threonine acceptance ratio and a 10% increase in the proline to threonine acceptance ratio was observed in tRNA from E. coli B cells grown in the presence of the tyrosine phosphonate. With this tRNA, no changes were observed in the phenylalanine to threonine or tyrosine to threonine acceptance ratios. It is interesting that the three amino acid acceptance ratios which did change, all changed the same amount (approximately 11%). With tRNA from

p-fluorophenylalanine treated cells, no changes in acceptance ratios were observed for those tRNAs accepting valine, tyrosine, phenylalanine and serine. The proline to threonine acceptance ratio increased 9%. tRNA from NF162 cells grown in the presence of O-methyl-DL-threonine showed decreases in the acceptance ratios for valine (-12%), tyrosine (-25%), proline (-10%), and serine (-15%) but no change in the ratio for phenylalanine acceptance. These last results, coupled with the threonine acceptance per A_{260}, suggests that in fact $tRNA^{Thr}$ and $tRNA^{Phe}$ levels increased relative to most others in O-methyl-DL-threonine treated cells. Oddly, the tyrosine to threonine acceptance ratio is decreased in the tRNA from both O-methyl-DL-threonine treated cells and α-amino-n-butyrate treated cells to essentially the value obtained with tRNA from E. coli B cells. tRNA from E. coli NF161 ($Arg^{-}Met^{-}rel^{+}$) has the same ratio for tyrosine (0.80) under control growth conditions as does that from E. coli NF162. α-Amino-n-butyrate treated E. coli NF162 cells had tRNA which showed no significant changes in acceptance ratios for those tRNAs accepting valine, phenylalanine and serine but showed a 15% decrease in tyrosine and a 10% decrease in the proline acceptance ratios. tRNA from arginine depleted E. coli NF162 show negligible changes in arginine and phenylalanine acceptance ratios, a 21% increase in the valine acceptance and decreases of 13% for serine, 18% for proline and 24% for tyrosine acceptance ratios. tRNA from methionine depleted E. coli NF162 showed an 11% increase in arginine acceptance, negligible changes in the valine and methionine acceptances and 10% and 19% decreases respectively in proline and tyrosine acceptance ratios.

In addition, amino acid acceptance ratios were determined in several instances which have not been included in the tables. No change in the glycine acceptance was observed in tRNA from tyrosine phosphonate treated cells relative to the control. This tRNA did show an 11% decrease in alanine acceptance. With tRNA from p-fluorophenylalanine treated cells, an 18% increase in the alanine acceptance was observed but there was no difference in the arginine acceptance relative to the control. No change in the arginine acceptance was observed in tRNA from α-amino-n-butyrate treated cells.

Since great care was taken to ensure rapid saturation of the tRNA acceptors, to normalize for variations of acceptance for different [^{14}C]amino acid mixes and to do most acceptance ratios at least in quadruplicate, the acceptance ratios expressed here are relatively accurate measurements of the tRNA populations from cells grown under the various conditions described. One can conclude that the acceptance data are not random but are characteristic of the particular growth condition.

Interpretation of the data of tables 2 and 3 is very difficult. It is obvious that tRNA populations are not strictly dependent upon the growth rate. It is interesting that growth of E. coli B in the presence of the tyrosine phosphonate seems to divide the tRNA population into two groups; a group consisting of those tRNAs which accept threonine, glycine, tyrosine and phenylalanine which stay "constant" and a group consisting of those tRNAs which accept valine, serine, and proline which all "increase" by 10 to 12%. However this larger grouping may not

be significant since it does not hold under all other growth conditions. Nevertheless the amounts of tRNA which accept threonine and phenylalanine are almost parallel in the five experimental examples for which sufficient information is available. The data argue against the cell maintaining specific ratios of tRNA populations although it is acknowledged that less than half of the tRNA acceptance ratios have been measured from each growth condition. Correlations might become apparent if more acceptance data were available. Since recent work (31,35) has shown that in E. coli tRNA precursors are considerably larger than mature tRNA molecules and that a single transcript may contain precursors for tRNAs capable of accepting several different amino acids, it is probably more important to measure the levels of individual tRNA isoacceptor species. Major changes in the levels of minor isoacceptors could result in only small changes in the total acceptance for a particular amino acid and would not be detected by the methods reported here. Thus, it is important to establish if the 10 to 25% changes in acceptance ratios observed in tables 2 and 3 are the result of changes in the levels of only certain isoacceptors or if all isoacceptor tRNAs for a particular amino acid are equally affected. To obtain this data the tRNAs were fractionated on RPC-5 anion exchange columns.

RPC-5 Chromatography

Radioactively labelled aminoacyl-tRNAs were prepared and fractionated on RPC-5 columns as described in Methods. The procedure, column and buffer systems are hereafter referred to as

standard procedures, standard analytical column and standard buffer systems. In all the profiles shown the radioactivity of every fraction was measured. Background due to deacylation was subtracted before the data were plotted. All profiles were run at least twice to check on the reproducibility of charging and column fractionation. The slight variability noted is usually due to deterioration in resolving power of the column after a large number of experiments such that poorly resolved peaks overlap giving a distorted picture of the amount of particular isoacceptors actually present. The recovery of radioactive label in the gradient varies somewhat (70-90%). This depends on several factors including the amount of free amino acid carried through from the preliminary chromatography of the labelled aminoacyl-tRNA on DEAE-cellulose and the extent of deacylation during handling, freezing, thawing, etc.

Several significant changes in the valine to threonine acceptance ratios were noted previously. Figs. 1-10 show the results of chromatography on RPC-5 of [^{3}H]Val-tRNA^{Val} from E. coli grown in the presence of amino acid analogues or in the absence of an essential amino acid. Figs. 1 and 5 are the controls for [^{3}H]Val-tRNA^{Val} from E. coli B and E. coli NF162 respectively. Both profiles show the presence of the major valine tRNA isoacceptors 1, 2a and 2b and the presence of one or more very minor valine accepting tRNA species. E. coli B contains an additional valine isoacceptor, here after called tRNA$_3^{Val}$, which elutes early on RPC-5 and which is present in significant but somewhat variable amounts in all the control samples examined. Fig. 2 shows

Figure 1. The RPC-5 profile of [^{3}H]Val-tRNA^{Val} (B). In this experiment, 7.8 A_{260} units of crude tRNA of E. coli B grown under controlled conditions at 37°C as described in Methods and containing 4.6 x 10^4 cpm [^{3}H]Val (4% counting efficiency) were run on a standard RPC-5 column and eluted with the standard buffers within a linear NaCl gradient of 0.45 to 0.65 M. Approximately 90% of the [^{3}H] applied to the column was recovered in the gradient. The specific activity of the [^{3}H] used in the aminoacylation reaction was 800 μCi/μmole.

Figure 2. The RPC-5 profile of [^{3}H]Val-tRNA^{Val} (B-tyrosine phosphonate). In this experiment 5.0 A_{260} units of crude tRNA of E. coli B grown in the presence of the tyrosine phosphonate and containing 7.6 x 10^4 cpm [^{3}H]Val (10% counting efficiency) were run as described in Fig. 5. Approximately 85% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H]valine used in the aminoacylation reaction was 1330 μCi/μmole.

Figure 1

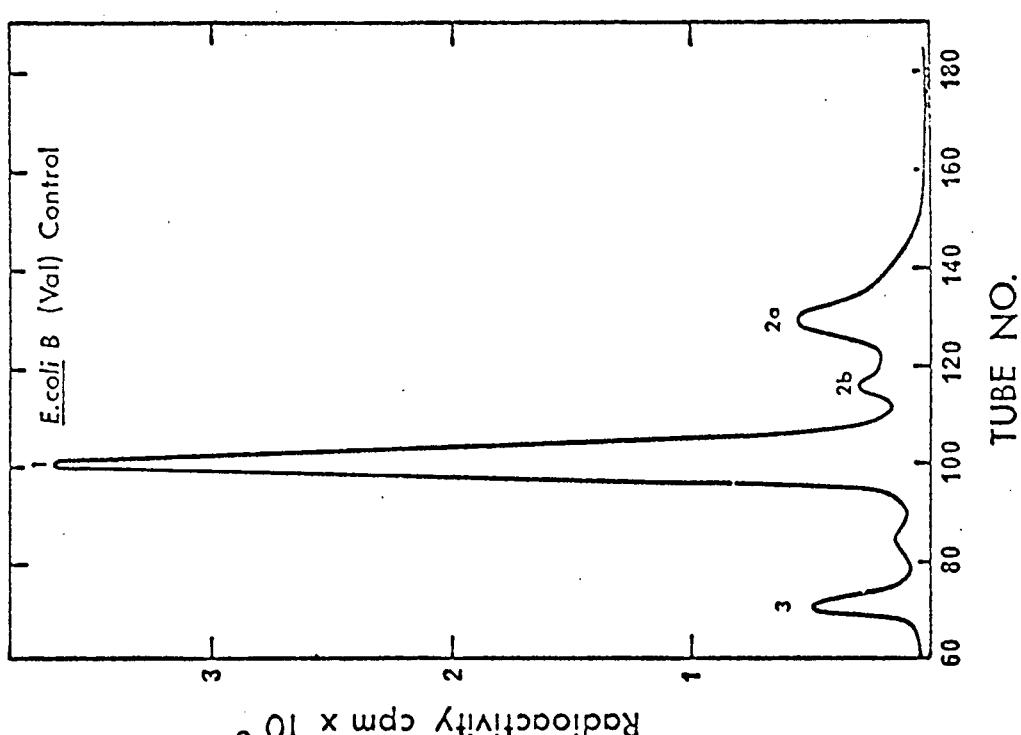
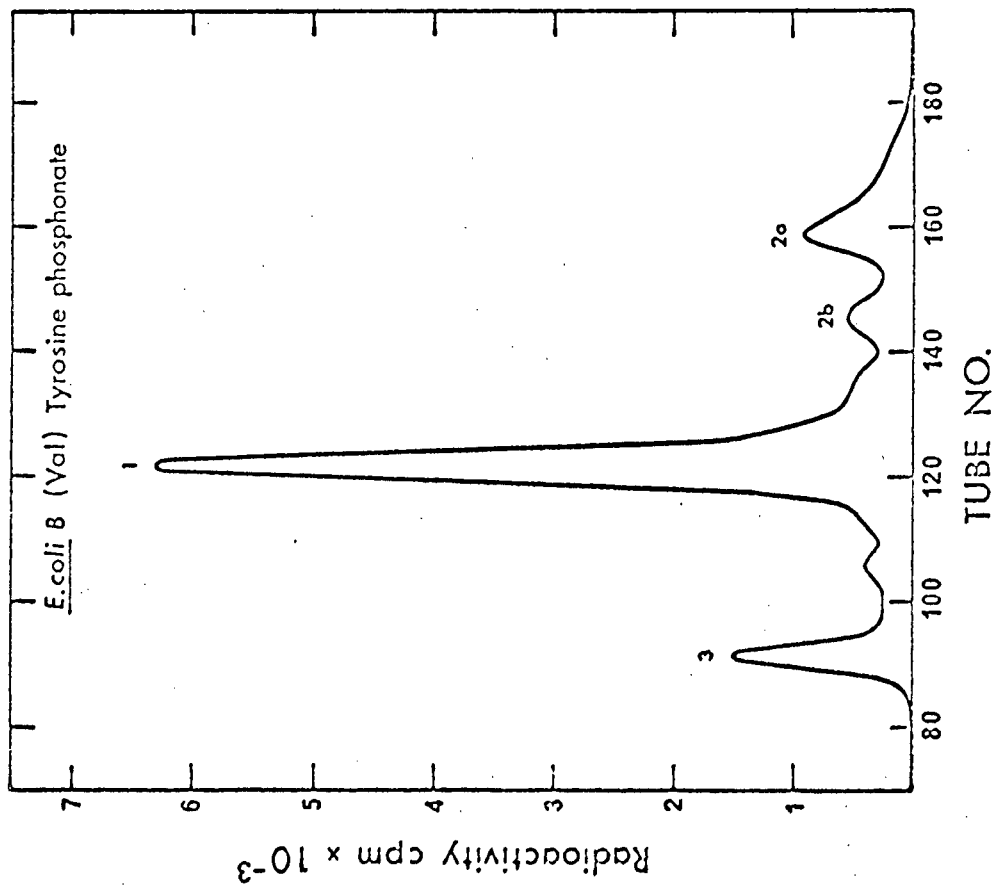


Figure 2



the [^{3}H]Val-tRNA^{Val} profile of crude tRNA of E. coli B grown in the presence of tyrosine phosphonate. An attempt has been made to quantitate the amounts of individual tRNA isoacceptors by measuring peak areas. This is difficult, and hence subject to error, where individual tRNA isoacceptors are not completely resolved.

It is clear from such measurements that in Fig. 2 there is approximately a 100% increase in the amount of Val-tRNA$_3^{Val}$ in cells grown in the presence of the tyrosine phosphonate. The percentage of the total valine acceptance for the isoacceptors 2a and 2b is similar to that of the control while the amount of the major isoacceptor Val-tRNA$_1^{Val}$ is decreased. However, the sum of the peak areas of the isoacceptors 1 and 3 is the same in control and tyrosine phosphonate treated cells (72-74%). Fig. 3, showing the [^{3}H]Val-tRNA^{Val} profile of cells grown in the presence of p-fluorophenylalanine, was analyzed in the same manner. A significant decrease in the amount of Val-tRNA$_3^{Val}$ is noted. Again the sum of the peak areas of isoacceptors 3 and 1 is essentially that of the control (74% of the total valine acceptance). The peak areas of isoacceptors 2a and 2b are very similar to those of Fig. 1. Fig. 4 shows the [H]Val-tRNA^{Val} profile of E. coli B cells grown into the stationary phase. In such cells Val-tRNA$_3^{Val}$ is absent. The relative percent of the total acceptance of Val-tRNA$_1^{Val}$ (73%) is equal to the sum of the isoacceptors 1 and 3 of Fig. 1. That isoacceptors 3 and 1 can sum consistently to a constant value suggests that both could be transcripts of the same gene(s). This possibility will be

Figure 3. The RPC-5 profile of [^{3}H]Val-tRNA^{Val} (B; p-fluorophenylalanine). In this experiment, 7.2 A_{260} units of crude tRNA of E. coli B grown in the presence of p-fluorophenylalanine and containing 1.87 x 10^5 cpm [^{3}H]valine (10% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 83% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H]valine used in the aminoacylation reaction was 1330 μCi/μmole.

Figure 4. The RPC-5 elution profile of [^{3}H]Val-tRNA^{Val} (B - stationary state). In this experiment 6.8 A_{260} units of crude tRNA of E. coli B grown into the stationary state and containing 1.5 x 10^5 cpm [^{3}H]Val (10% counting efficiency) were run as described in Fig. 5. Approximately 80% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H]valine used in the aminoacylation reaction was 1300 μCi/μmole.

Figure 3

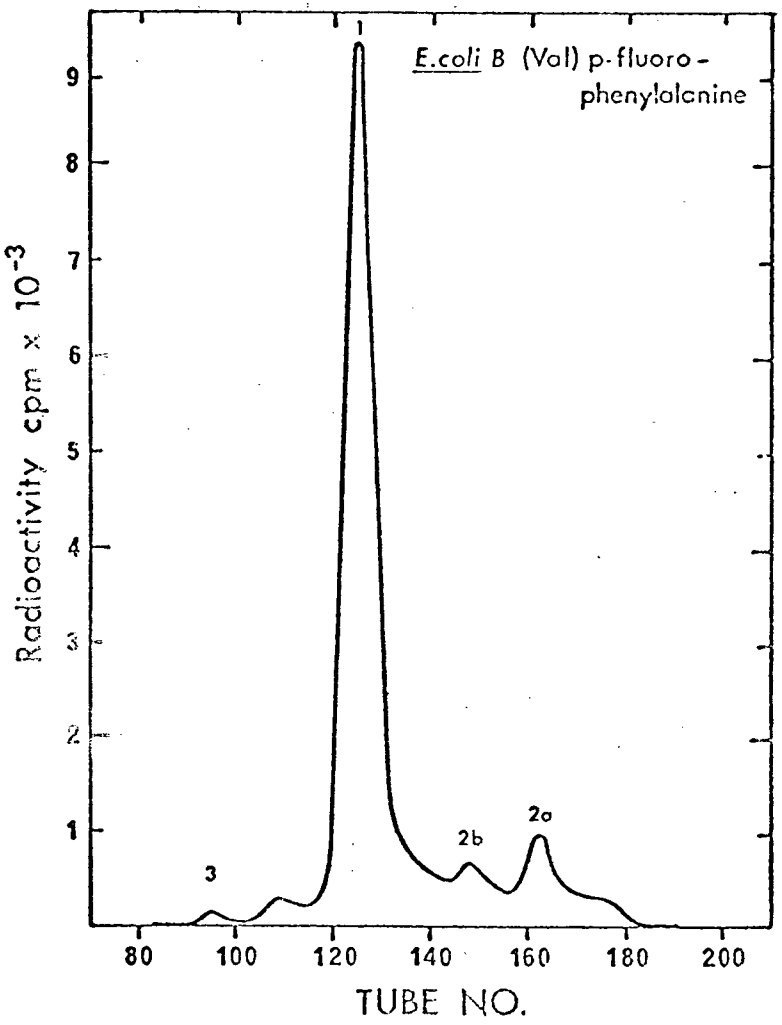
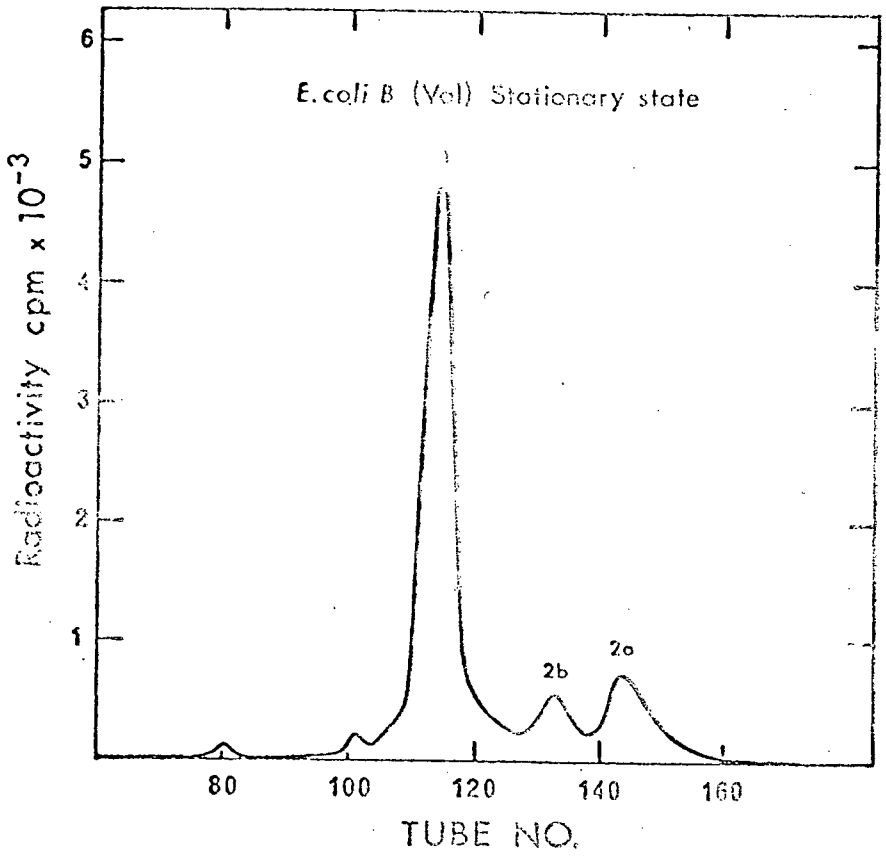


Figure 4



discussed at length later in this thesis. The isoacceptor Val-tRNA$_3^{Val}$ may be a physically altered form of Val-tRNA$_1^{Val}$ or it may be an undermodified form of Val-tRNA$_1^{Val}$ lacking particular modified nucleosides. If this hypothesis is correct, then the 11% increase in the valine to threonine acceptance ratio in tyrosine phosphonate treated cells is accomplished without a selective alteration in the relative transcription of those genes coding for the valine isoacceptors 1, 2a and 2b. If Val-tRNA$_3^{Val}$ is a unique gene product, i.e. transcribed from a gene(s) other than those for the isoacceptors 1, 2a and 2b, then selective synthesis of the valine isoacceptors 3, 2a and 2b could account for the increased acceptance ratio observed in the tyrosine phosphonate treated cells. This is considered a less likely possibility.

As shown in Fig. 6, E. coli NF162 cells grown in the presence of O-methyl-DL-threonine do accumulate a small amount of Val-tRNA$_3^{Val}$. The relative valine tRNA isoacceptor distribution is the same as that of the control although a decrease of 12% in the valine to threonine acceptance ratio was noted. Differential transcription of individual tRNA^{Val} isoacceptors does not account for the altered amino acid acceptance ratio. Fig. 7 shows the RPC-5 profile of [^{3}H]Val-tRNA^{Val} from α-amino-n-butyrate treated E. coli NF162 cells. Val-tRNA$_3^{Val}$ is present in large amounts (accounting for up to 13% of the total valine acceptance). The valine isoacceptors 1, 2a and 2b are present although it is difficult to estimate the relative quantities because of the presence of two novel valine isoacceptors 1β and 2aβ. Val-tRNA$_{2a\beta}^{Val}$ accounts for

Figure 5. The RPC-5 elution profile of [^{3}H]Val-tRNA^{Val} (NF162). In this experiment 7.0 A_{260} units of crude tRNA of E. coli NF162 grown under standard conditions and containing 1.1 x 10^5 cpm [^{3}H]Val (10% counting efficiency) were applied to a standard analytical column and eluted with standard buffers within a linear NaCl gradient from 0.50 to 0.65 M. Approximately 95% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H]valine used in the aminoacylation reaction was 1300 μCi/μmole.

Figure 6. The RPC-5 profile of [^{3}H]Val-tRNA^{Val} (NF162; O-methyl-DL-threonine). In this experiment, 5.8 A_{260} units of crude tRNA of E. coli NF162 grown in the presence of O-methyl-DL-threonine and containing 1.30 x 10^5 cpm [^{3}H]Val (10% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 86% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H]valine used in the aminoacylation reaction was 1330 μCi/μmole.

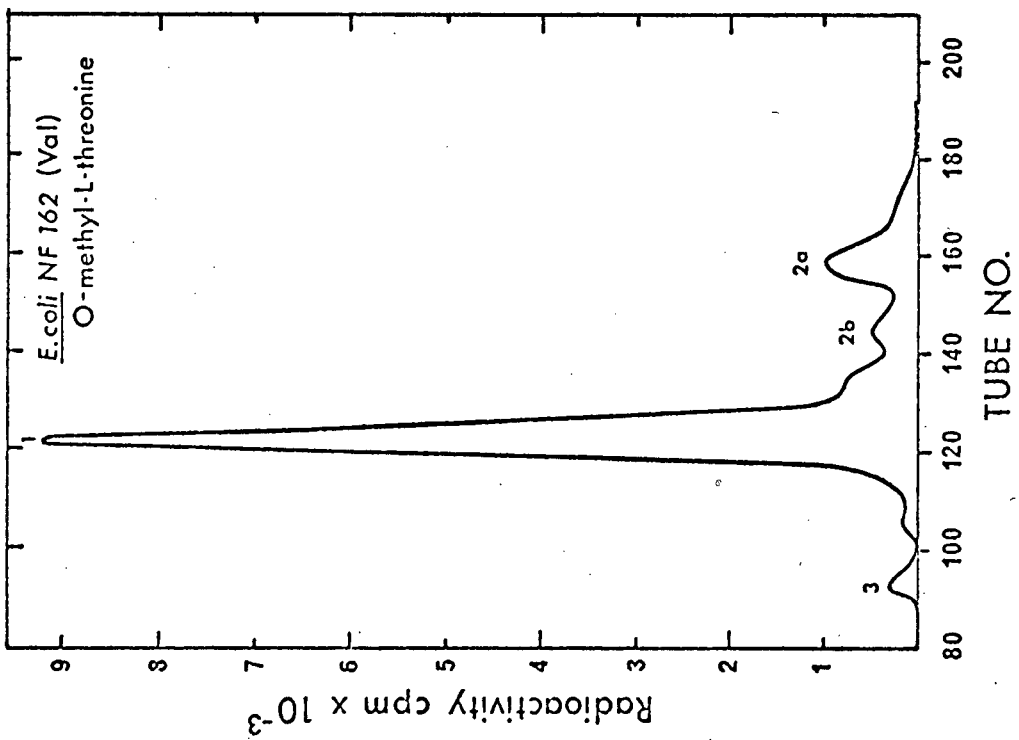


Figure 6

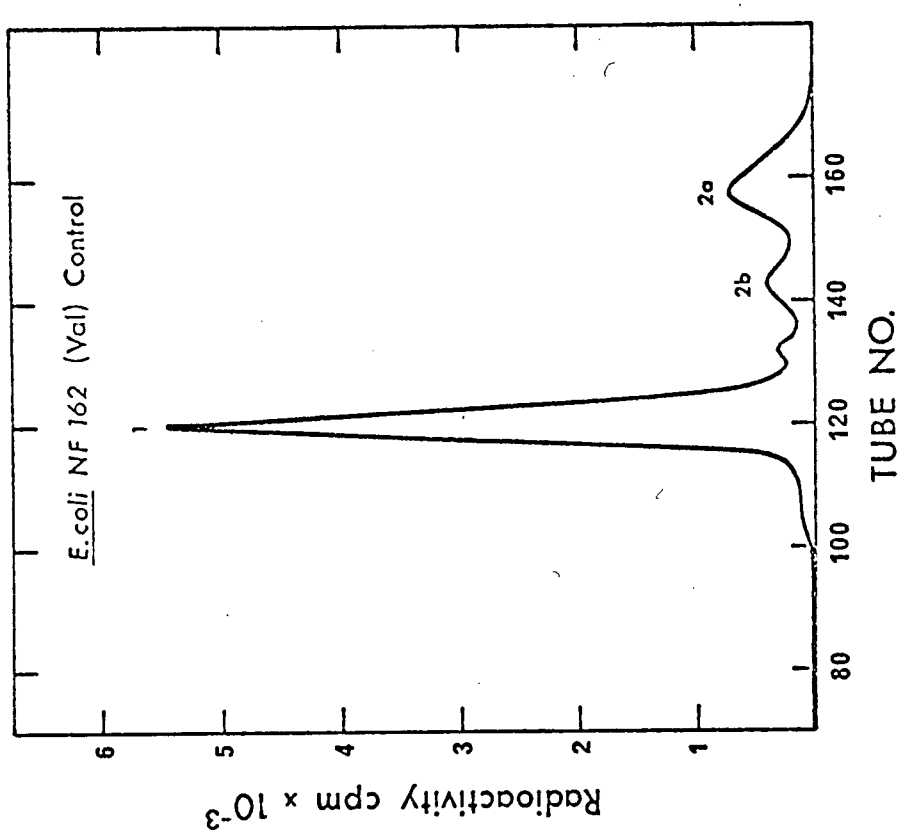


Figure 5

Figure 7. The RPC-5 profile of [^{3}H]Val-tRNA^{Val} (NF162; α-amino-n-butyrate). In this experiment 8.0 A_{260} of crude tRNA of E. coli NF162 grown in the presence of α-amino-n-butyrate and containing 2.20 x 10^{5} cpm [^{3}H]Val (10% counting efficiency) were run as described in Fig. 5. Approximately 85% of the applied radioactivity was recovered in the gradient. The [^{3}H]valine used in the aminoacylation reaction had a specific activity of 1300 μCi/μmole.

Figure 8. The cochromatography on RPC-5 of [^{14}C]Val-tRNA^{Val} (B) and [^{3}H]Val-tRNA^{Val} (162-Arg). In this experiment 10.4 A_{260} units of crude tRNA of E. coli B containing 3.30 x 10^{5} cpm [^{14}C] Val (80% counting efficiency) plus 6.27 A_{260} units of crude tRNA of E. coli NF162 depleted of arginine and containing 2.64 x 10^{5} cpm [^{3}H]Val (37% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 76% of the applied [^{14}C] and 72% of the applied [^{3}H] radioactivity was recovered in the gradient. The [^{3}H]valine and [^{14}C]valine used in the aminoacylation reactions had specific activities of 1300 μCi/μmole and 266 μCi/μmole respectively.

Figure 7

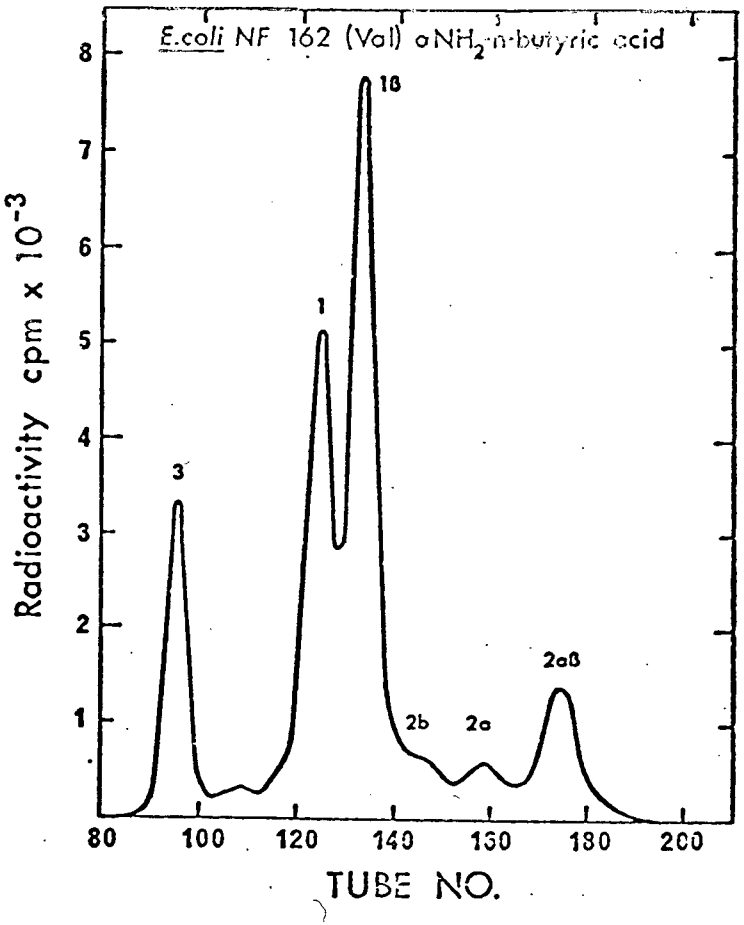
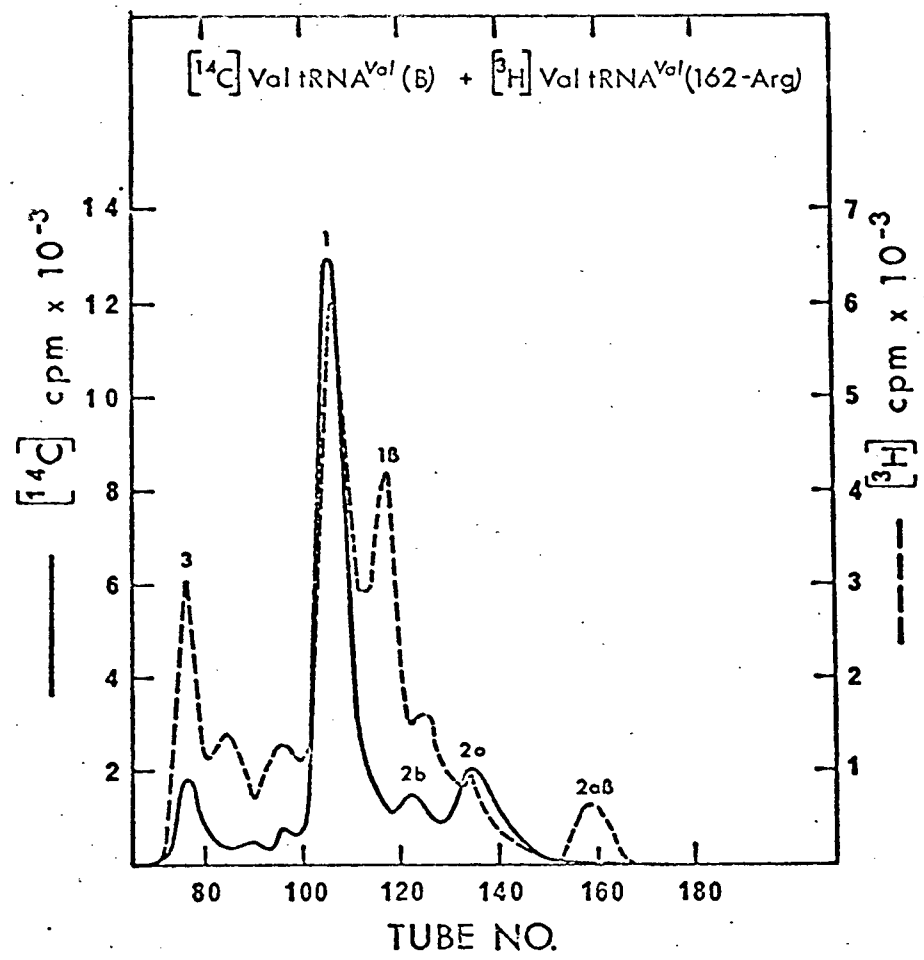


Figure 8



approximately 10% of the total valine acceptance. It is likely that the novel isoacceptors 1β and 2aβ represent undermodified forms of the major isoacceptors normally observed. Since the identity of these peaks and the valine isoacceptor 3 previously discussed is not known, it is not possible to estimate the relative quantities of the transcription products of the particular $tRNA^{Val}$ genes. Although no overall change in the valine to threonine acceptance ratio was observed it remains to be proven that altered transcription of particular $tRNA^{Val}$ genes did not occur.

Figures 8 to 10 show the labelled Val-$tRNA^{Val}$ profiles from arginine depleted and methionine depleted E. coli NF162. Cochromatography has been used in order that a comparison can be made of the valine isoacceptors present under the different growth conditions. This is especially important because of the complexity of the isoacceptor distributions observed. Cochromatography with [^{14}C]Val-$tRNA^{Val}$ (B) has been used instead of cochromatography with [^{14}C]Val-$tRNA^{Val}$ (NF162) because the former contains, in addition to those valine isoacceptors observed in E. coli NF162, Val-$tRNA_3^{Val}$ which serves as a reference peak for early eluting Val-$tRNA^{Val}$ isoacceptors. In figure 8, it can be seen that arginine depleted E. coli NF162 cells have a Val-$tRNA^{Val}$ profile on RPC-5 very much like that for α-amino-n-butyrate treated E. coli B cells. The major valine isoacceptors 1, 2a and 2b are present as are the novel valine isoacceptors 3, 1β and 2aβ previously observed. Val-$tRNA_3^{Val}$ is again present in very significant quantities. The amount of 1β present in the arginine

Figure 9. The cochromatography on RPC-5 of [^{14}C]Val-tRNA^{Val} (B) and [^{3}H]Val-tRNA^{Val} (162-Met). In this experiment 5.9 A_{260} units of crude tRNA of E. coli B containing 1.55 x 10^5 cpm [^{14}C]Val (80% counting efficiency) plus 7.5 A_{260} units of crude tRNA of E. coli NF162 depleted of methionine and containing 4.28 x 10^5 cpm [^{3}H]Val (37% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 82% of the applied [^{3}H] label and 85% of the applied [^{14}C] label were recovered in the gradient. The specific activities of the [^{3}H]valine and [^{14}C]valine used in the aminoacylation reaction were 1300 μCi/μmole and 266 μCi/μmole respectively.

Figure 10. The cochromatography on RPC-5 of [^{3}H]Val-tRNA^{Val} (NF162-Arg) and [^{14}C]Val-tRNA^{Val} (NF162-Met). In this experiment 6.3 A_{260} units of crude tRNA of methionine depleted E. coli NF162 containing 1.1 x 10^5 cpm [^{14}C]Val (80% counting efficiency) plus 9.5 A_{260} units of crude tRNA of arginine depleted E. coli NF162 containing 9.1 x 10^5 cpm [^{3}H]Val (37% counting efficiency) were run as described in Fig. 5. Approximately 82% of the applied [^{3}H] and 96% of the applied [^{14}C] were recovered in the gradient. The specific activities of the [^{3}H]valine and [^{14}C]valine used in the aminoacylation reaction was 1300 μCi/μmole and 266 μCi/μmole respectively.

Figure 9

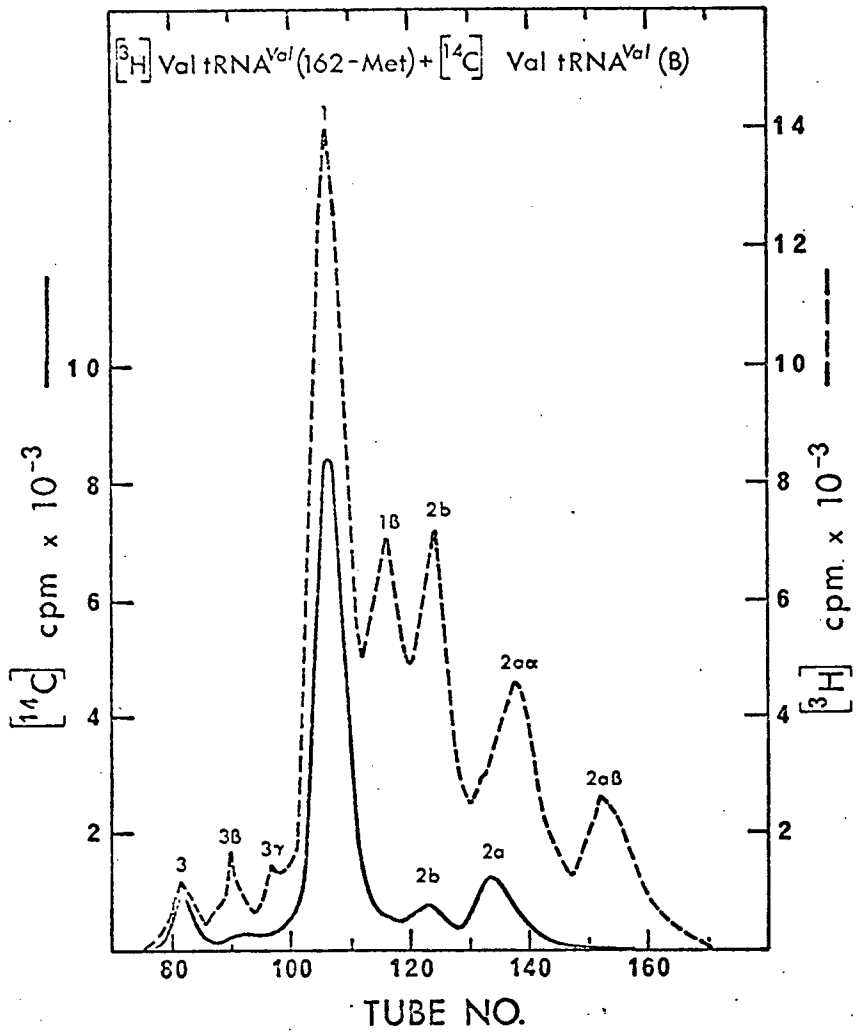
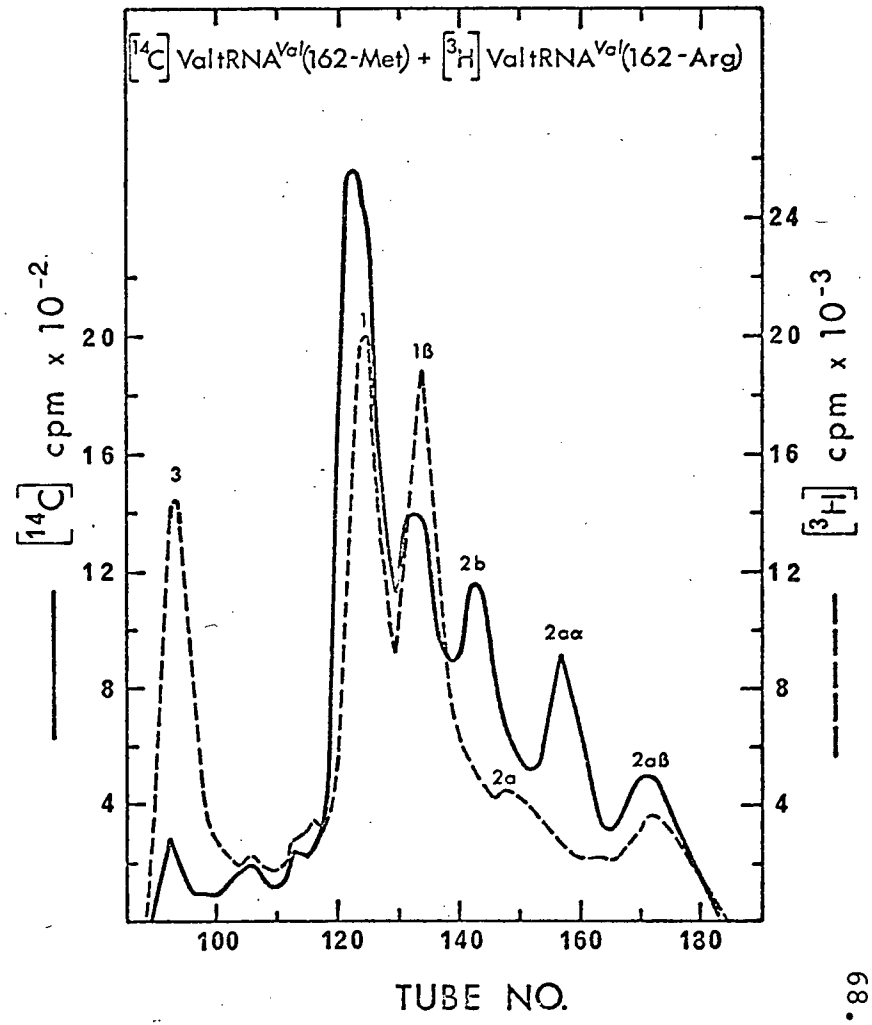


Figure 10



depleted cells is considerably less than that observed in α-amino-n-butyrate treated cells. It is interesting that such very different growth conditions give rise to the same novel valine isoacceptors. This argues against the novel isoacceptors being formed in response to specific environmental stress i.e. valine analogue treated cells do not have specific novel valine tRNA isoacceptors. It is also noted that arginine depleted E. coli NF162 accumulate small amounts of valine isoacceptors which elute between Val-tRNA$_3^{Val}$ and Val-tRNA$_1^{Val}$. Methionine depleted E. coli NF162 give quite a different tRNA profile on RPC-5. A small amount of Val-tRNA$_3^{Val}$ is observed. New valine isoacceptors 3β and 3γ are observed in small amounts. Val-tRNA$_1^{Val}$ and Val-tRNA$_{2b}^{Val}$ are both present in significant quantities. As in arginine depleted cells the valine isoacceptors 1β and 2aβ are observed. It is difficult to estimate the amount of Val-tRNA$_{2a}^{Val}$ present because of the presence of the new valine isoacceptor 2aα. Fig. 10 confirms the results of the previous two figures and makes more obvious some of the quantitative differences in individual valine isoacceptors. Since the identity of the novel valine isoacceptors is not known it is not possible to discuss the results obtained in terms of the amount of transcription of particular tRNA^{Val} genes. The novel valine isoacceptors observed are in themselves interesting. They are likely undermodified forms of the valine isoacceptors normally observed.

Figures 11 and 12 show the RPC-5 profiles of [^{3}H]Ser-tRNA^{Ser} from control and tyrosine phosphonate treated cells respectively. Significant increases in the amounts of two of the serine iso-

Figure 11. The RPC-5 profile of [^{14}C]Ser-tRNA^{Ser} (B). In this experiment 6.2 A_{260} units of crude tRNA of E. coli B containing 1.05×10^5 cpm (76% counting efficiency) were applied to a standard analytical column and eluted with standard buffers within a linear NaCl gradient of 0.45 to 1.5 M. Approximately 80% of the applied radioactivity was recovered in the gradient. The [^{14}C]serine used in the aminoacylation reacting had a specific activity of 149 µCi/µmole.

Figure 12. The RPC-5 profile of [^{14}C]Ser-tRNA^{Ser} (B-Tyrosine Phosphonate). In this experiment 4.0 A_{260} units of crude tRNA of E. coli B grown in the presence of the tyrosine phosphonate and containing 6.9×10^4 cpm [^{14}C]Ser (76% counting efficiency) were run as described in Fig. 11. Approximately 90% of the applied radioactivity was recovered in the gradient. The [^{14}C] serine used in the aminoacylation reaction had a specific activity of 149 µCi/µmole.

Figure 11

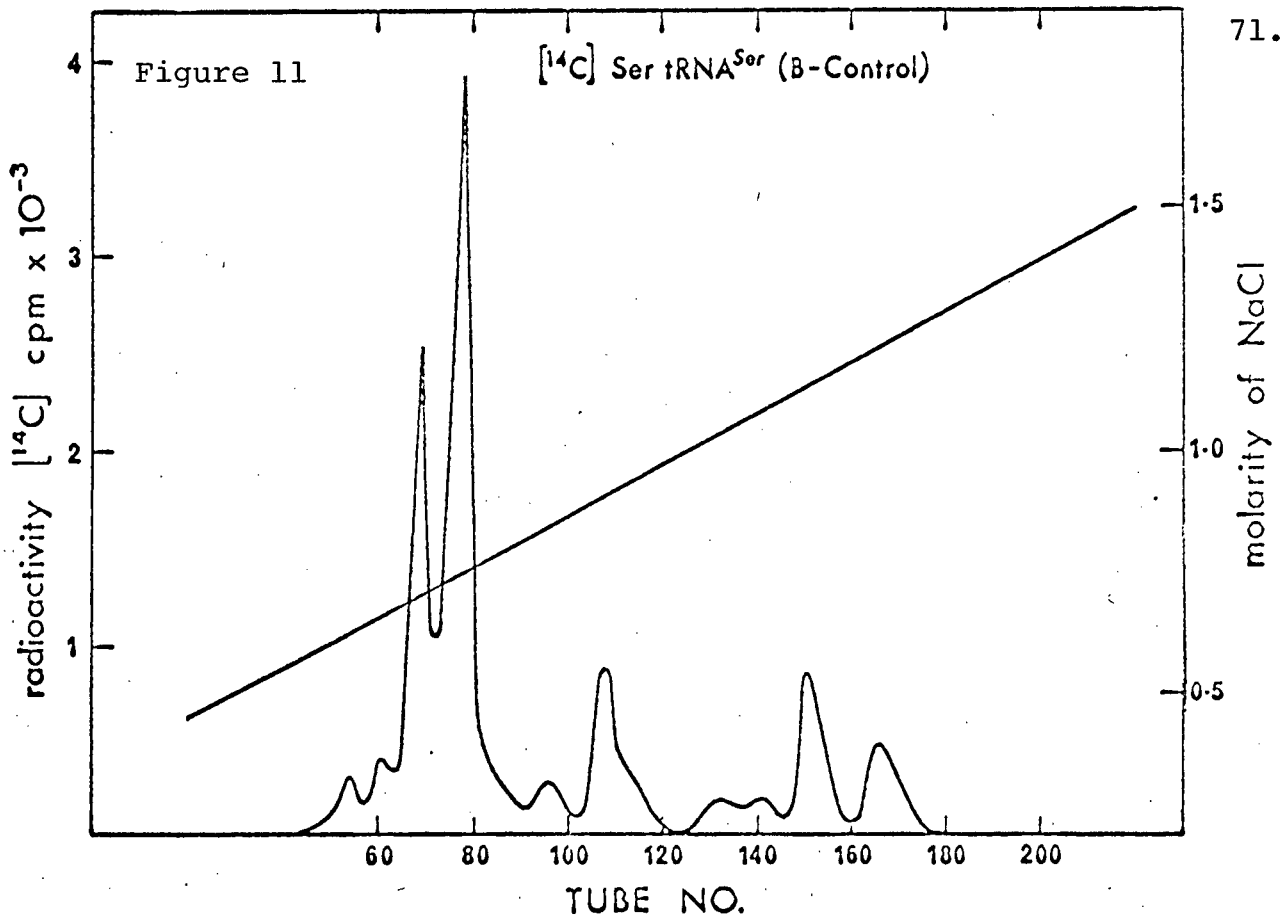
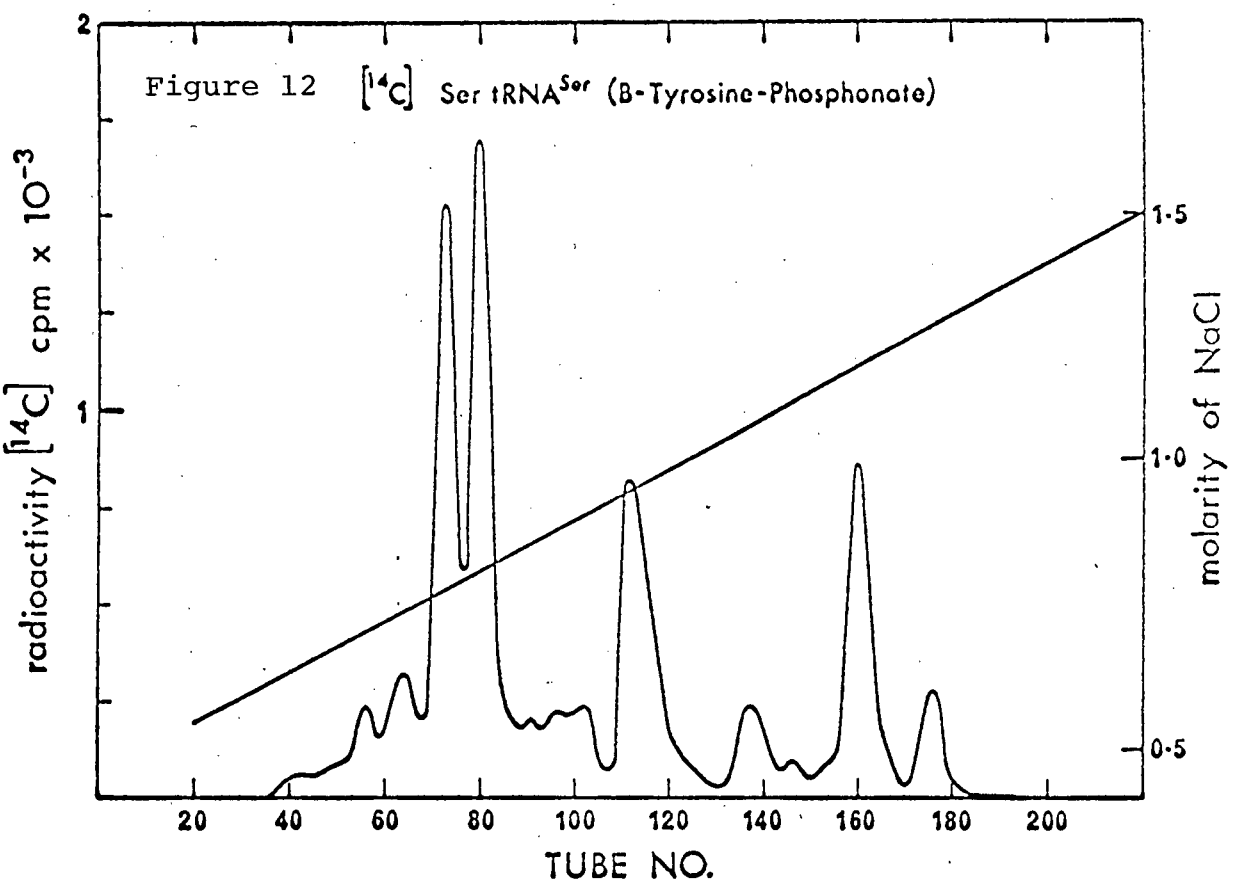


Figure 12 [14C] Ser tRNA^{Ser} (B-Tyrosine-Phosphonate)



acceptors are noted in Fig. 12. The increases in these peaks, (the 3rd & 4th major serine isoacceptors in order of elution from RPC-5 in the control experiment) from approximately 15% of the total acceptance to approximately 21% of the total acceptance for the 3rd major isoacceptor, and from approximately 10% of the total acceptance to approximately 14% of the total acceptance for the 4th major isoacceptor could conceivably account for the 12% increase in the serine to threonine acceptance ratio observed. It is possible in this instance that a specific synthesis of two serine isoacceptors occurred in response to growth inhibition by tyrosine phosphonate. Why or how such a specific change could have occurred is not obvious. Figures 13 to 15 show the [^{3}H]Ser-tRNA^{Ser} profiles for control NF162 cells, NF162 cells treated with α-amino-n-butyrate and NF162 cells depleted of arginine. Figures 13 and 14 show superior resolution to that of Figures 11, 12 and 15 because RPC-5 of 50 mesh sieve size was used in the latter experiments while RPC-5 of 200 mesh sieve size was used in all other RPC-5 profiles in this thesis. Fig. 14 shows a slightly altered Ser-tRNA^{Ser} profile. A number of indistinct serine isoacceptors are observed in fractions 90 to 130. There appears to be less material in the final two major serine isoacceptors. The earlier eluting species may in fact be precursors of the later eluting ones. No overall change in the serine acceptance ratio was observed as previously indicated. α-Amino-n-butyrate treatment causes a much more drastic alteration in isoacceptor distribution of Val-tRNA^{Val} than Ser-tRNA^{Ser}. The [^{3}H]Ser-tRNA^{Ser} (162-Arg) (Fig. 15) profile differs from that

Figure 13. The RPC-5 profile of [^{3}H]Ser-tRNA^{Ser} NF162. In this experiment 6.7 A_{260} units of crude tRNA from E. coli NF162 containing 4.2 x 10^5 cpm [^{3}H]Ser (10% counting efficiency) were run as described in Fig. 11. Approximately 90% of the applied radioactivity was recovered in the gradient. The [^{3}H]serine used in the aminoacylation reaction had a specific activity of 3370 μCi/μmole.

Figure 14. The RPC-5 profile of [^{3}H]Ser-tRNA^{Ser} (NF162; α-amino-n-butyrate). In this experiment 10.0 A_{260} units of crude tRNA from E. coli NF162 grown in the presence of the analogue α-amino-n-butyrate and containing 4.5 x 10^5 cpm [^{3}H]Ser (10% counting efficiency) were run as described in Fig. 11. Approximately 90% of the applied radioactivity was recovered in the gradient. The [^{3}H]serine used in the aminoacylation reaction had a specific activity of 3370 μCi/μmole.

Figure 13

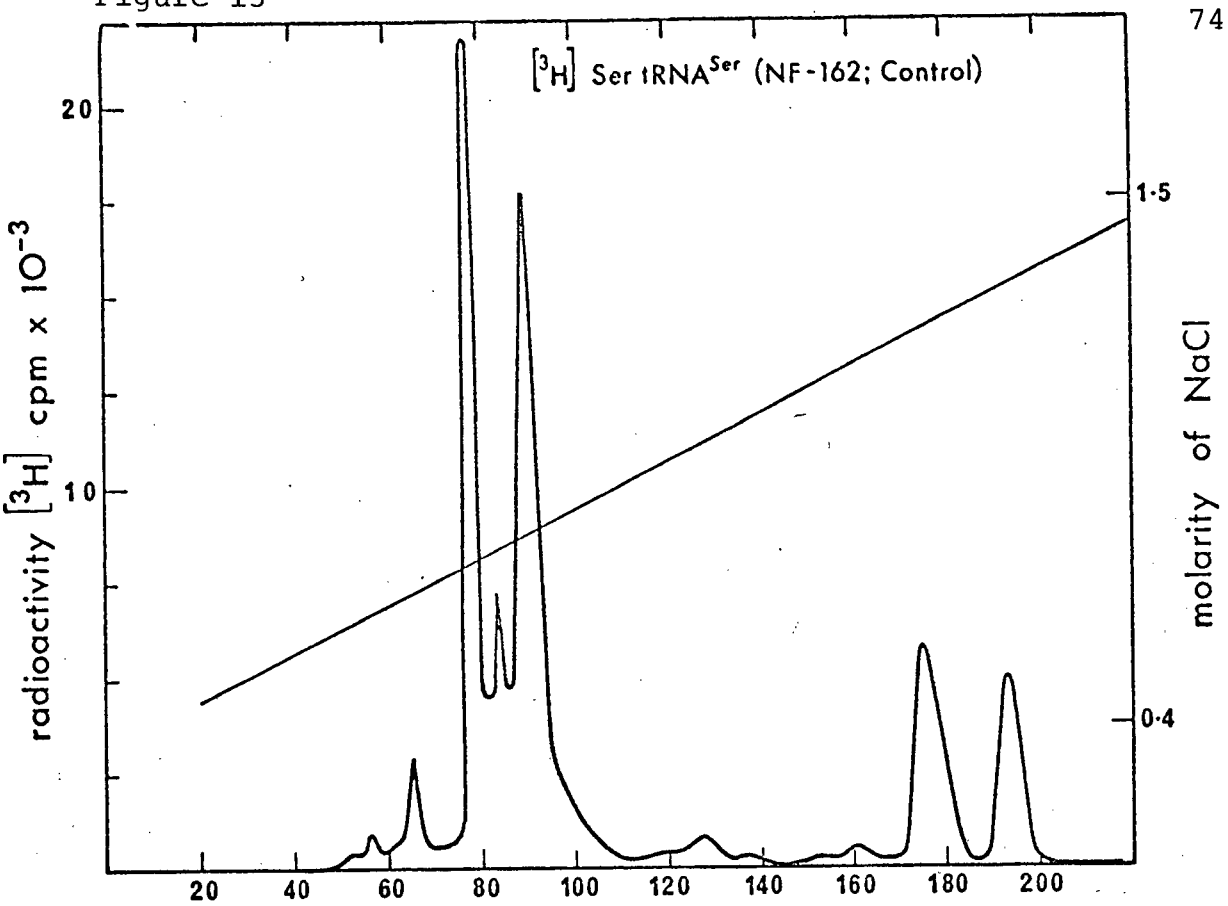
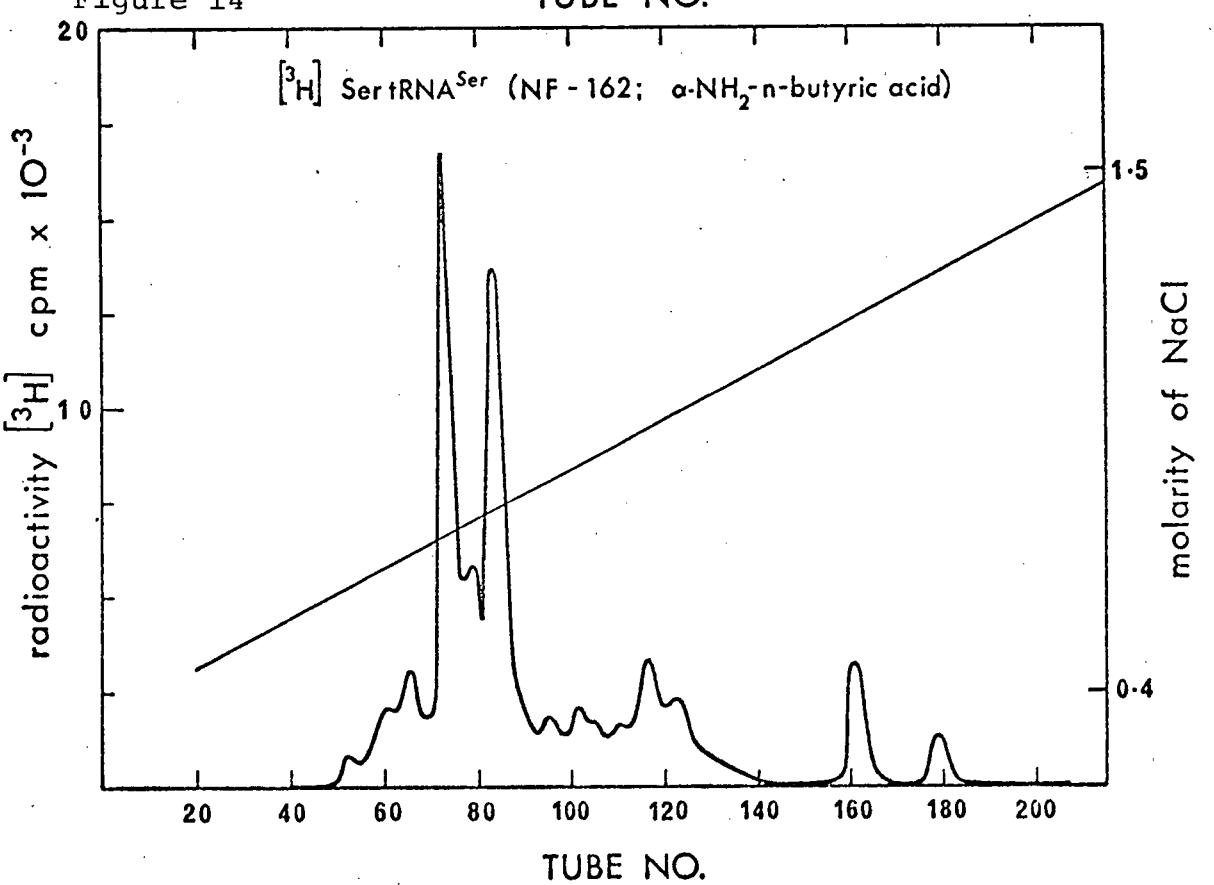


Figure 14



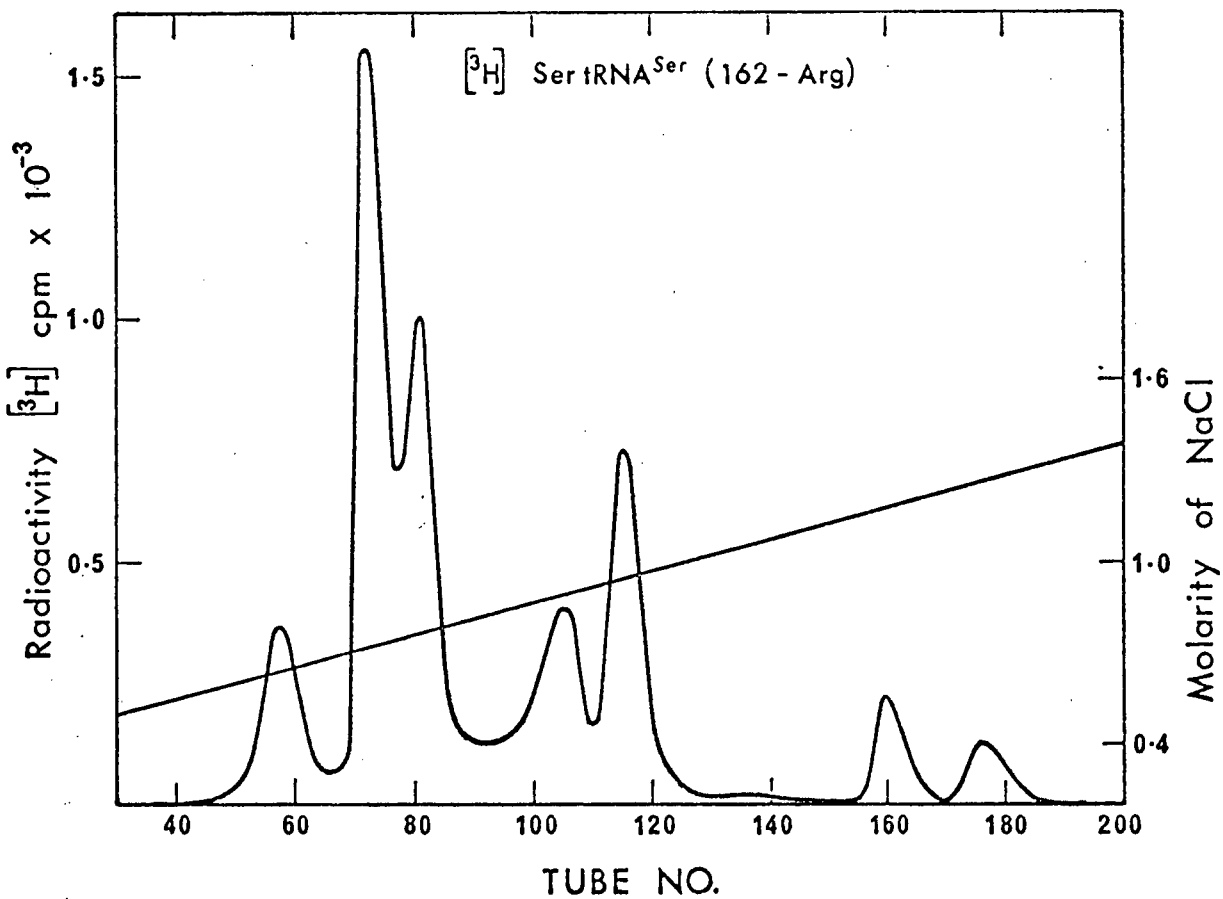


Figure 15. The RPC-5 profile of [^{3}H]Ser-tRNA^{Ser} (NF162-Arg). In this experiment 9.0 A_{260} units of crude tRNA of E. coli NF162 deprived of arginine, containing 4.0 x 10^5 cpm [^{3}H]Ser (10% counting efficiency) were run as described in Fig. 11. Approximately 90% of the applied radioactivity was recovered in the gradient. The [^{3}H]serine used in the aminoacylation reaction had a specific activity of 3370 µCi/µmole.

of the control Fig. 13, particularly in the region fraction 100 to fraction 120. There appears to be an increase of material in this latter region and a decrease in the size of the later eluting peaks. The region containing the two major isoacceptors is reduced to approximately 50% of the total serine acceptance from the control value of approximately 66%. If we assume that the tRNA in the region of fractions 100 to 120 is transcribed from the same genes as the final eluting serine isoacceptors then the 13% decrease in the serine to threonine acceptance ratio is easily accounted for by a specific decrease in the synthesis of the major serine isoacceptor of fractions 65 to 90 of Fig. 15. This argument is tenuous and serves to point out the need to identify the exact gene origin of all the tRNA isoacceptors for a particular amino acid.

Fig. 16 shows the [^{3}H]Leu-tRNA^{Leu} (NF162) profile on RPC-5. This profile is essentially the same as that of the [^{14}C]Leu-tRNA^{Leu} (B-control) used for cochromatography in Figures 17 and 18. Figures 17 and 18 show the cochromatography of [^{14}C]Leu-tRNA^{Leu} (B-control) and [^{3}H]Leu-tRNA^{Leu} of α-amino-n-butyrate treated NF162 cells and arginine depleted NF162 cells respectively. These profiles are included to demonstrate the extensive formation of novel leucine isoacceptors under these growth conditions. Obviously different tRNAs have a different potential to form novel isoacceptors in a particular growth condition.

The literature contains several publications related to this work. Wong et al. (190) suggest that when growth of either a leucine or tryptophan auxotroph was limited by the supply of

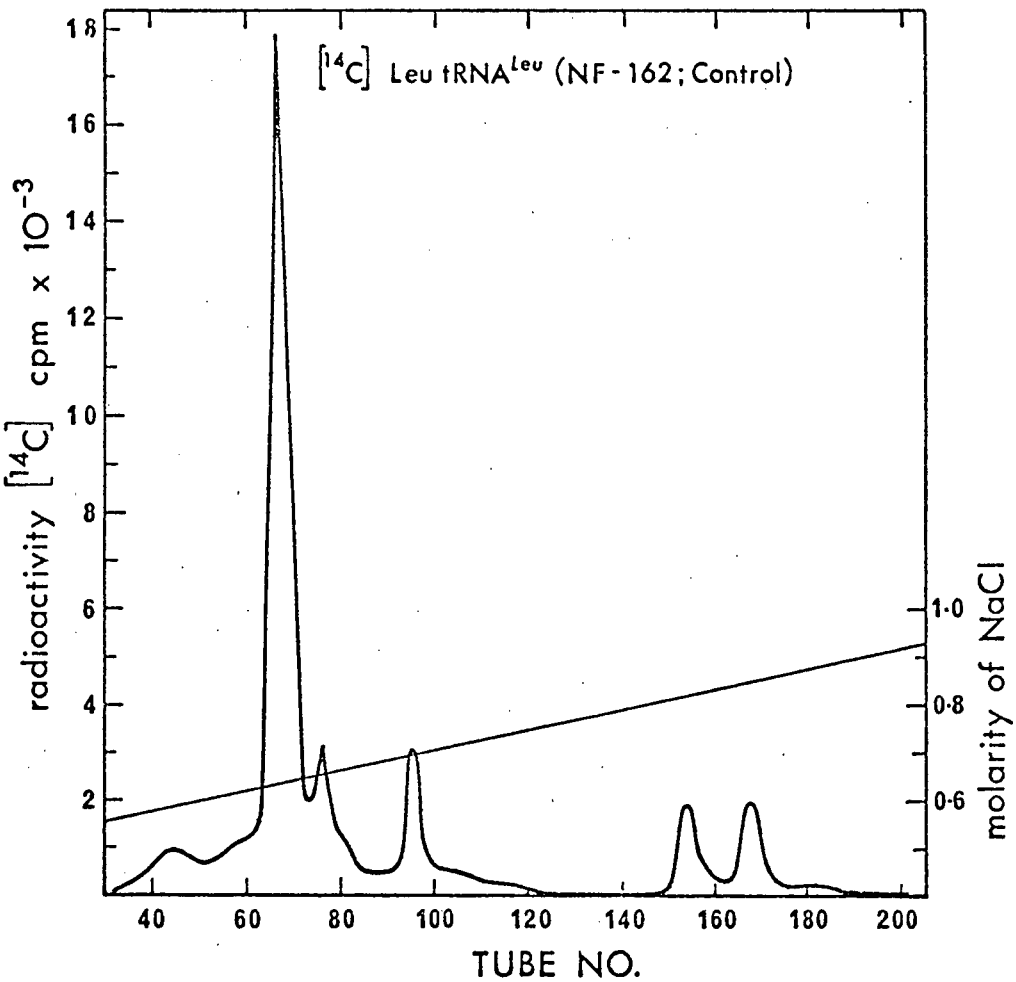


Figure 16. The RPC-5 elution profile of [^{14}C]Leu-tRNA^{Leu} (NF162). In this experiment 6.4 A_{260} units of crude tRNA of E. coli NF162 containing 2.9 x 10^5 cpm [^{14}C]Leu (80% counting efficiency) were run on a standard analytical column and eluted with standard buffers within a linear NaCl gradient of 0.50 to 0.95 M. Approximately 71% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{14}C] leucine used in the aminoacylation reaction was 294 μCi/μmole.

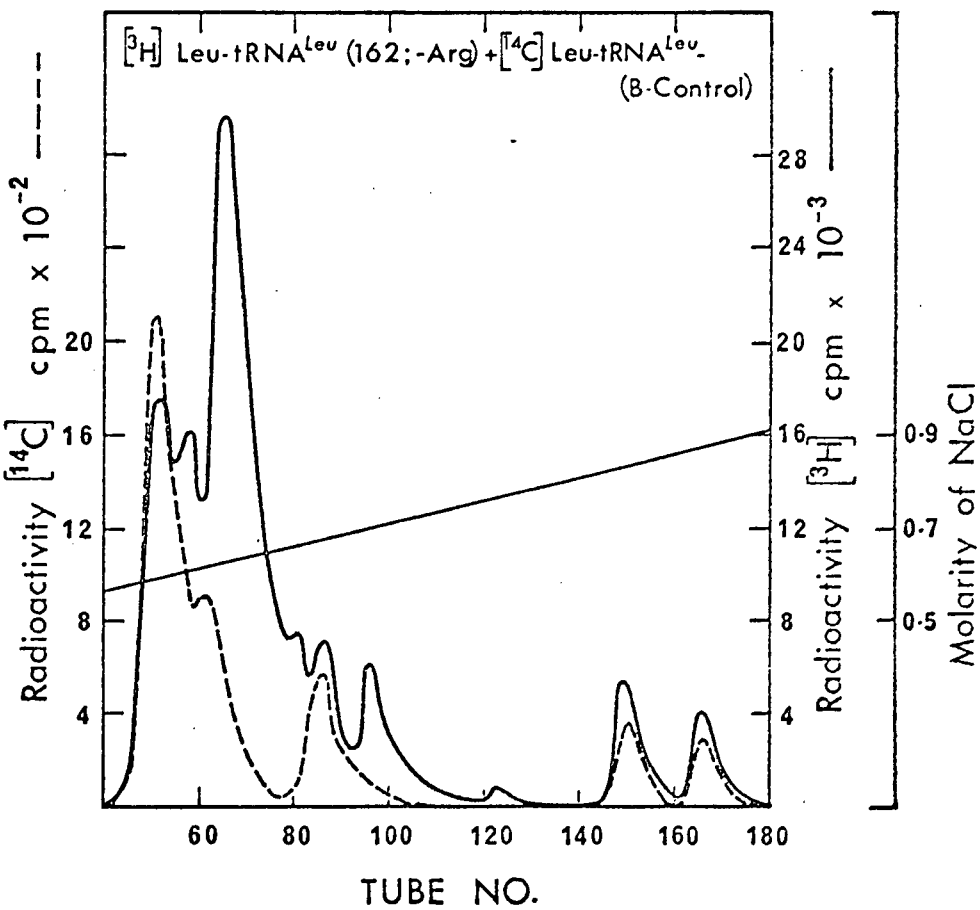


Figure 17. The cochromatography of [^{3}H]Leu-tRNA^{Leu} (NF162: α-amino-n-butyrate) and [^{14}C]Leu-tRNA^{Leu} (B). In this experiment, crude tRNA of E. coli NF162 grown in the presence of the valine analogue α-amino-n-butyrate and containing 1.35 x 10^5 cpm [^{3}H]Leu (37% counting efficiency) and crude tRNA of E. coli B containing 9.5 x 10^4 cpm [^{14}C]Leu (80% counting efficiency) were run as described in Fig. 16. Approximately 85% of the applied [^{3}H] and 82% of the applied [^{14}C] were recovered in the gradient. The specific activities of the [^{3}H]leucine and [^{14}C] leucine used in the aminoacylation reactions were 5,000 μCi/μmole and 294 μCi/μmole respectively. The leucine to threonine acceptance ratio was 1.60 for the crude tRNA of (NF162; α-amino-n-butyrate).

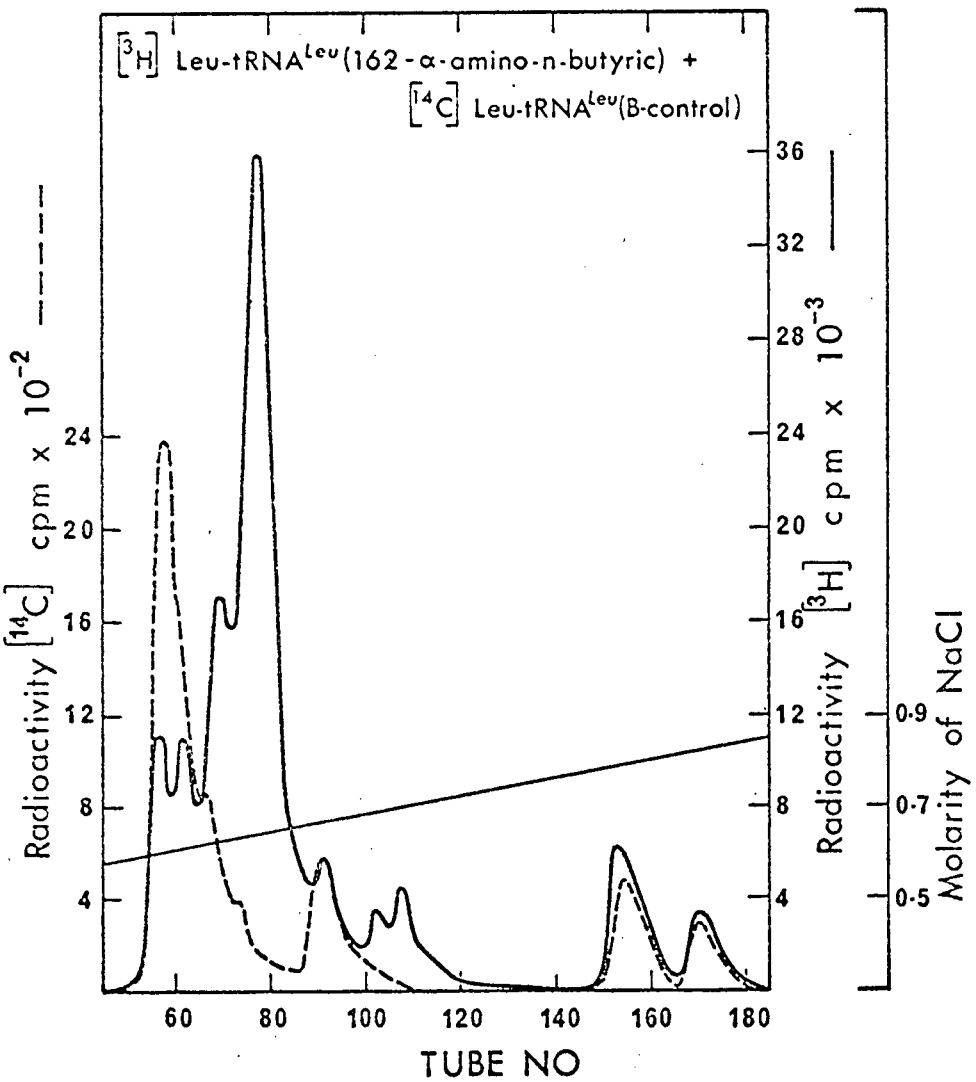


Figure 18. The cochromatography of [^{3}H]Leu-tRNA^{Leu} (NF162:-Arg) and [^{14}C]Leu-tRNA^{Leu} (B). In this experiment, crude tRNA of E. coli NF162 depleted of arginine and containing 1.33 x 10^5 cpm [^{3}H]Leu (37% counting efficiency) and crude tRNA of E. coli B containing 9.5 x 10^4 cpm [^{14}C]Leu (80% counting efficiency) were run as described in Fig. 16. Approximately 85% of the applied [^{3}H] and 84% of the applied [^{14}C] was recovered in the gradient. The specific activities of the [^{3}H]leucine and [^{14}C]leucine used in the aminoacylation reactions were 5,000 µCi/µmole and 294 µCi/µmole respectively. The [^{14}C]leucine acceptance to [^{14}C] threonine acceptance ratio was 1.85 for the crude tRNA of (NF162-Arg).

amino acid no specific changes were observed in the tRNA levels corresponding to the limiting amino acid. The design of their experiments was such that it is possible that specific tRNA control might not be observed under the experimental conditions they used and thus we sought to confirm or refute their observations in a more clearly defined system. Our results confirm the results previously reported in that we likewise observed no specific changes in tRNA levels when E. coli NF162 cells were depleted of arginine or methionine. We also attempted to extend the previous work by analyzing individual isoacceptor distribution. Large changes in the phenylalanine to lysine acceptance ratio have been reported in E. coli (rel^-Arg^-) starved of arginine (270). Differences in response of stringent and relaxed cells to arginine deprivation were also noted.

In an interesting experiment Wong et al. (190) plotted tRNA content versus growth rate over a wide range of growth rates. Straight lines of different slopes were obtained for $tRNA^{Leu}$, $tRNA^{Ile}$, and $tRNA^{Try}$. Since the slopes were not identical, this suggests that different growth rates have quite different tRNA populations (one tRNA relative to another). This argues for specific control of tRNA biosynthesis. It also poses the possibility that in amino acid analogue treated cells, the tRNA population is more characteristic of the growth rate than the growth inhibitor. This, however, is disproved by the fact that tyrosine phosphonate treated cells and p-fluorophenylalanine treated cells, having the same growth rate, show very significant differences in their tRNA populations. The identical 10% increase

in the $tRNA^{Pro}$ content in each experiment could be because of the identical change in growth rate. More than one tRNA control mechanism may be operative under any particular set of growth conditions.

Skjold et al. (298) observe no changes in relative isoacceptor distribution for all the tRNAs examined for tRNA from slow and fast growing E. coli. They postulated that isoacceptors are regulated as a group. Our results suggest the same idea. Significant differences were observed in the serine to valine acceptance ratio of cells grown on acetate as a carbon source (0.55) compared to cells grown on nutrient broth (1.17). Numerous other changes in acceptance ratios were also observed suggesting some form of tRNA control as a function of growth rate.

Changes in amino acid acceptance ratios and altered amounts of particular tRNA isoacceptors can result from a differential rate of nuclease digestion of particular tRNAs. It has been reported that mutations which destabilize tRNA structure rendering those tRNAs more susceptible to nuclease attack (268,269) or mutations which result in slower processing of tRNA precursors (268,269) allowing for nonspecific nuclease cleavage, result in correspondingly decreased tRNA levels. It is conceivable that tRNAs deficient in certain modified nucleosides have an altered susceptibility to nuclease action. Hence tRNA levels and isoacceptor distribution can change without a significant change in the actual rate of gene transcription. Uncleaved precursor tRNAs lacking modified nucleosides are not processed more slowly than those having modified nucleosides (275). An interesting

hypothesis (274) has been put forward suggesting that the increased 2'-O-methylation observed in Bacillus stearothermophilus grown at high temperature results in a stabilization of the tRNAs against nuclease attack when the tRNAs have a more open structure. If the hypothesis is correct, differential 2'-O-methylation could significantly alter individual amino acid acceptance and relative isoacceptor distribution.

It is also possible that tRNAs may undergo specific loss of their CCA termini. This possibility has not been examined in these experiments. Turnover of the CCA end has been shown (61) to virtually cease in amino acid starved E. coli.

Novel tRNA isoacceptors have been reported in E. coli grown under a wide variety of adverse growth conditions. Starvation of a leucine auxotroph for leucine resulted in the formation of novel tRNA isoacceptors for leucine, histidine, arginine, valine and phenylalanine (292). Starvation of a histidine auxotroph for histidine did not yield novel isoacceptors for leucine or arginine although starvation of an arginine auxotroph for arginine did (292). In our hands, starvation of an arginine auxotroph for arginine resulted in the formation of novel isoacceptors for valine and leucine and changes in isoacceptor distribution for serine. Starvation of a phenylalanine auxotroph for phenylalanine resulted in novel isoacceptor formation for $tRNA^{Phe}$ (173). Starvation of a relaxed control methionine auxotroph for methionine has been shown to be a particularly effective means of obtaining undermodified tRNA. Methyl deficient tRNA isoacceptors for phenylalanine (186,188,293,294,295),

leucine (296), valine (179,180) and methionine (246) have been studied.

Growth of E. coli in the presence of chloramphenicol has been used as a means of obtaining undermodified tRNAs for phenylalanine (172,173,175,291), leucine (172,173), and methionine, arginine, lysine, isoleucine and serine (173). Novel $tRNA^{Phe}$ isoacceptors have been observed in E. coli grown on a low phosphate medium at a slow growth rate (171). E. coli grown in a medium with less than 10^{-7} M iron or grown under limiting aeration in an aged medium show novel $tRNA^{Phe}$ isoacceptors (170). It has been shown that the latter novel isoacceptors lack ms^2i^6A and i^6A (176). The novel $tRNA^{Phe}$ isoacceptors of low phosphate media are thought to lack these same modifications (171) as are the $tRNA^{Phe}$ isoacceptors of chloramphenicol treated cells (175). Chloramphenicol treated cells have been shown also to be deficient in the minor nucleosides hU, s^4U, m^2A and m^7G, m^1G (173,290,291). A pseudouridine deficiency in tRNA was noted in E. coli treated with 2-thiouracil (297). tRNA of methionine starved methionine auxotrophs is methyl deficient (179,180,186,188,293-296). Other nucleoside modifications are reduced as well (173) and not all methyl modified nucleosides are equally methyl deficient (173,185). Most likely the novel isoacceptors observed in the experiments described in this thesis lack modified nucleosides. It is interesting that amino acid analogues give rise to many of the same novel isoacceptor tRNAs observed under conditions of amino acid starvation. To clarify the literature it would be helpful if attempts were made to relate the various novel isoacceptors from the different growth conditions.

In several instances it has been shown that undermodified tRNAs can have different functional properties (137 and references cited therein). In one instance it has been suggested that undermethylated tRNA has a reduced capacity to accept amino acids (247). Other laboratories have suggested that methyl deficient tRNA accepts amino acids although at a somewhat reduced rate, the final level of acylation obtained being the same (246). In the experiments described in this thesis sufficient amino-acyl-tRNA synthetase was used to ensure rapid saturation of the acceptors. Acceptance was measured at 5, 10 at 20 minutes to demonstrate complete acylation. Those undermodified forms capable of accepting amino acids were completely acylated. However, undermodified tRNAs incapable of any aminoacylation obviously would not be detected.

It is not possible to predict the precise nature of the modified nucleoside deficiency based on elution position on RPC. Several tRNAs lacking ψ elute at higher salt concentration on RPC than the fully modified forms (310,249). Undermethylated tRNAs have been shown to elute both earlier (179,246) and later (183) than the fully mature tRNA. tRNAs lacking hydrophobic modified nucleosides such as i^6A and ms^2i^6A elute at lower salt concentrations (170,175). tRNA from E. coli grown in the presence of chloramphenicol is deficient in ms^2i^6A, hU, s^4U, m^2A and m^1G (173,290,291). Several of the resulting undermodified tRNA including those which accept leucine, lysine, isoleucine and arginine elute at higher salt concentrations than the fully modified forms while others such as those accepting tyrosine

and phenylalanine elute at lower salt concentrations (173). The absence of ms^2i^6A/i^6A in these latter tRNAs probably accounts for the shift to the earlier elution position. Reaction of Q containing tRNAs with CNBr, which eliminates the positive charge of Q, causes a dramatic shift in elution position to higher salt (B. White-personal communication). The loss of the positive charge, in tRNAs lacking m^7G of m^1A, would result in a tRNA eluting at a higher salt concentration. Conversely tRNAs deficient in uridine-5-oxyacetic acid would have one less negative charge and should elute earlier than the mature form. If $tRNA_3^{Val}$ is an undermodified form of $tRNA_1^{Val}$, its elution position is easily explained in terms of the absence of uridine-5-oxyacetic acid.

The experimental results described in this thesis argue against any simple role for aminoacyl-tRNA in the specific control of individual tRNA biosynthesis. The results confirm that tRNA populations are not static. It would appear that changes in total acceptance can occur without any significant changes in relative isoacceptor distribution. An excellent example of this is seen in the analysis of tRNA of E. coli Str^D discussed later in the thesis. This would suggest that there is a control mechanism capable of maintaining the relative isoacceptor distribution. One might ask how such a mechanism functions when several other tRNAs form part of the transcript. For $tRNA^{Ser}$ the results suggested that the levels of particular isoacceptors could be selectively increased or decreased. There was no obvious relationship between growth condition and the changes observed. It is

possible that $tRNA^{Ser}$ isoacceptors are under specific individual control while the $tRNA^{Val}$ isoacceptors are subject to quite different control mechanisms. The data are made more complicated by the presence of novel isoacceptors whose gene origin is not known. Mechanisms for the control of tRNA biosynthesis appear to exist but attempts to understand and interpret them will require the accumulation of considerably more data.

Effect of Growth Temperature on E. coli tRNA

The results obtained using amino acid depletion and amino acid analogues to obtain altered tRNA isoacceptor profiles suggested that the more slowly the cells grew the more altered the tRNA profiles were likely to be. Since E. coli grow over a wide temperature range, often at very slow growth rates (273), it was decided that growth at extremes of growth temperature might be a useful tool to obtain novel tRNA isoacceptors. E. coli B cells were therefore grown over a range of temperatures in the Biogen as described in Methods and the tRNA was isolated and analyzed.

As shown in table 4, numerous changes in amino acid acceptance ratios were observed as a function of growth temperature. In most instances this ratio decreased suggesting that the relative threonine acceptance was in fact increasing. The percent decreases, however, were quite variable. tRNA from cells grown at 44°C showed a 64% decrease in the tyrosine to threonine acceptance ratio but only a 10% decrease in the proline to threonine acceptance ratio. tRNA from cells grown at 17°C showed increases in several amino acid acceptance ratios including a 13%

Table 4. Amino acid acceptance of crude tRNA of E. coli B cells grown at various temperatures

Growth Temperature	Growth Rate dph	Acceptance $\frac{[^{14}C]Val}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Pro}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Tyr}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Leu}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Ser}{[^{14}C]Thr}$	Picomole $[^{14}C]$ Thr acceptance / A_{260} unit
17°	0.24	1.44	1.65	0.80	1.91	1.30	26
20°	0.30	1.17	1.51	0.54	1.90	1.18	52
30°	1.0	0.87					55
37°	1.0	1.28	1.57	0.65	2.10	1.19	65
41°	1.0	1.06					60
44°	0.20	0.83	1.42	0.29	1.42	0.93	62

increase in the valine to threonine acceptance ratio. No specific overall pattern in amino acid acceptance ratios can be deduced from the data presented in table 4 nor perhaps is there reason to expect one.

Radioactively labelled Val-tRNA^{Val} from each growth temperature was run on RPC-5 as shown in Figures 19-26. Cochromatography with Val-tRNA^{Val} (B-37°) and Val-tRNA^{Val} (NF162-Arg) in most instances allows one to relate growth conditions possessing similar Val-tRNA^{Val} isoacceptors. As can be seen from Figures 19 and 20, Val-tRNA^{Val} (B-44°) differs dramatically from the Val-tRNA^{Val} profiles described thus far in that Val-tRNA$_1^{Val}$ is not the major valine isoacceptor. Val-tRNA$_3^{Val}$ is present as is Val-tRNA$_{1\beta}^{Val}$. Val-tRNA$_{2a\beta}^{Val}$ is certainly absent. There are several nondistinct peaks in the Val-tRNA$_{2a}^{Val}$ and Val-tRNA$_{2b}^{Val}$ region. Two new isoacceptors, Val-tRNA$_{3\alpha}^{Val}$ and Val-tRNA$_{3\gamma}^{Val}$ are present. Val-tRNA$_{3\gamma}^{Val}$ is present in such large amounts that one can suggest that it is an undermodified form of Val-tRNA$_1^{Val}$.

Fig. 21 shows the RPC-5 profile of [^{3}H]Val-tRNA^{Val} (B-41°). The profile suggests that the valine isoacceptors 3, 3α and 3γ are present in cells grown at 41°C. It is interesting that these cells possessed a doubling time the same as cells grown at 37°C.

Fig. 22 shows the RPC-5 profile of [^{3}H]Val-tRNA^{Val} (B-30°). The valine isoacceptor distribution of 30°C is essentially that observed at 37°C except for a slight increase in the amount of Val-tRNA$_3^{Val}$.

Figure 19. The cochromatography on RPC-5 of [^{14}C]Val-tRNA^{Val} (B-37°) and [^{3}H]Val-tRNA^{Val} (B-44°). In this experiment 10.4 A_{260} units of crude tRNA of E. coli B grown at 37° and containing 3.30×10^5 cpm [^{14}C]Val (80% counting efficiency) plus 7.36 A_{260} units of crude tRNA of E. coli B grown at 44°C and containing 6.48×10^5 cpm [^{3}H]Val (37% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 76% of each of the applied labels was recovered in the gradient. The [^{3}H]valine and [^{14}C]valine used in the aminoacylation reactions had specific activities of 1300 μCi/μmole and 266 μCi/μmole respectively.

Figure 20. The cochromatography of [^{14}C]Val-tRNA^{Val} (B-44°) and [^{3}H]Val-tRNA^{Val} (NF162-Arg). In this experiment 4.5 A_{260} units of crude tRNA of E. coli B grown at 44°C and containing 1.15×10^5 cpm of [^{14}C]Val (80% counting efficiency) plus 8.1 A_{260} units of crude tRNA of E. coli NF162 depleted of arginine and containing 7.8×10^5 cpm [^{3}H]Val (37% counting efficiency) were run as described in Fig. 5. Approximately 98% of each of the applied labels was recovered in the gradient. The specific activities of the [^{3}H]valine and [^{14}C]valine used in the aminoacylation reactions were 1300 μCi/μmole and 266 μCi/μmole respectively.

Figure 19

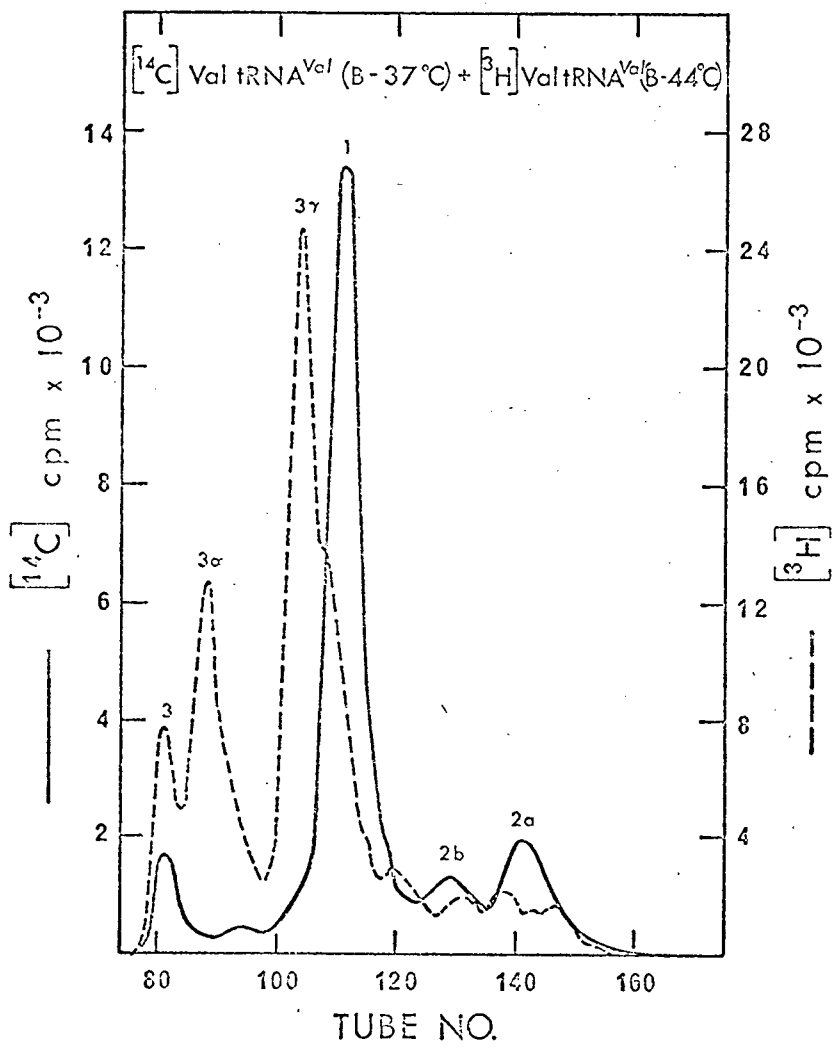
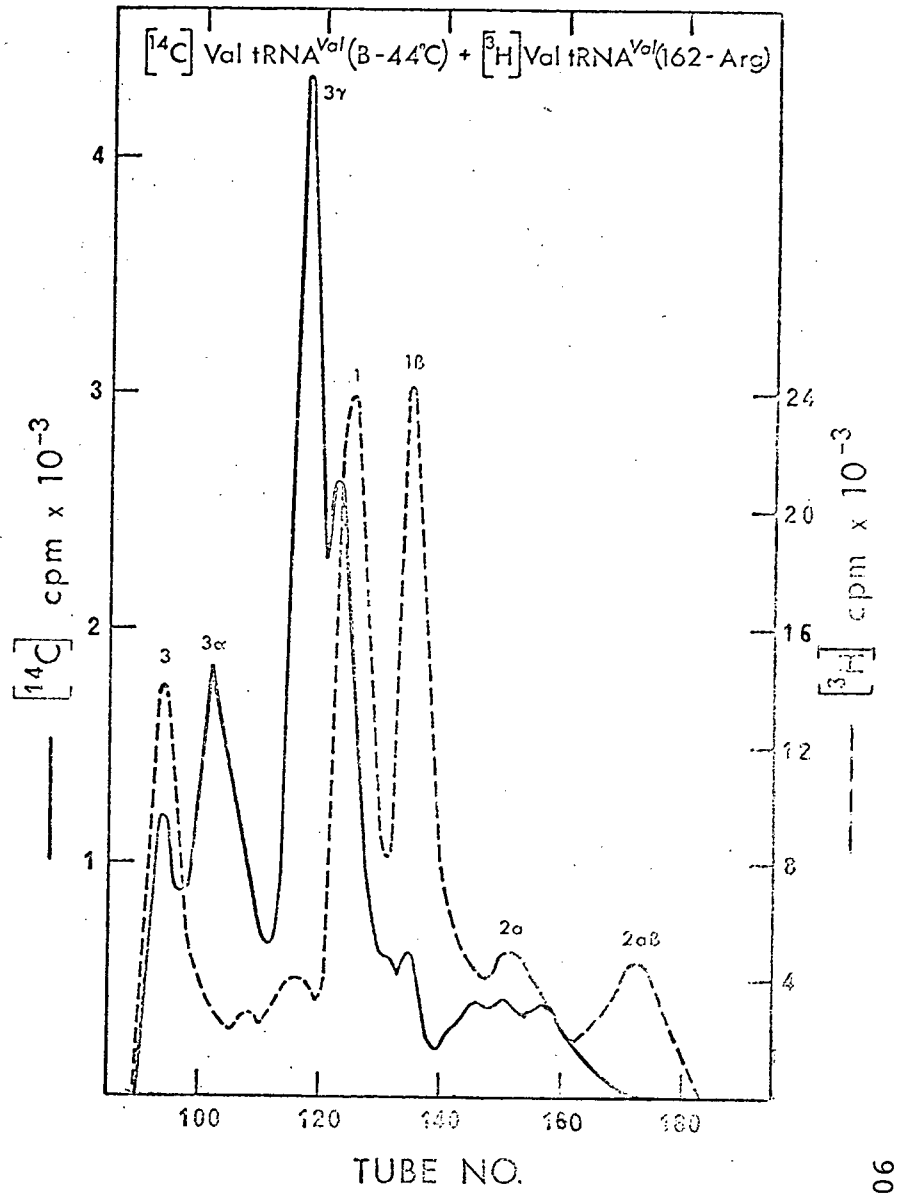


Figure 20



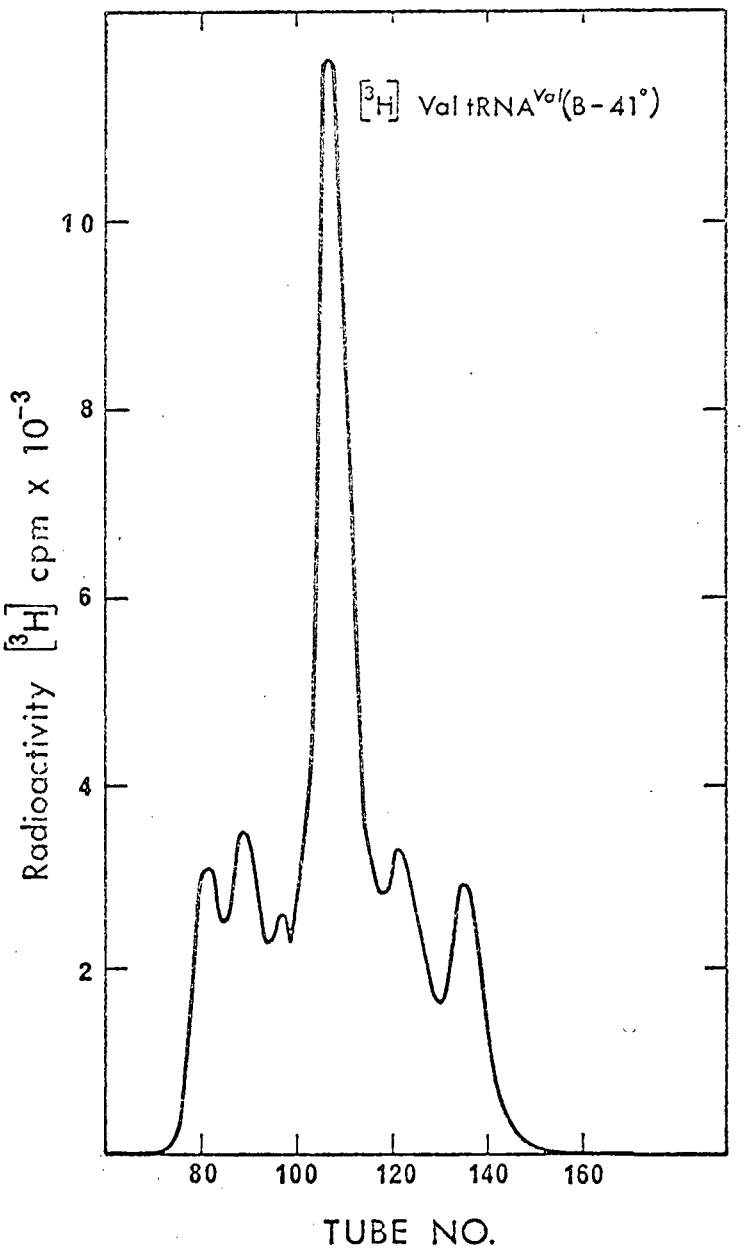


Figure 21. The RPC-5 elution profile of [^{3}H]Val-tRNA^{Val} (B-41°). In this experiment 10.5 A_{260} units of crude tRNA of E. coli B grown at 41° and containing 8.5 x 10^5 cpm [^{3}H] Val (37% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 70% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H] valine used in the aminoacylation reaction was 1300 µCi/µmole.

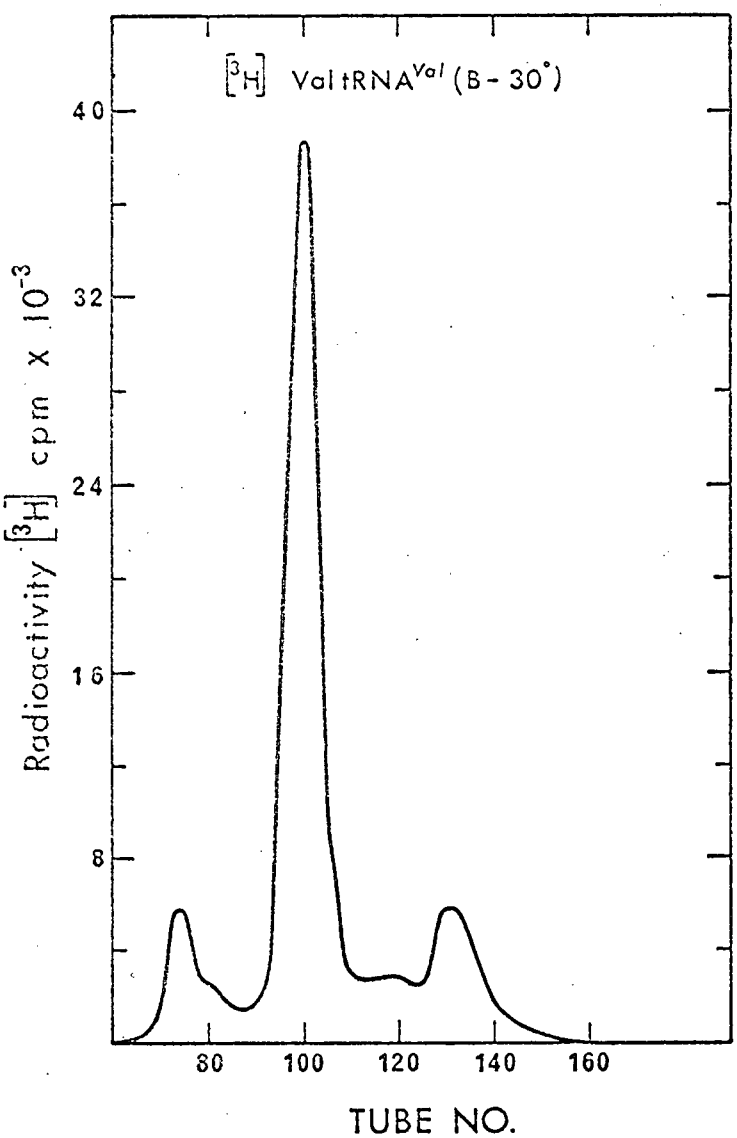


Figure 22. The RPC-5 elution profile of [^{3}H]Val-tRNA^{Val} (B-30°). In this experiment 8.1 A_{260} units of crude tRNA of E. coli B grown at 30° and containing 5.3 x 10^5 cpm [^{3}H]Val (37% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 82% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H]valine used in the aminoacylation reaction was 1300 μCi/μmole.

Significant changes in the profiles are observed in cells grown at 25°C or at lower temperatures. From Figures 23 and 24, it can be seen that Val-tRNA^{Val} (B-20°) contains the commonly observed valine isoacceptors 3 and 1. This tRNA lacks the valine isoacceptor 2aβ but contains 2aα and some 2a. New valine isoacceptors 2bα and 1α are observed. The valine isoacceptors 3α and 3γ are present as they are in cells grown at 44°C although in considerably different proportions. A major new valine isoacceptor, 3β, is also observed in cells grown at 20°C.

Val-tRNA^{Val} (B-17°) is quite similar to Val-tRNA^{Val} (B-20°) as can be seen in Figures 25 and 26. Absent from these profiles are the valine isoacceptors 2a, 2b and 2aβ. Val-tRNA$^{Val}_{3\alpha}$ is essentially absent. The valine isoacceptors 3, 3β, 3γ, 1, 1α, 2aα and 2bα are present although the proportion of each is changed. Val-tRNA$^{Val}_{3\gamma}$ is present in large amounts in cells grown at 17°C as it is in cells grown at 44°C. As the doubling time of the cells grown at 17°C and 44°C are very similar it would appear that the amount of the valine isoacceptor 3γ observed is dependent upon the growth rate of the cells.

It has been demonstrated by Lindahl et al. (300) that certain tRNAs are activated or renatured by heating to 60°C in the presence of Mg^{2+}. None of the valine isoacceptor profiles described in this thesis were altered when the crude tRNA preparations were treated in this way prior to aminoacylation and RPC-5 chromatography. Similarly no changes in isoacceptor profiles on RPC-5 were observed when any of the labelled Val-tRNA^{Val} samples were prepared in the presence of the other nineteen unlabelled amino acids.

Figure 23. The RPC-5 elution profile of [^{14}C]Val-tRNA^{Val} (B-37°) and [^{3}H]Val-tRNA^{Val} (B-20°). In this experiment 9.0 A_{260} units of crude tRNA of E. coli B grown at 37°C and containing 2.8 x 10^5 cpm [^{14}C]Val (80% counting efficiency) plus 4.8 A_{260} units of crude tRNA of E. coli B grown at 20°C and containing 4.2 x 10^5 cpm [^{3}H]Val (37% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 85% of the applied [^{14}C] and 82% of the applied [^{3}H] was recovered in the gradient. The [^{3}H] valine and [^{14}C]valine used in the aminoacylation reactions had specific activities of 1300 µCi/µmole and 266 µCi/µmole respectively.

Figure 24. The cochromatography of [^{14}C]Val-tRNA^{Val} (B-20°) and [^{3}H]Val-tRNA^{Val} (NF162-Arg). In this experiment 6.25 A_{260} units of crude tRNA of E. coli B grown at 20°C and containing 1.3 x 10^5 cpm of [^{14}C]Val (80% counting efficiency) plus crude tRNA from arginine depleted E. coli NF162 as per Fig. 20 were run as described in Fig. 5. Approximately 90% of the applied [^{14}C] and 96% of the applied [^{3}H] were recovered in the gradient. The specific activities of the [^{3}H]valine and [^{14}C]valine used in the aminoacylation reactions were 1300 µCi/µmole and 266 µCi/µmole respectively.

Figure 24

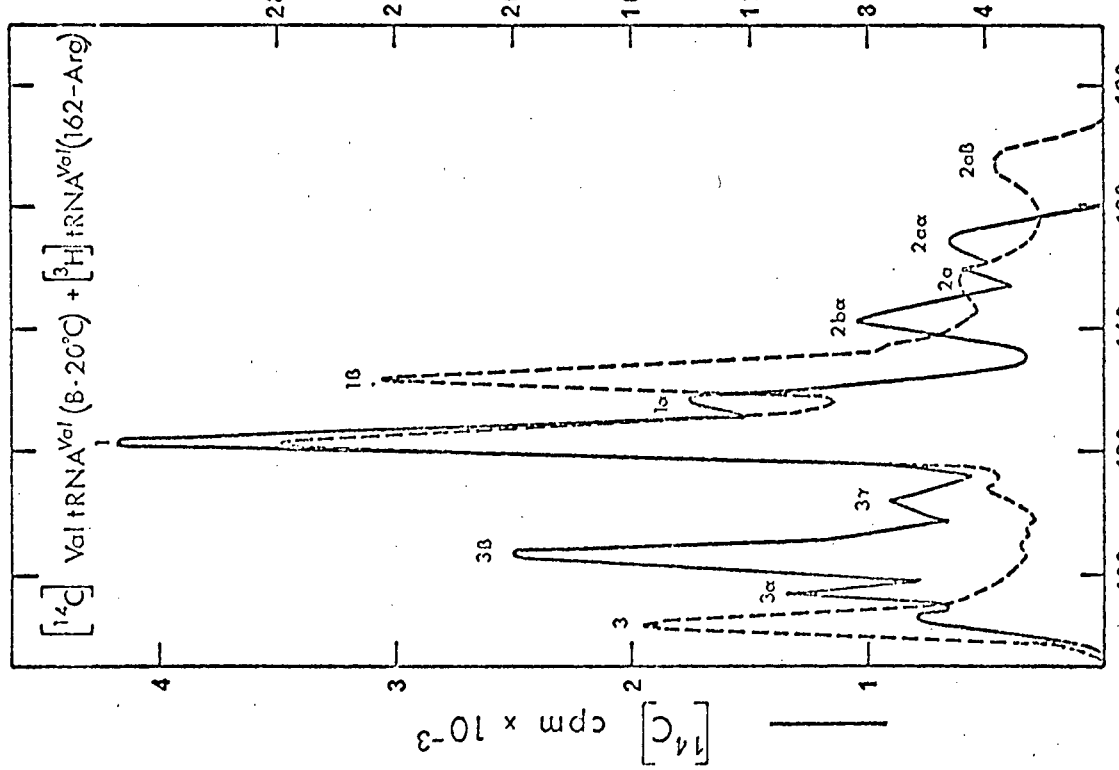


Figure 23

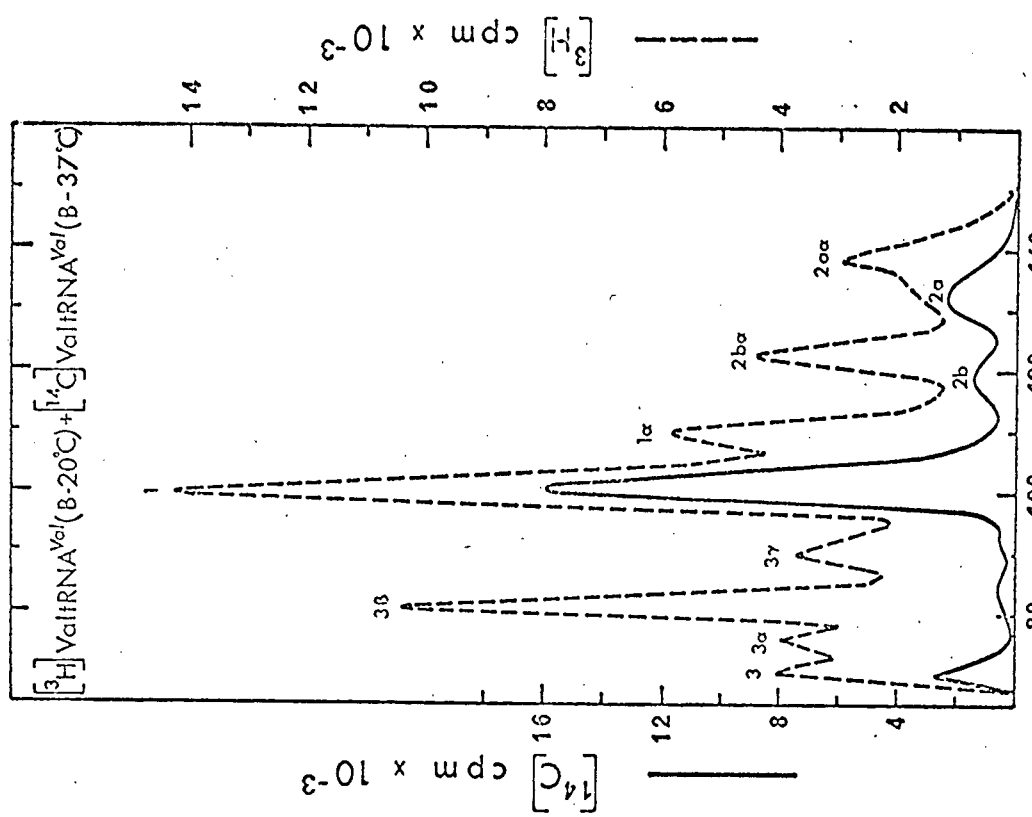


Figure 25. The RPC-5 elution profile of [^{14}C]Val-tRNA^{Val} (B-37°) and [^{3}H]Val-tRNA^{Val} (B-17°). In this experiment 9.0 A_{260} units of crude tRNA of E. coli B grown at 37°C and containing 2.8 x 10^5 cpm [^{14}C]Val (80% counting efficiency) plus 7.1 A_{260} units of crude tRNA of E. coli B grown at 17°C and containing 2.8 x 10^5 cpm [^{3}H]Val (37% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 79% of the applied [^{14}C] and 78% of the applied [^{3}H] radioactivity was recovered in the gradient. The [^{3}H]valine and [^{14}C]valine used in the aminoacylation reactions had specific activities of 1300 µCi/µmole and 266 µCi/µmole respectively.

Figure 26. The cochromatography on RPC-5 of [^{3}H]Val-tRNA^{Val} (B-17°) and [^{14}C]Val-tRNA^{Val} (NF162-Arg). In this experiment 11.4 A_{260} units of crude tRNA of E. coli B grown at 17°C and containing 5.87 x 10^5 cpm [^{3}H]Val (37% counting efficiency) plus crude tRNA of arginine depleted E. coli NF162 cells containing 7.0 x 10^4 cpm [^{14}C]Val (80% counting efficiency) were run as described in Fig. 5. Approximately 80% of each of the applied labels was recovered in the gradient. The specific activities of the [^{3}H] valine and [^{14}C]valine used in the aminoacylation reactions were 1330 µCi/µmole and 266 µCi/µmole respectively.

Figure 25

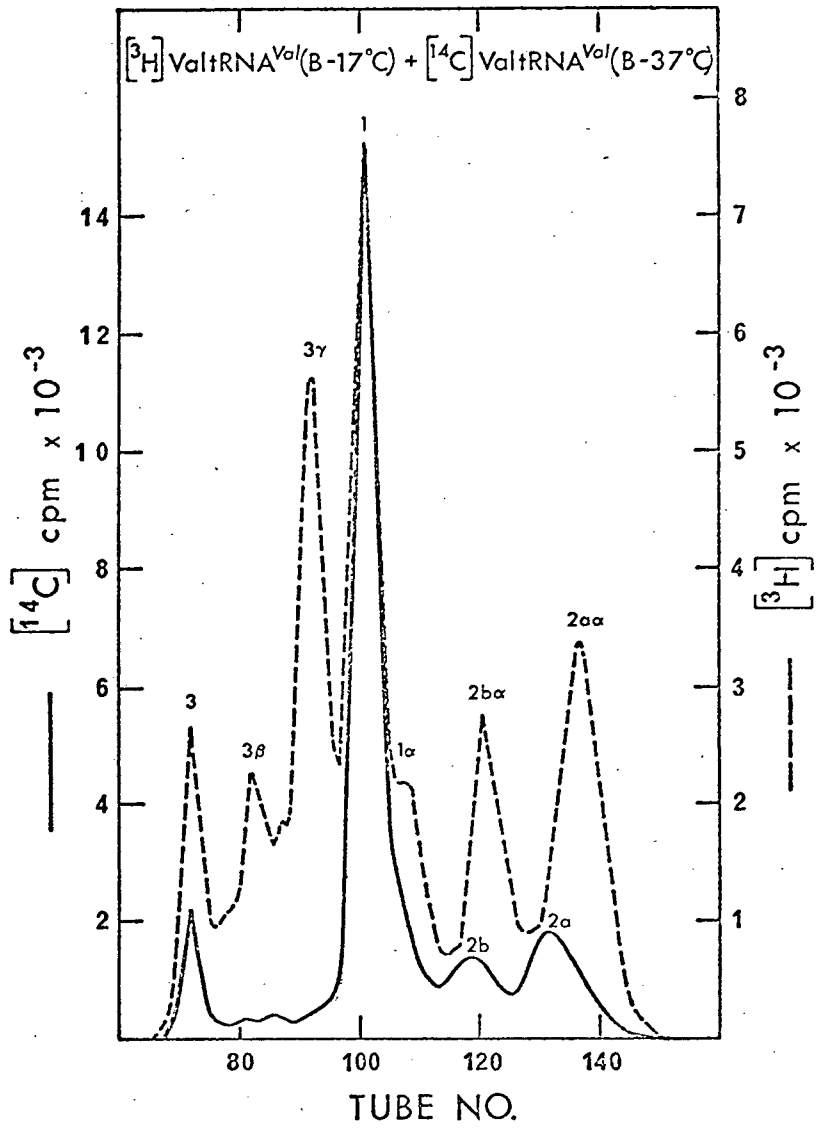
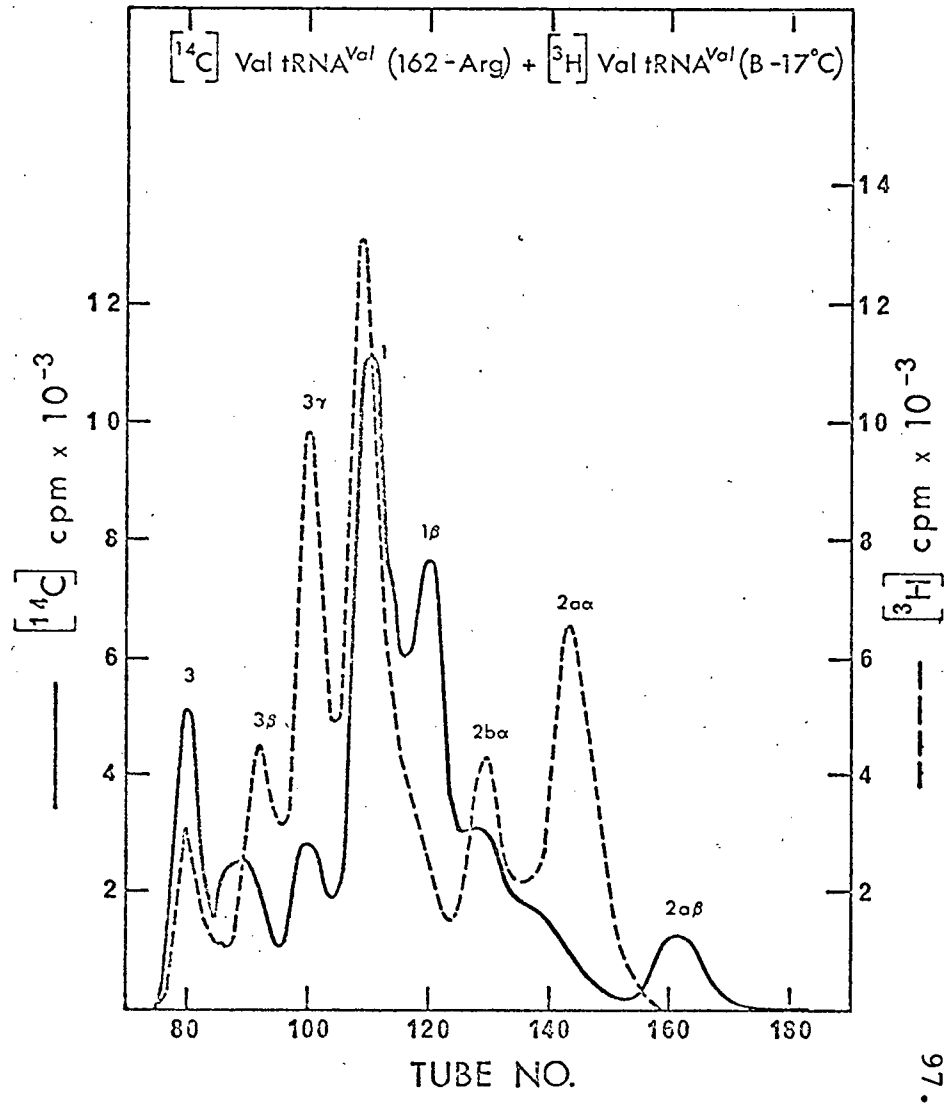


Figure 26



To check on the generality of novel isoacceptor formation, the tRNA of the various growth temperatures was analyzed with respect to the isoacceptor distributions for those tRNAs accepting leucine, serine and threonine. Figures 27 to 29 and Fig. 13 show the RPC-5 profiles of labelled Leu-tRNA^{Leu} from E. coli B grown at 17°C, 20°C, 37°C and 44°C. At both high and low temperatures novel leucine isoacceptors are observed. It was shown by cochromatography that the first and the last two leucine isoacceptors of the Leu-tRNA^{Leu} (B-17°) profile are the same isoacceptors observed at 37°C. All other leucine isoacceptors observed in E. coli grown at 17°C appear to be novel and characteristic of the extreme growth temperature. Figures 30 and 31 show the [^{3}H]Ser-tRNA^{Ser} profiles of E. coli grown at 17°C and 44°C respectively. Both high and low extremes of growth temperature result in the formation of novel serine tRNA isoacceptors. Figs. 32 to 34 show the results of cochromatography on RPC-5 of [^{14}C]Thr-tRNA^{Thr} (B-37°) and [^{3}H]Thr-tRNA^{Thr} of cells grown at 17°C, 20°C and 44°C respectively. In contrast to the results obtained for valine, leucine and serine tRNA isoacceptors, no novel threonine tRNA isoacceptors are observed in cells grown at either 17°C or 20°C. Thus not all tRNA populations are equally capable of giving rise to novel tRNA isoacceptors in E. coli grown at low temperatures. There are some quantitative changes in the relative threonyl isoacceptor distribution. In agreement with previous results, growth at 44°C results in the formation of novel threonine isoacceptors and as in the case of valine isoacceptors from that growth temperature, the major

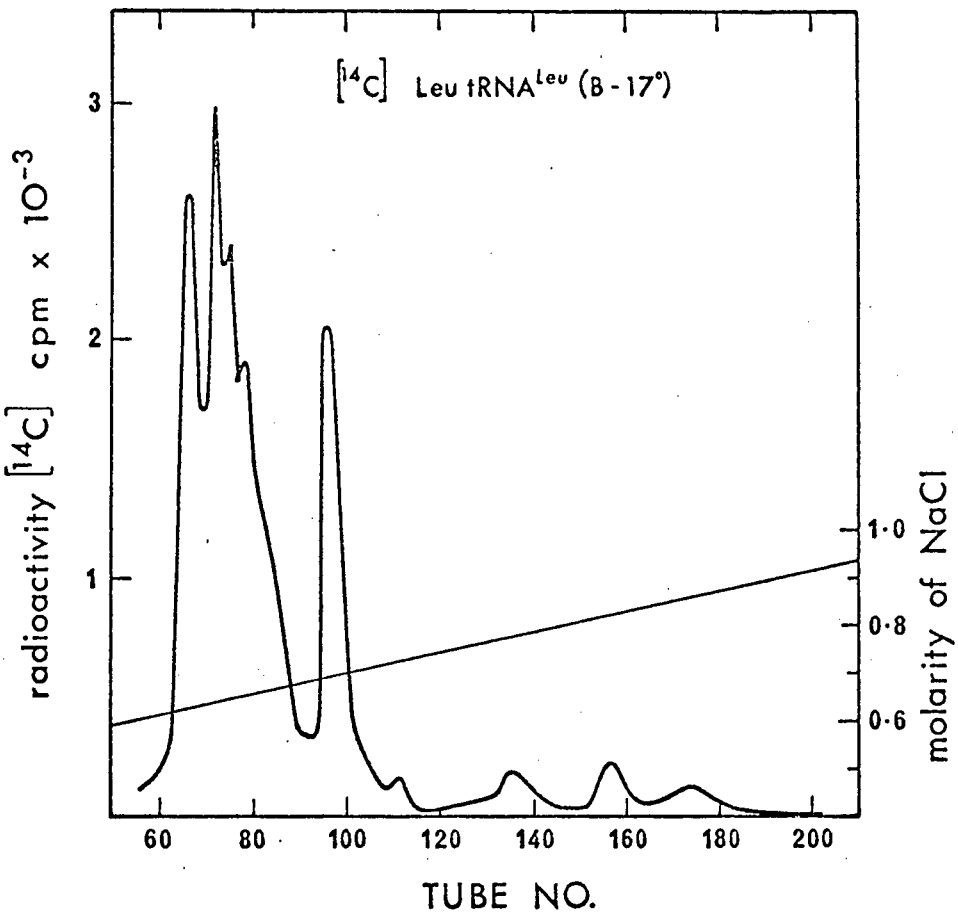


Figure 27. The RPC-5 elution profile of [^{14}C]Leu-tRNA^{Leu} (B-17°). In this experiment 6.6 A_{260} units of crude tRNA of E. coli B grown at 17°C and containing 1.32 x 10^5 cpm [^{14}C] Leu (80% counting efficiency) were run on RPC-5 as described in Fig. 16. Approximately 86% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{14}C]leucine used in the aminoacylation reaction was 294 μCi/μmole.

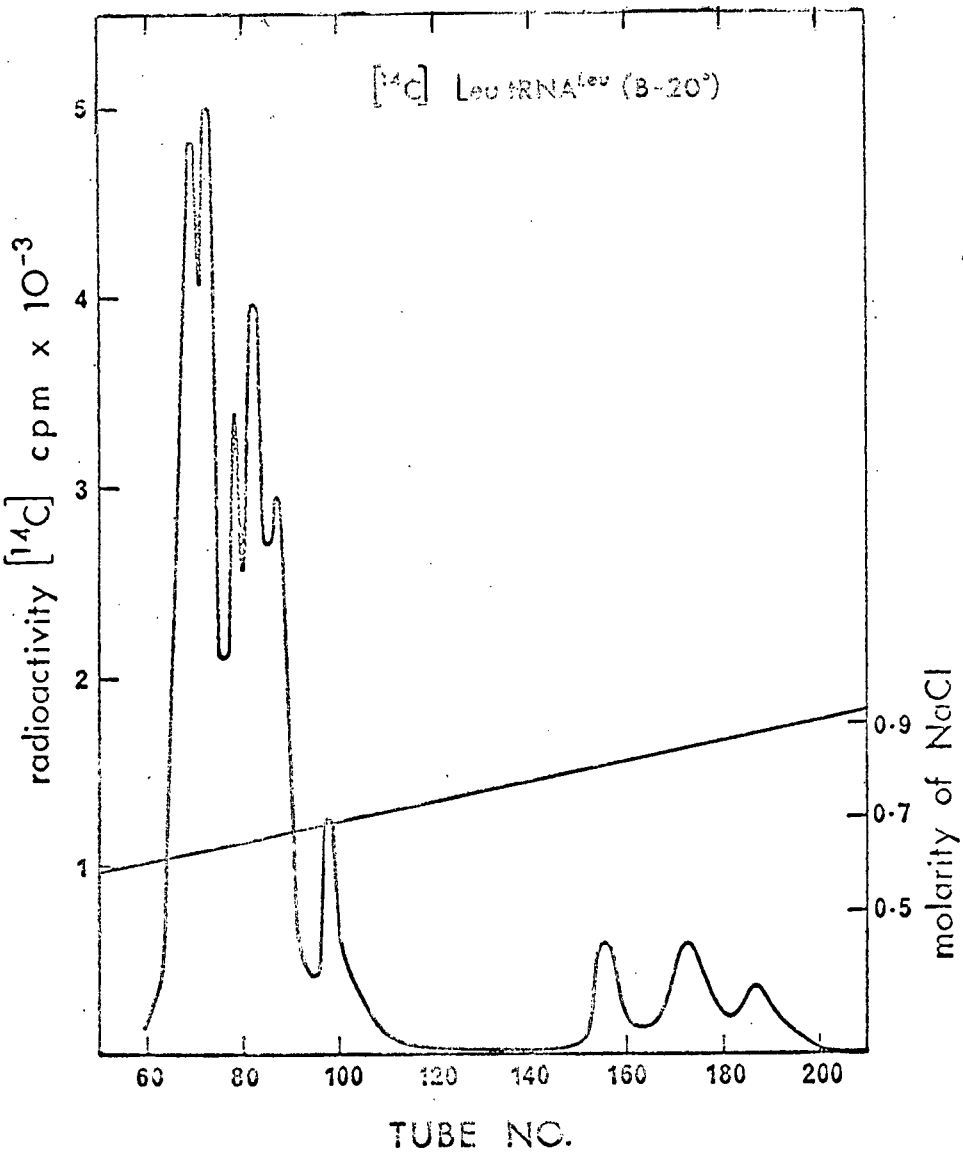


Figure 28. The RPC-5 elution profile of [^{14}C]Leu-tRNA^{Leu} (B-20°). In this experiment 7.4 A_{260} units of crude tRNA of E. coli B grown at 20°C and containing 2.7 x 10^5 cpm [^{14}C] Leu (80% counting efficiency) were applied to a standard analytical column and eluted with standard buffers within a linear NaCl gradient from 0.525 to 0.95 M. Approximately 77% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{14}C]leucine used in the aminoacylation reaction was 294 μCi/μmole.

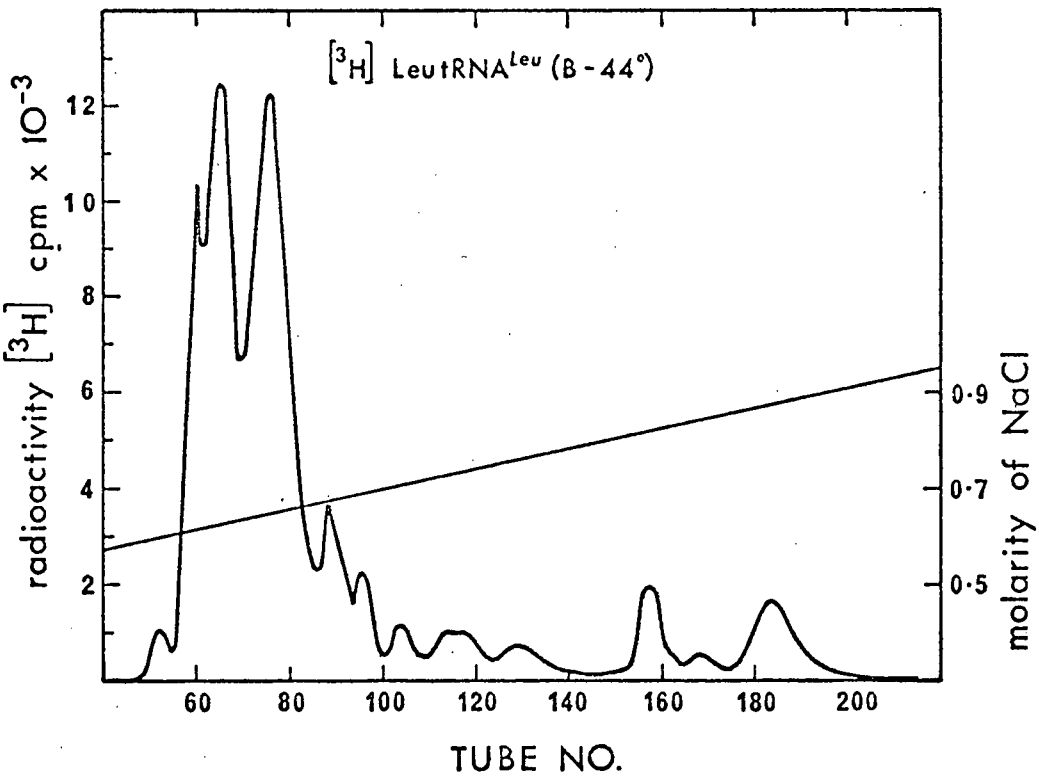


Figure 29. The RPC-5 profile of [^{3}H]Leu-tRNA^{Leu} (B-44°). In this experiment 3.0 A_{260} units of crude tRNA from E. coli B grown at 44°C and containing 10.0 x 10^5 cpm [^{3}H]Leu (37% counting efficiency) were run as described in Fig. 16. Approximately 75% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H]leucine used in the aminoacylation reaction was 5,000 μCi/μmole.

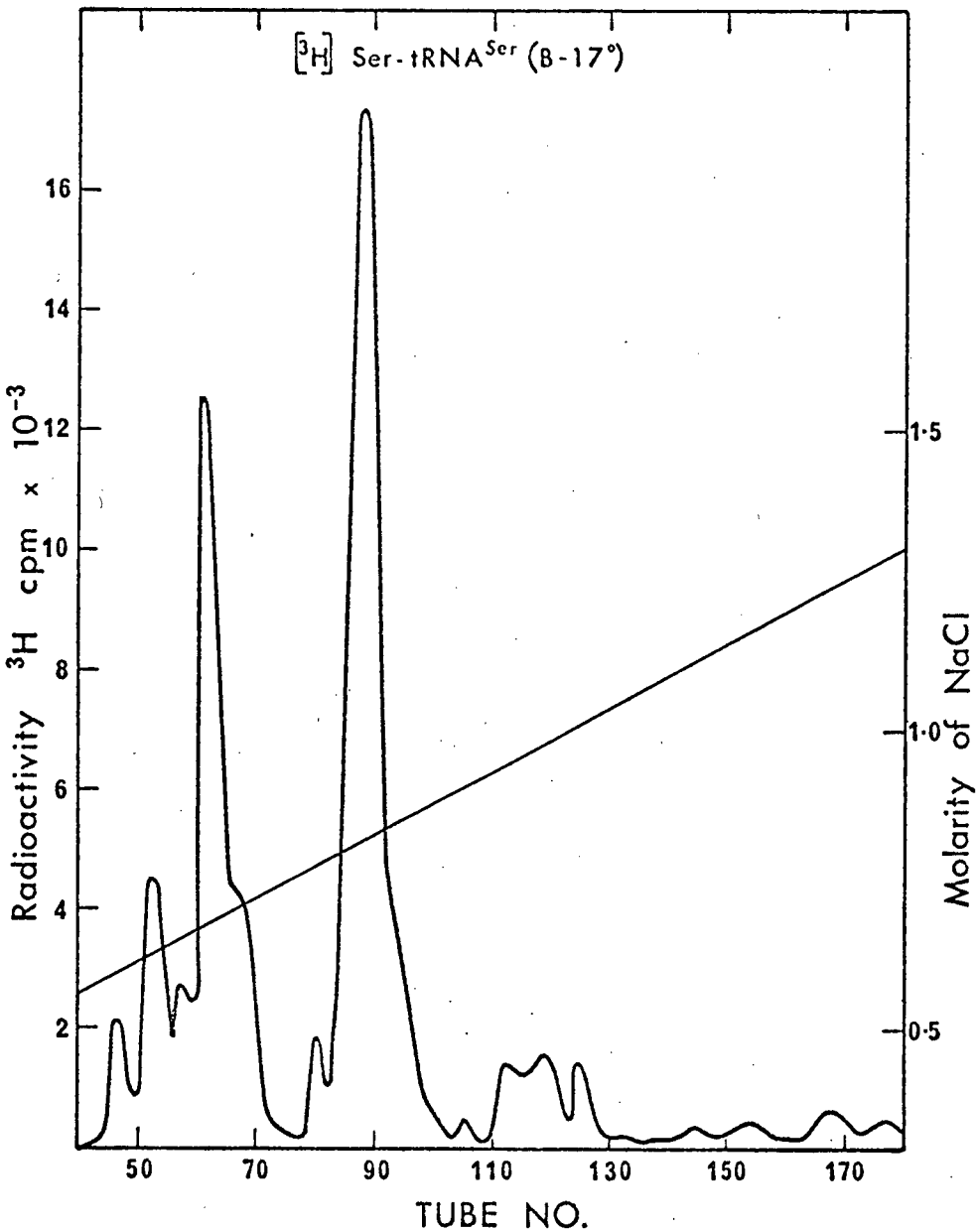


Figure 30. The RPC-5 profile of [^{3}H]Ser-tRNA^{Ser} (B-17°). In this experiment 8.0 A_{260} units of crude tRNA of E. coli B grown at 17° and containing 7.1 x 10^5 cpm (37% counting efficiency) was run as described in Fig. 11. Approximately 90% of the applied radioactivity was recovered in the gradient. The [^{3}H] serine used in the aminoacylation reaction had a specific activity of 3370 μCi/μmole.

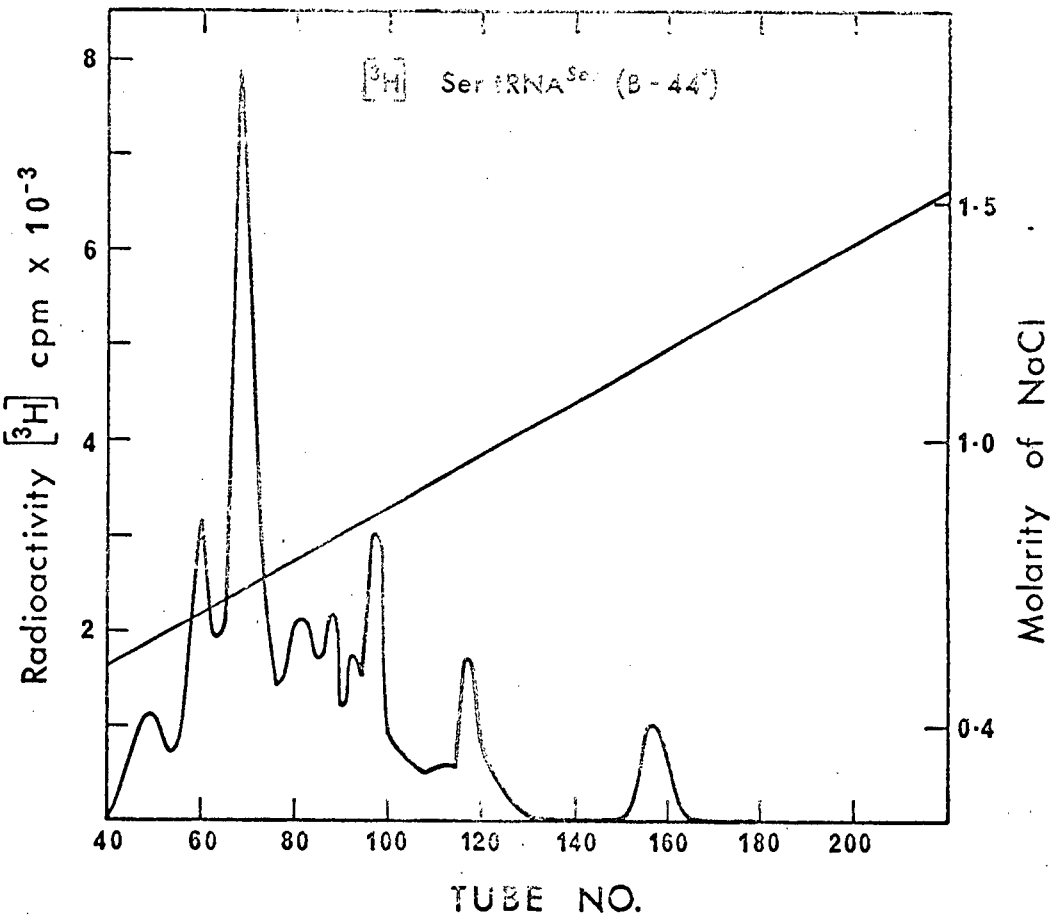


Figure 31. The RPC-5 profile of [^{3}H]Ser-tRNA^{Ser} (B-44°). In this experiment 3.0 A_{260} of crude tRNA of E. coli B grown at 44°C and containing 5.0 x 10^5 cpm [^{3}H]Ser (37% counting efficiency) was run as described in Fig. 11. Approximately 84% of the applied radioactivity was recovered in the gradient. The [^{3}H]serine used in the aminoacylation reaction had a specific activity of 3370 µCi/µmole.

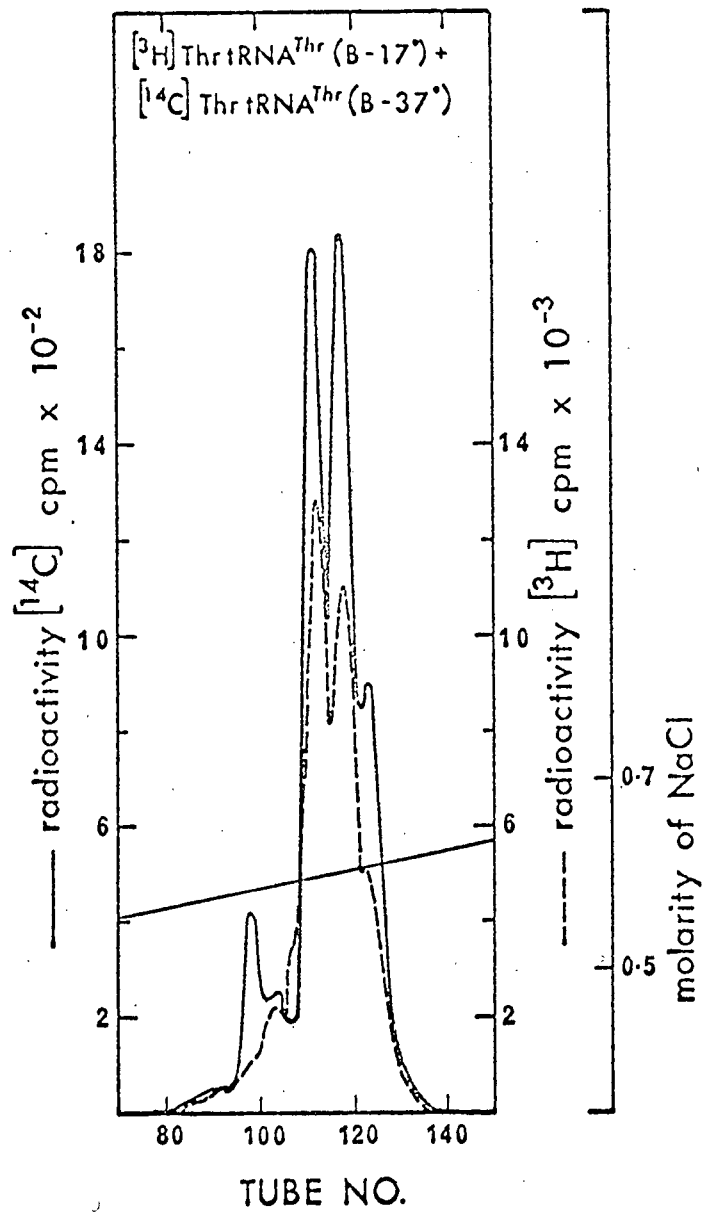


Figure 32. The cochromatography on RPC-5 of [^{14}C]Thr-tRNA^{Thr} (B-37°) and [^{3}H]Thr-tRNA^{Thr} (B-17°). In this experiment, 3.0 A_{260} units of crude tRNA of E. coli B grown at 37°C and containing 5.7 x 10^4 cpm [^{14}C]Thr (80% counting efficiency) plus 7.5 A_{260} units of crude tRNA of E. coli B grown at 17°C and containing 3.1 x 10^5 cpm [^{3}H]Thr (37% counting efficiency) were applied to a standard analytical column and eluted within a linear NaCl gradient 0.50 to 0.70 M. In the gradient, 84% of the [^{14}C] and 75% of the [^{3}H] radioactive labels were recovered. The [^{3}H]threonine and [^{14}C]threonine used in the aminoacylation reaction had specific activities of 2390 µCi/µmole and 205 µCi/µmole respectively.

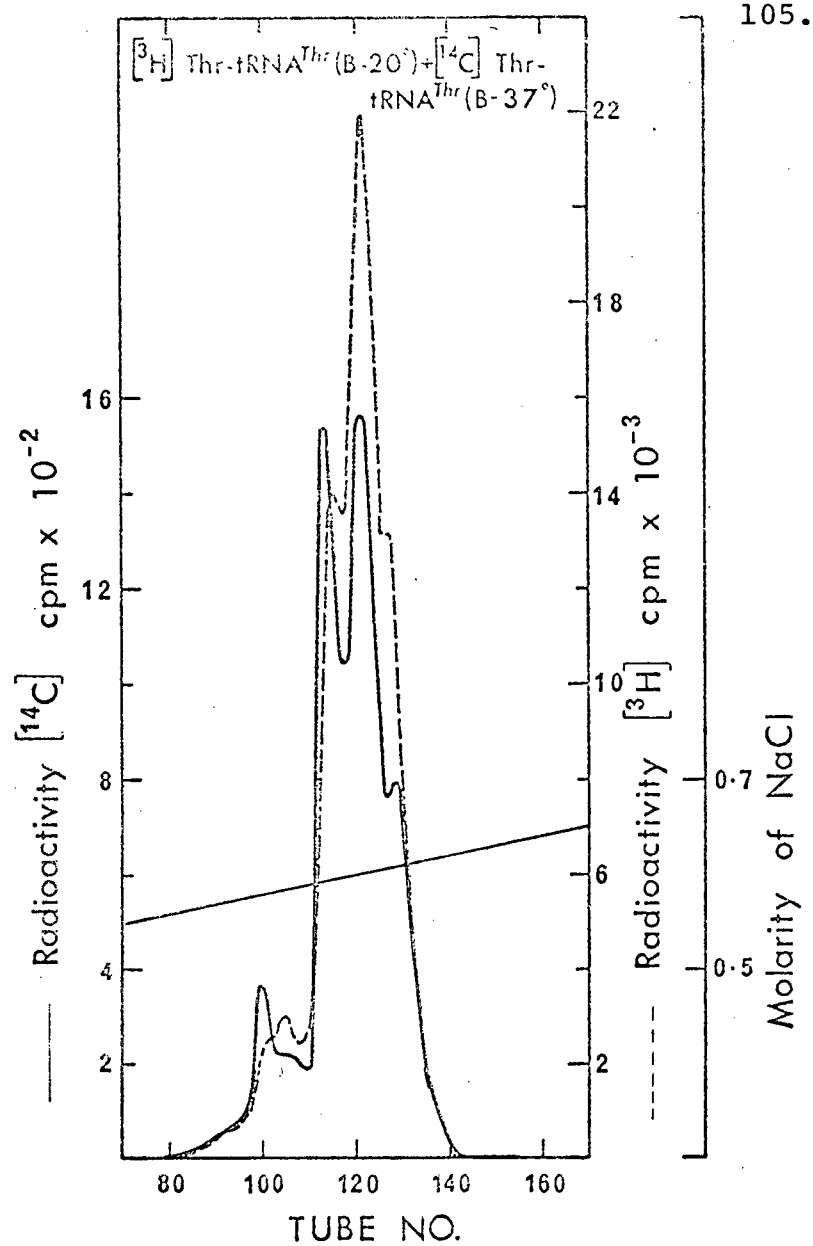


Figure 33. The cochromatography on RPC-5 of [^{14}C]Thr-tRNA^{Thr} (B-37°) and [^{3}H]Thr-tRNA^{Thr} (B-20°). In this experiment, tRNA from E. coli B grown at 37° as described in Fig. 32 plus 7.2 A_{260} units of crude tRNA of E. coli B grown at 20°C and containing 5.8 x 10^5 cpm [^{3}H]Thr (37% counting efficiency) were run on an analytical column as described in Fig. 32. In the gradient 80% of the [^{14}C] and 75% of the [^{3}H] radioactive labels were recovered. The specific activities of the amino acids used were those of Fig. 32.

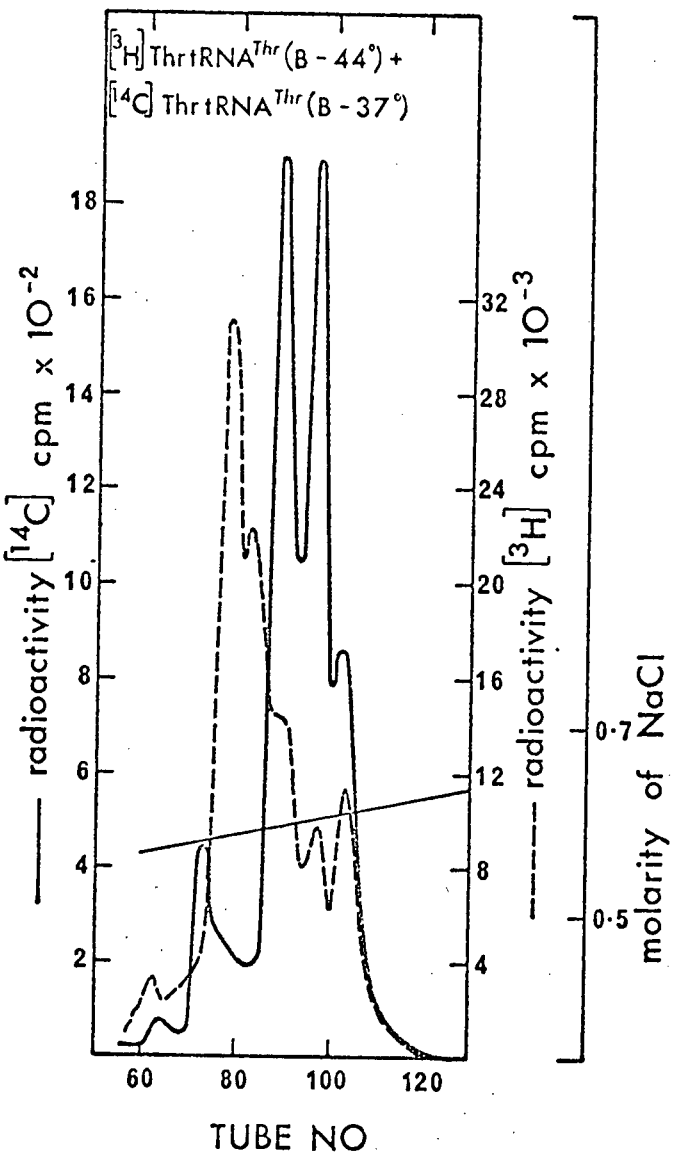


Figure 34. The cochromatography on RPC-5 of [^{14}C]Thr-tRNA^{Thr} (B-37°) and [^{3}H]Thr-tRNA^{Thr} (B-44°). In this experiment, tRNA from E. coli B grown at 37° as described in Fig.32 plus 9.85 A_{260} units of crude tRNA of E. coli B grown at 44°C and containing 6.9 x 10^5 cpm [^{3}H]Thr (37% counting efficiency) were applied to a standard analytical column and eluted within a linear NaCl gradient 0.525 to 0.725 M. In the gradient 87% of the [^{14}C] and 91% of the [^{3}H] radioactive labels were recovered. The specific activities of the amino acids used were those of Fig. 32.

threonine isoacceptors at 44°C growth temperature are not the commonly observed threonine isoacceptors of the 37°C growth temperature.

In order to investigate further the possible origin of the novel tRNA isoacceptors several experiments were planned involving supplementation of the growth media at the extreme growth temperatures with selected metal salts, amino acids etc. To speed this "screening" process these experiments were carried out in flasks in a water bath shaker as described in Methods. When E. coli B was grown up in this manner in the same media as used in the Biogen and at 17°C, no novel Val-tRNA^{Val} isoacceptors were observed. Fig. 35 shows [^{3}H]Val-tRNA^{Val} (B-12°) on RPC-5 in which E. coli B was grown at 12°C at a growth rate of 0.08 dph in a flask. Only minor differences between this profile and that characteristic of growth at 37°C in flasks are observed. Also, Val-tRNA$_3^{Val}$ is not observed in flask grown cells at 37°C. These results suggest that the novel isoaccepting tRNAs are not formed as a simple consequence of the growth rate or the growth temperature. Other factors obviously are involved. These results argue against simple temperature inactivation of tRNA modification enzymes. They suggest that the novel tRNA isoacceptors form as a result of temperature aggravation of a physiological or nutritional problem (at extremes of growth temperature).

E. coli B was grown in flasks at 41.5°C. Fig. 36 shows [^{3}H]Val-tRNA^{Val} (B-41.5°) on RPC-5. The valine isoacceptor distribution observed in flask grown cells is very much like

Figure 35. The RPC-5 elution profile of [^{3}H]Val-tRNA^{Val} (B-12°). In this experiment 4.9 A_{260} units of crude tRNA of E. coli B grown at 12°C in flasks as described in methods and containing 1.74×10^5 cpm (37% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 85% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H]valine used in the aminoacylation reaction was 1300 μCi/μmole.

Figure 36. The RPC-5 profile of [^{3}H]Val-tRNA^{Val} (B-41.5°). In this experiment 4.7 A_{260} units of crude tRNA of E. coli B grown at 41.5° in a flask and containing 4.3×10^4 cpm [^{3}H]Val (10% counting efficiency) were run as described in Fig. 5. Approximately 87% of the applied radioactivity was recovered in the gradient. The [^{3}H]valine used in the aminoacylation reaction had a specific activity of 1300 μCi/μmole.

Figure 35

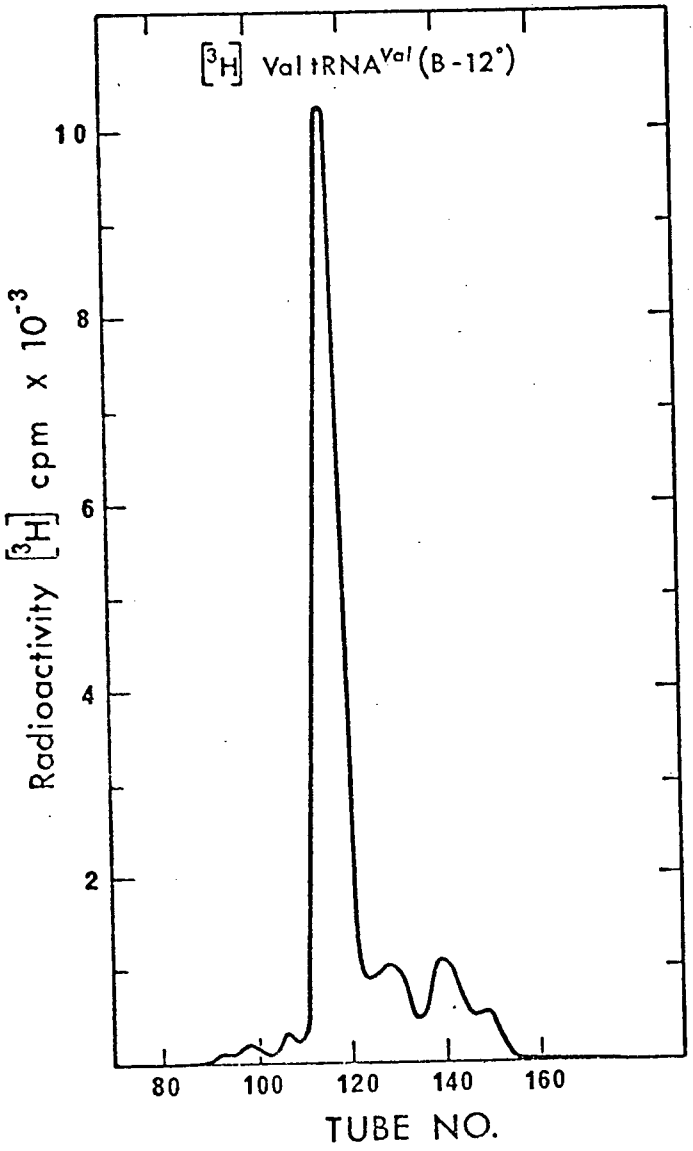
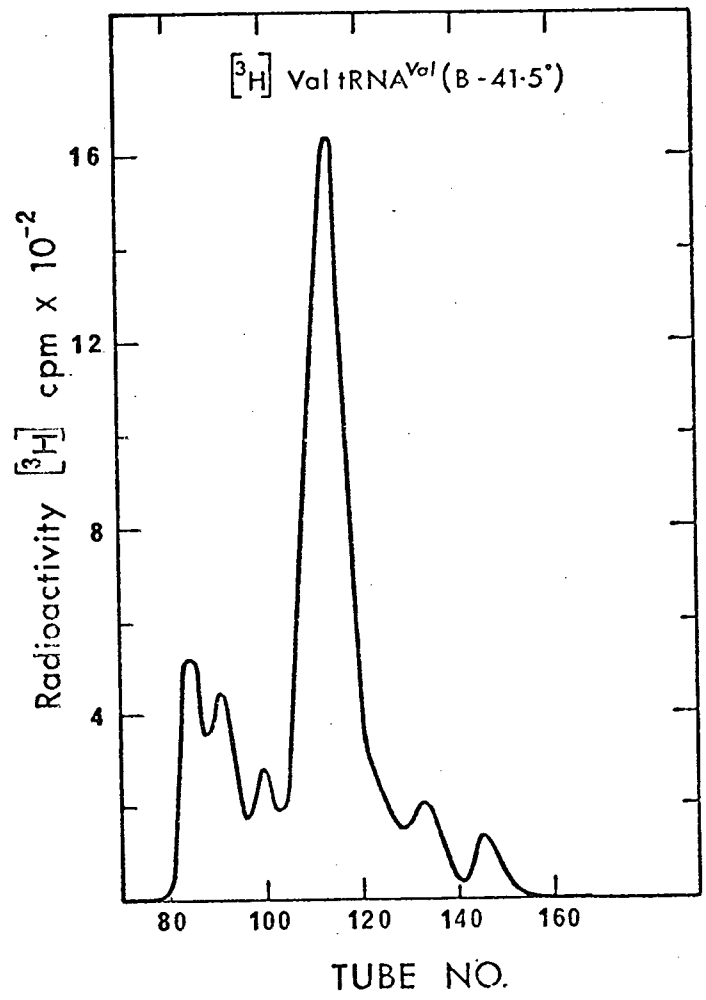


Figure 36



that observed at the similar temperature in Biogen grown cells (see Fig. 21). Although novel isoacceptors are formed at both high and low growth temperatures, in fact the same novel isoacceptors can be formed e.g. Val-tRNA$^{Val}_{3\gamma}$, the mechanisms for the formation of these novel isoacceptors are different, the low temperature results not being obtained in flask grown cultures. E. coli B was grown in flasks at 42°C on the enriched medium-YT broth. Fig. 37 shows the [^{3}H]Val-tRNA^{Val} (B-42°) profile on RPC-5. Although the growth rate was slower than for the E. coli B previously grown in flasks at 41.5°C (Fig. 36) a considerable reduction in the early eluting novel valine isoacceptors is observed. The formation of novel isoacceptors is therefore partially reversible at high temperature by supplementing the media with one or more factors. It has been reported in the literature (271) that high growth temperature (44.5°) results in a methionine deficiency. Simple methionine starvation (Fig. 9) did not give the early eluting novel valine isoacceptors. This suggests that the early eluting novel valine isoacceptors are not just simple undermethylated tRNAs (although they could be that as well as being undermodified in some other aspect). It also suggests that methionine is not the factor necessary to inhibit formation of the early eluting novel isoacceptors. Some of the data for these last experiments is summarized in Table 5. Table 5 shows that the valine to threonine acceptance ratios for tRNA from cells grown in the range 41° to 42°C are all decreased relative to the control and these ratios are in fact very similar under the slightly different growth conditions used. The

Figure 37. The RPC-5 elution profile of [^{3}H]Val-tRNA^{Val} (B-42°). In this experiment 5.8 A_{260} units of crude tRNA of E. coli B grown at 42°C in flasks on YT broth as described in methods and containing 3.8 x 10^5 cpm [^{3}H]Val (37% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 86% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H]valine used in the aminoacylation reaction was 1300 µCi/µmole.

Figure 38. The RPC-5 elution profile of [^{3}H]Val-tRNA^{Val} (W-21°). In this experiment 10.0 A_{260} units of crude tRNA of E. coli W grown at 21° and containing 6.9 x 10^5 cpm [^{3}H]Val (37% counting efficiency) were run on RPC-5 as described in Fig. 5. Approximately 88% of the applied radioactivity was recovered in the gradient. The specific activity of the [^{3}H]valine used in the aminoacylation reaction was 1300 µCi/µmole.

Figure 37

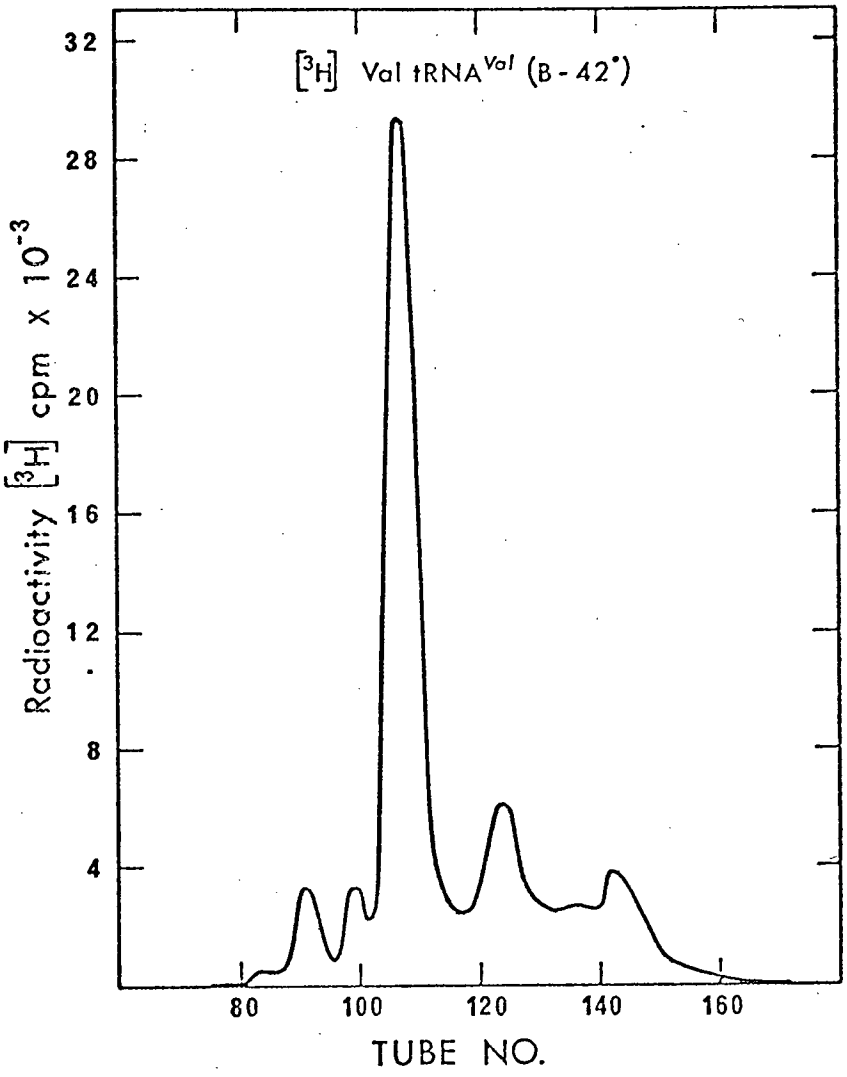


Figure 38

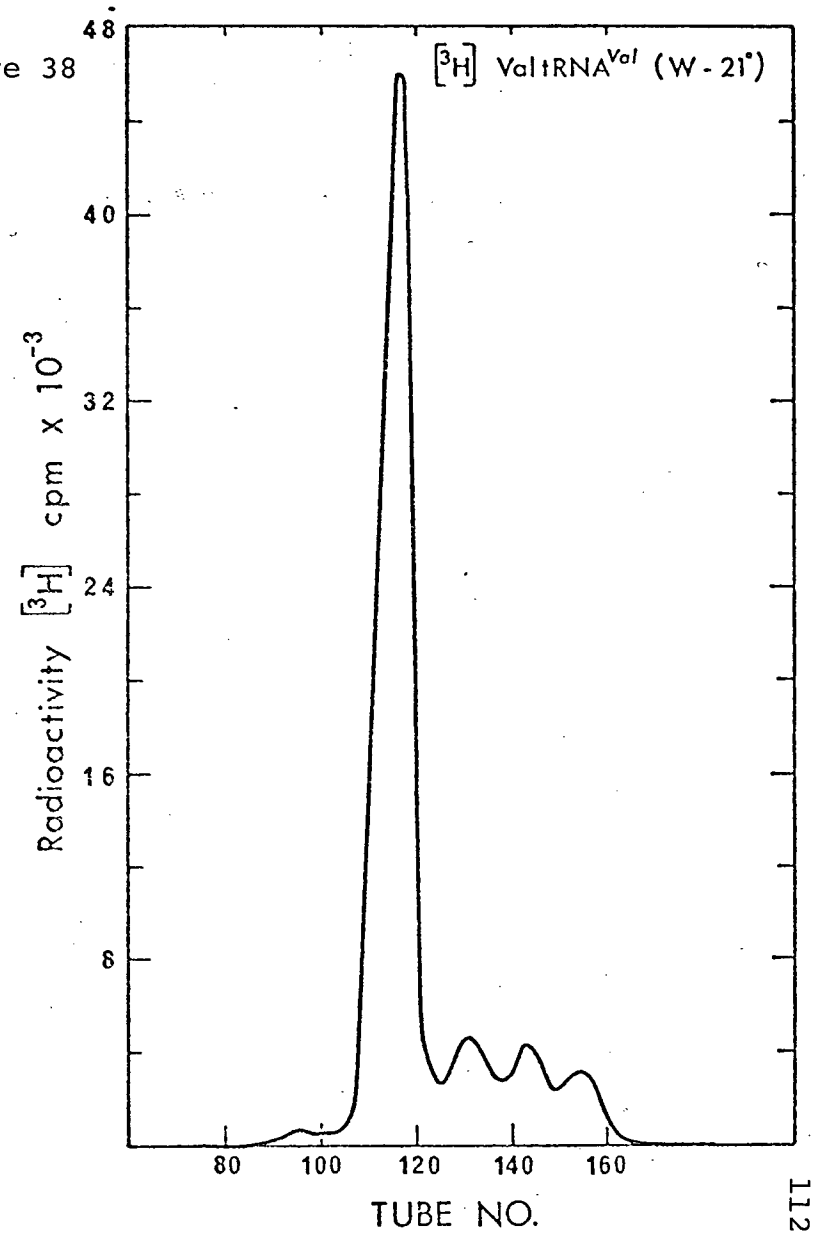


Table 5. Amino acid acceptance of crude tRNA of E. coli B cells grown at high temperature

Growth Condition	Growth Rate dph	Acceptance $\frac{[^{14}C]Val}{[^{14}C]Thr}$	Picomoles $[^{14}C]$ Thr acceptance per A_{260} unit
41°C Biogen	1.0	1.06	60
41.5°C Flask	0.43	0.97	41
42.0°C Flask + YT Broth	0.30	1.12	60
37°C Biogen	1.0	1.28	65

similarity in these ratios may suggest that there is a specific tRNA population characteristic of growth in the range 41° to 42°C.

To determine whether the different isoaccepting tRNAs are characteristic of given strains of E. coli, various strains were tested. E. coli B was obtained from an entirely different source, grown at 20°C and shown to contain the novel valine isoacceptors. E. coli W was grown in the Biogen at 21°C (0.27 dph) and the tRNA isolated and analyzed. The valine to threonine acceptance ratio was shown to be 1.23 where the crude tRNA accepted 53 picomoles of [^{14}C]threonine per A_{260} unit. This [^{3}H]Val-tRNA^{Val} (W-21°) was run on RPC-5 as shown in Fig. 38. The profile obtained is very similar to that of E. coli B grown at 37°C in a flask. None of the early eluting novel valine isoacceptors are observed. Only the late-eluting valine isoacceptor is unusual. Work must be done to determine if that isoacceptor is characteristic of the strain or the growth condition. It is interesting that a smaller but similar peak is observed for E. coli B grown in a flask at 12°C (see Fig. 35). At a growth temperature of 21°C E. coli B grown in the Biogen has many novel valine isoacceptors. It will be interesting to determine if E. coli W forms such novel isoacceptors under more adverse growth conditions.

Additional experimentation is necessary to define the mechanism(s) of formation of novel isoacceptor tRNAs at extremes of growth temperature in the Biogen.

As was indicated in Methods it was not mechanically feasible to sterilize the Biogen. All other precautions were taken to prevent contamination by other organisms and contamination was strictly monitored. In one growth experiment it was necessary to discard the cells because of this problem. The novel tRNA isoacceptors observed do not represent tRNA forms of other organisms. Relating to this problem a number of points are stressed:

(1) The inoculum was free of contaminating organisms and was obtained by growing up colonies from single cells.

(2) Growth curves were monitored. Potentially composite curves were not observed.

(3) At the time of harvesting the cells were diluted in sterile media and plated. The plates were incubated for 48 hrs and then checked for the number of colonies, colony morphology and sensitivity to phage T7.

(4) Auxotrophic strains were checked to demonstrate the absence of growth in unsupplemented media.

(5) Only some tRNAs give rise to novel isoacceptors under each adverse growth condition.

(6) Both high and low growth temperatures give the same novel valine isoacceptors.

(7) The same novel valine isoacceptors are observed at high temperature in sterilized flasks and in the nonsterilized Biogen.

All these points strongly argue against contamination being a factor in these experiments.

It is likely that extremes of growth temperature interfere with some enzyme(s) involved in the proper nutritional maintenance of the cell. Permease enzymes involved in the uptake of ions etc. from the medium are considered likely candidates. It is tempting to speculate that the extremes of growth temperature act by altering lipid structure in the membrane thereby inactivating certain proteins which are very dependent on adjacent lipids for activity. If these proteins were limiting under particular growth conditions, then the additional aggravation of growth at extremes of temperature would result in cells seriously deficient in the product of the function of these particular proteins. Such a deficiency of, e.g. particular ions, would have wide ranging effects on a number of cellular processes including modified nucleoside biosynthesis. E. coli grown in flasks at low temperature grew more quickly than E. coli grown in the Biogen at the same low temperature suggesting that one or more factors were more limiting in the Biogen than in the flask. Growth in the Biogen and in flasks differed at high temperature also in that growth in the latter was much slower at any particular high growth temperature. Conceivably certain key biosynthetic enzymes could also be very temperature dependent for activity. For example, it is known that homoserine trans-succinylase the first enzyme of the methionine biosynthetic pathway (271) is sensitive to inactivation at 44° and the resultant loss of methionine could limit the amount of methylated bases formed.

It has been shown that E. coli grown in a medium with less than 10^{-7} M iron contains novel $tRNA^{Phe}$ isoacceptors (170) as mentioned earlier. The same novel isoacceptors are observed in bacteria growing under limiting aeration where the iron is present as the insoluble hydroxide (170). Since we observe novel isoacceptors for most tRNAs including those lacking ms^2i^6A we may be observing a similar but not identical phenomenon. It has been recently demonstrated (171) that growth of E. coli in low phosphate media at slow growth rates results in novel $tRNA^{Phe}$ formation. The presence of the novel isoacceptors is dependent on both the phosphate concentration, and the growth rate. The novel isoacceptors we observe appear to be dependent on two or more factors also; growth temperature and one or more nutritional factors.

Characterization of $tRNA_3^{Val}$

Experiments previously described suggested that it was necessary to establish the identity of $tRNA_3^{Val}$ so that changes in valine isoacceptor distributions on RPC-5 could be interpreted. The first experiments towards this end involved the characterization of the 3'OH terminal [^{3}H]valine oligonucleotide obtained from RNase T_1 digestion of [^{3}H]Val-$tRNA_3^{Val}$. [^{3}H]Val-$tRNA^{Val}$ (B) and [^{3}H]Val-$tRNA^{Val}$ (162-Arg) were prepared in the standard manner and run individually on RPC-5 as described in Methods. Aliquots were counted on a scintillation counter and the radioactivity distribution on the columns determined. Based upon these results the individual Val-$tRNA_3^{Val}$,

Val-$tRNA_1^{Val}$ and Val-$tRNA_{2b}^{Val}$ isoacceptors were pooled and precipitated. The individual isoacceptors were then resuspended in buffer and treated with RNase T_1 as described in Methods. The oligonucleotides were chromatographed on DEAE-cellulose and eluted with an ammonium formate gradient as described in Methods. The sequences of the common valine isoacceptors (208) are such that the valine isoacceptors 2a and 2b both give rise to a 3'OH terminal pentanucleotide fragment (CpApCpCpA) while the major valine isoacceptor 1 yields a 13 nucleotide 3'OH terminal fragment (UpCpApUpCpApCpCpCpApCpCpA) upon treatment with RNase T_1. These are easily separated on DEAE-cellulose as shown in Fig. 39. The pentanucleotide fragment elutes at approximately 0.09 M ammonium formate pH 4.5 i.e. about tube 70 in Fig. 39 and the 13 nucleotide fragment elutes at about 0.20 M ammonium formate pH 4.5 i.e. about tube 150 in Fig. 39. The 3'OH terminal fragment of $tRNA_3^{Val}$ appears to be the same size as the fragment from $tRNA_1^{Val}$. At pH 4.5, RNase T_1 cleaves slowly after As, especially those that are in single stranded regions of RNA and hence easily accessible (R.C. Warrington-unpublished results). The first major peak off the columns of Fig. 39 is probably CpCpA-[^{3}H]Val. Evidence from chromatography of this early eluting material on DEAE-cellulose urea columns suggests that this fragment cannot be any larger than a trinucleotide (based upon its calculated charge). At higher RNase T_1 levels, additional cleavages after As are observed for [^{3}H]Val-$tRNA_1^{Val}$ giving rise to two additional [^{3}H]Val-oligonucleotide fragments of probable sequence UpCpApCpCpCpApCpCpA and CpCpCpApCpCpA.

Figure 39. DEAE-cellulose chromatography of RNase T_1 digests of individual [^{3}H]Val-tRNA^{Val} isoacceptors. Individual isoacceptors were prepared as described in Methods and incubated with RNase T_1 as indicated.

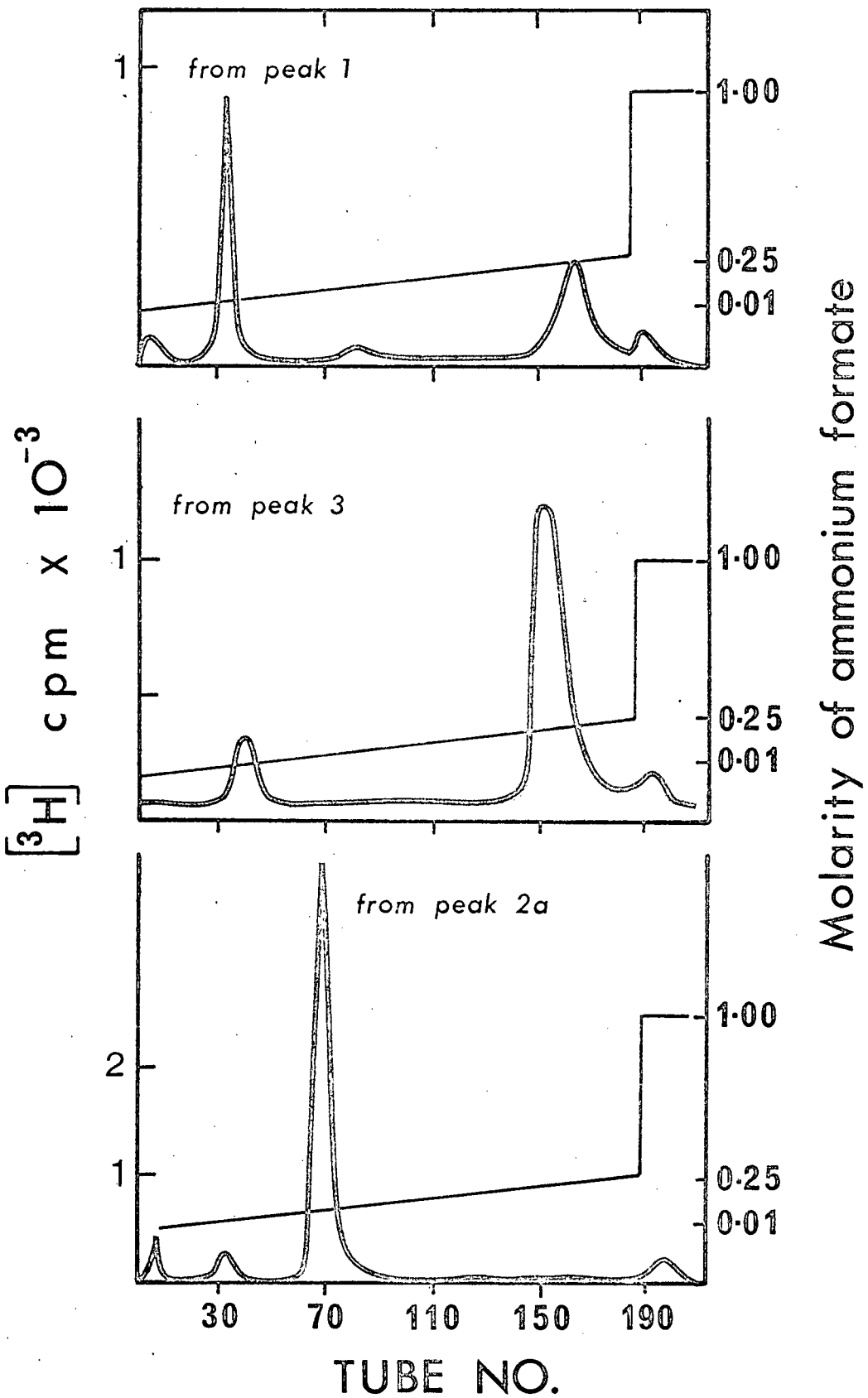
Top: 8.0×10^4 cpm [^{3}H]Val-tRNA$_1^{Val}$ (10% counting efficiency) in a total volume of 1.8 ml of digestion buffer to which was added 100 Sankyo units of RNase T_1. The mixture was incubated for 4 hrs at 37°C and run on the column as described in Methods.

Middle: 6.5×10^4 cpm [^{3}H]Val-tRNA$_3^{Val}$ (10% counting efficiency) in a total volume of 1.5 ml of digestion buffer to which was added 50 Sankyo units of RNase T_1. The mixture was incubated for 4 hrs at 37°C and run on the column as described in Methods.

Bottom: 14.8×10^4 cpm [^{3}H]Val-tRNA$_{2a}^{Val}$ (10% counting efficiency) in a total volume of 1.5 ml of digestion buffer to which was added 50 Sankyo units of RNase T_1. The mixture was incubated for 4 hrs at 37° and run on the column as described in Methods.

Figure 39

[^{3}H] Val-RNase T_1 Oligonucleotides on DEAE cellulose



In an analogous experiment, it has been shown that the same two additional fragments can be obtained from [^{3}H]Val-tRNA$_3^{Val}$. This strongly suggests that not only are the 3' terminal oligonucleotides of RNase treated valine isoacceptors 1 and 3 identical in size, they probably also have an identical sequence. This in turn argues that they are both transcribed from the same gene(s) and that Val-tRNA$_3^{Val}$ is probably an undermodified form of Val-tRNA$_1^{Val}$.

Purification of tRNA$_3^{Val}$

To test this possibility further, the nucleoside analysis of tRNA$_3^{Val}$ was required. Relatively pure tRNA$_3^{Val}$ was isolated as described in Methods. The RPC-5 elution profile for absorbance at 260 nm and [^{14}C]valine acceptance are shown in Fig. 40 for the final step of the purification procedures.

The column was assayed by aminoacylating 25 µl aliquots from each tube in a standard charging experiment having a total reaction volume of 100 µl. Aliquots of 50 µl were pipetted onto filter paper discs after 20 min incubation and the acid insoluble [^{14}C]valine determined. The charging conditions used insured complete saturation of the tRNA with the labelled amino acid. The specific activity of the [^{14}C]valine used in the aminoacylation reaction was 209 µCi/µmole. The efficiency of radioactive counting of the [^{14}C]valine on filter paper discs was found to be approximately 60% at the channel settings used.

The specific activity of the peak fraction #95 was 1733 picomoles of [^{14}C]valine acceptance per A_{260} unit. Based upon

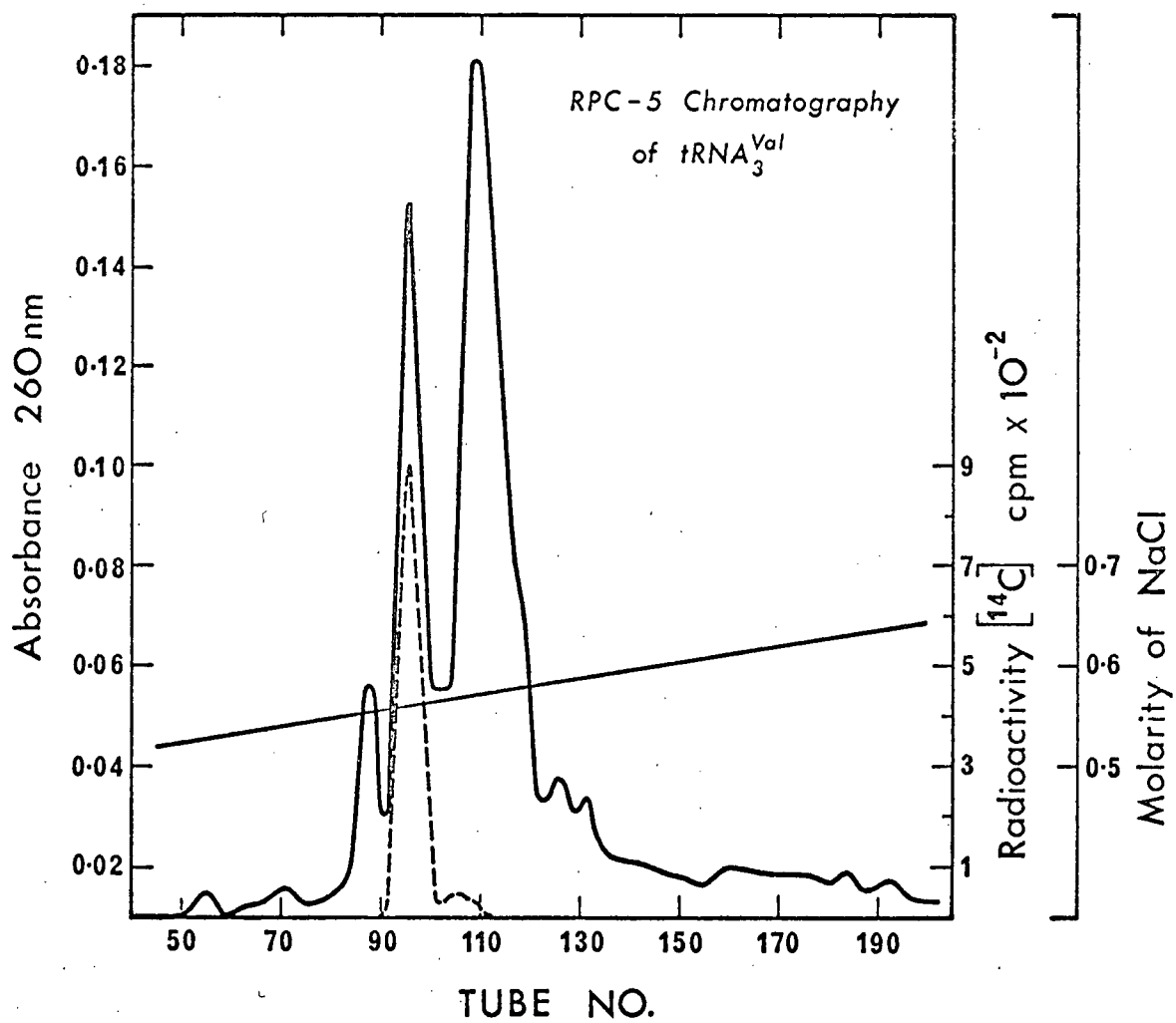


Figure 40. Final step in the purification of $tRNA_3^{Val}$. In this experiment 47.8 A_{260} units of tRNA enriched for $tRNA_3^{Val}$ were run on a 2.4 x 45 cm RPC-5 column (50 mesh sieve size) and eluted with 2 l. of buffer containing 10 mM tris-HCl pH 7.0, 1 mM 2-mercaptoethanol within a linear NaCl gradient of 0.50 to 0.65. The fraction size was 10 ml and the column flow rate was 180 ml/hr. The solid line indicates the absorbance profile at 260 nm while the dotted line refers to the [^{14}C]valine acceptance in a standard aminoacylation reaction.

the distribution of amino acid acceptance in relation to the absorbance at 260 nm, tubes 91 to 100 inclusive were pooled and considered a pure sample of $tRNA_3^{Val}$. The total yield was 11.4 A_{260} units after precipitation with ethanol and collection of the precipitate by Millipore filtration.

Spectral Characteristics of $tRNA_3^{Val}$

The $tRNA_3^{Val}$ was dissolved in 1.0 ml of distilled H_2O and an absorbance spectrum determined as shown in Fig. 41. The spectrum was compared with spectra of commercial crude E. coli tRNA and crude tRNA of control E. coli B growth experiments. Significant spectral differences were observed in the 330 to 340 nm region. Absorbance in this region is due to the presence of the minor nucleoside s^4U. The absence of any specific absorbance peak in this region in purified $tRNA_3^{Val}$ strongly suggests that s^4U is not present in $tRNA_3^{Val}$. A significant absorption is seen in this region for both commercial tRNA and E. coli B crude tRNA. The A_{335} to A_{260} ratios for these latter two samples are similar to those reported in the literature (173,301).

Qualitative Nucleotide Analysis of $tRNA_3^{Val}$

In a subsequent experiment 3.0 A_{260} units of the purified $tRNA_3^{Val}$ were RNase treated and a preliminary nucleotide analysis carried out as described in Methods. As shown in Fig. 42, present are the nucleotides Tp, Ap, Cp, Gp, Up, m^7Gp, Ψp, a breakdown product of m^7Gp (B.N. White-personal communication) and the nucleoside A of the 3' terminal CpCpA sequence. Present in any

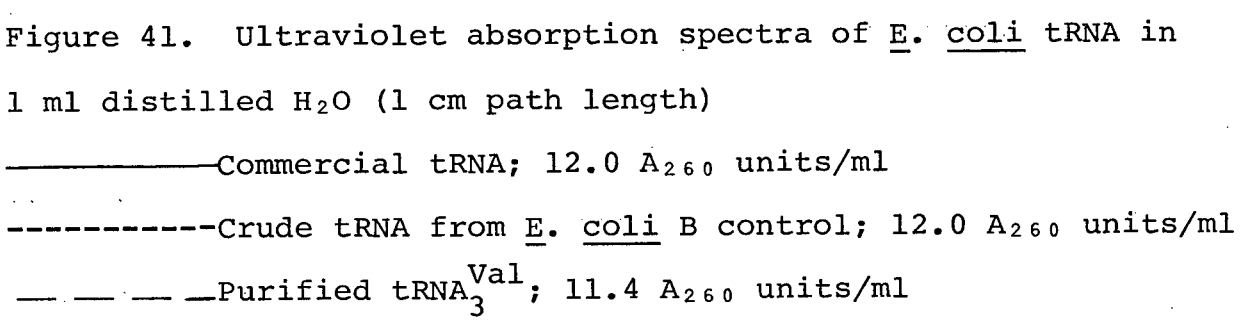


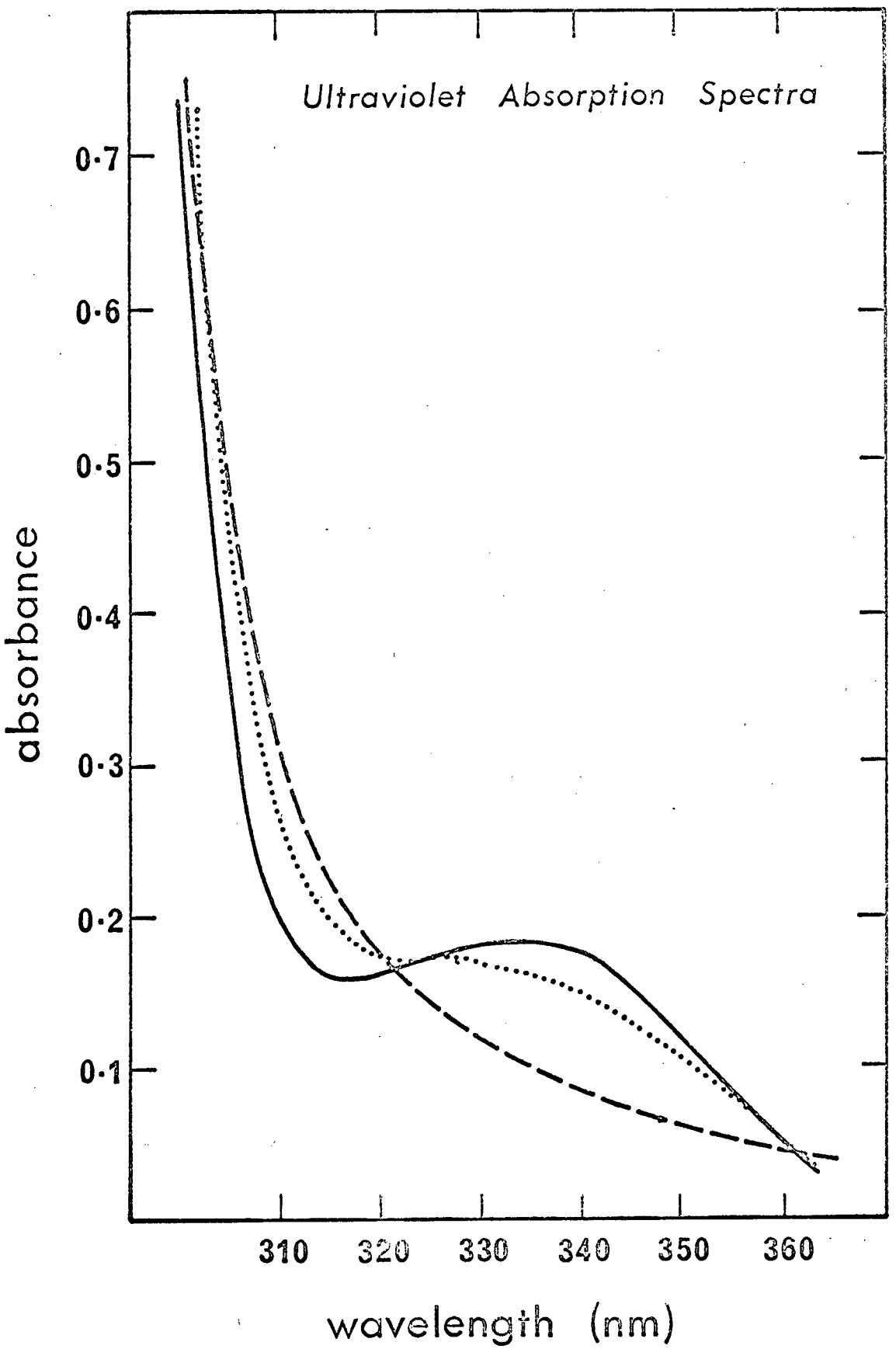
Figure 41. Ultraviolet absorption spectra of E. coli tRNA in 1 ml distilled H_2O (1 cm path length)

————————Commercial tRNA; 12.0 A_{260} units/ml

----------Crude tRNA from E. coli B control; 12.0 A_{260} units/ml

— — — —Purified $tRNA_3^{Val}$; 11.4 A_{260} units/ml

Figure 41


Ultraviolet Absorption Spectra
absorbance
0·7
0·6
0·5
0·4
0·3
0·2
0·1
310
320
330
340
350
360
wavelength (nm)

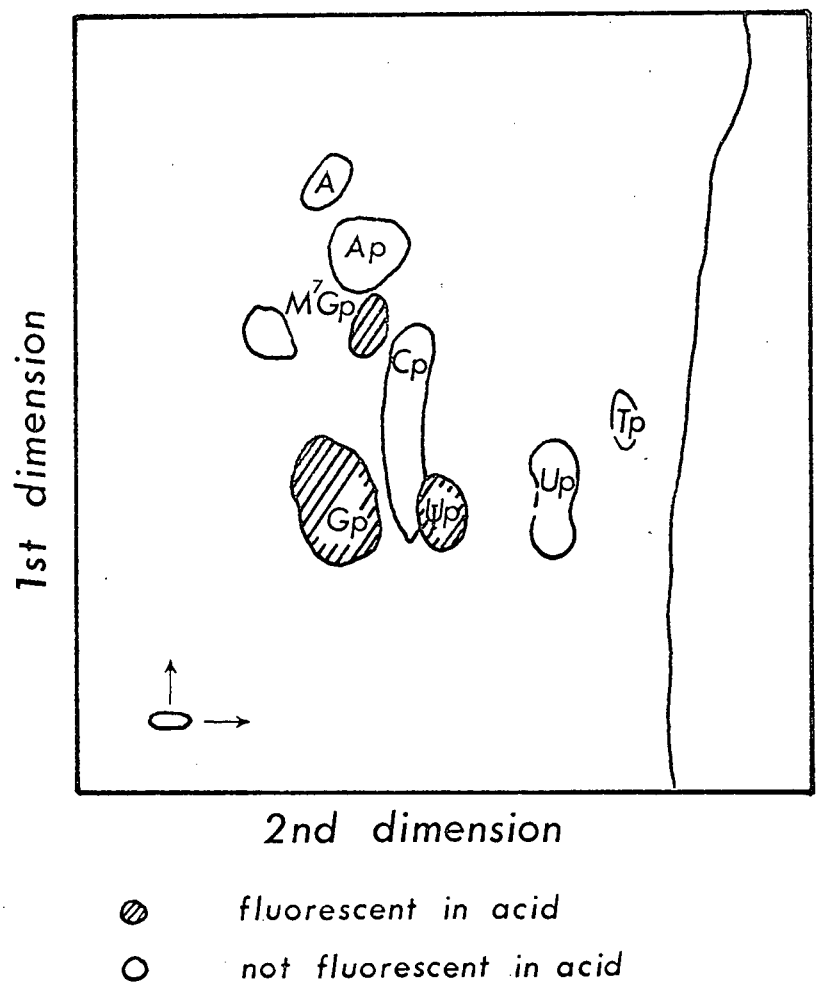


Figure 42. Two dimensional thin layer chromatogram of nucleotide digest of $tRNA_3^{Val}$. 3.0 A_{260} of digested material was applied to 10 cm x 10 cm cellulose on glass plates and chromatographed as described in Methods.

significant amounts are the nucleotides s^4Up and m^6Ap. It is not possible to comment on the presence or absence of Vp (the nucleotide of uridine-5-oxyacetic acid) or hUp because of the distortion of the Up spot particularly in the second dimension of chromatography. The absence of s^4Up confirms the earlier results of spectral analysis. Quantitative nucleoside analysis was subsequently performed by the Randerath tritium labelling technique.

Randerath Nucleoside Analysis of $tRNA_3^{Val}$

The total nucleoside analysis of $tRNA_3^{Val}$ was carried out by Randerath tritium labelling techniques as described in Methods. For comparison purposes, the nucleoside composition of a gift of purified $tRNA_1^{Val}$ was determined in a parallel experiment. Fig. 43 shows the results of the fluorograms of these experiments. Several qualitative differences are noted. $tRNA_3^{Val}$ lacks the nucleosides m^6A and V (uridine-5-oxyacetic acid) present in $tRNA_1^{Val}$ and X (1-[3-amino-3-carboxypropyl]uridine), present in $tRNA_{2a}^{Val}$ and $tRNA_{2b}^{Val}$. The reacted nucleoside spot for V was eluted and shown to have one negative charge by paper electrophoresis at pH 7.0. Differences in the uridine spots are due to the use of different batches of thin layer chromatography plates. Such differences have been noted previously in the literature (304). Individual spots were cut out and the radioactive nucleoside triols eluted and counted as described by Randerath. A quantitative nucleoside analysis was obtained for $tRNA_1^{Val}$ and $tRNA_3^{Val}$ as shown in Table 6. The experimental

Figure 43. Randerath nucleoside analysis of $tRNA_1^{Val}$ (A) and $tRNA_3^{Val}$ (B). Randerath nucleoside analysis was carried out as described in methods. Each TLC plate was spotted with 10 µl of the final reaction mix (.06 to .10 A_{260} units) and eluted first in solvent F and subsequently in solvent G (260). The fluorograms were developed after 72 hours exposure.

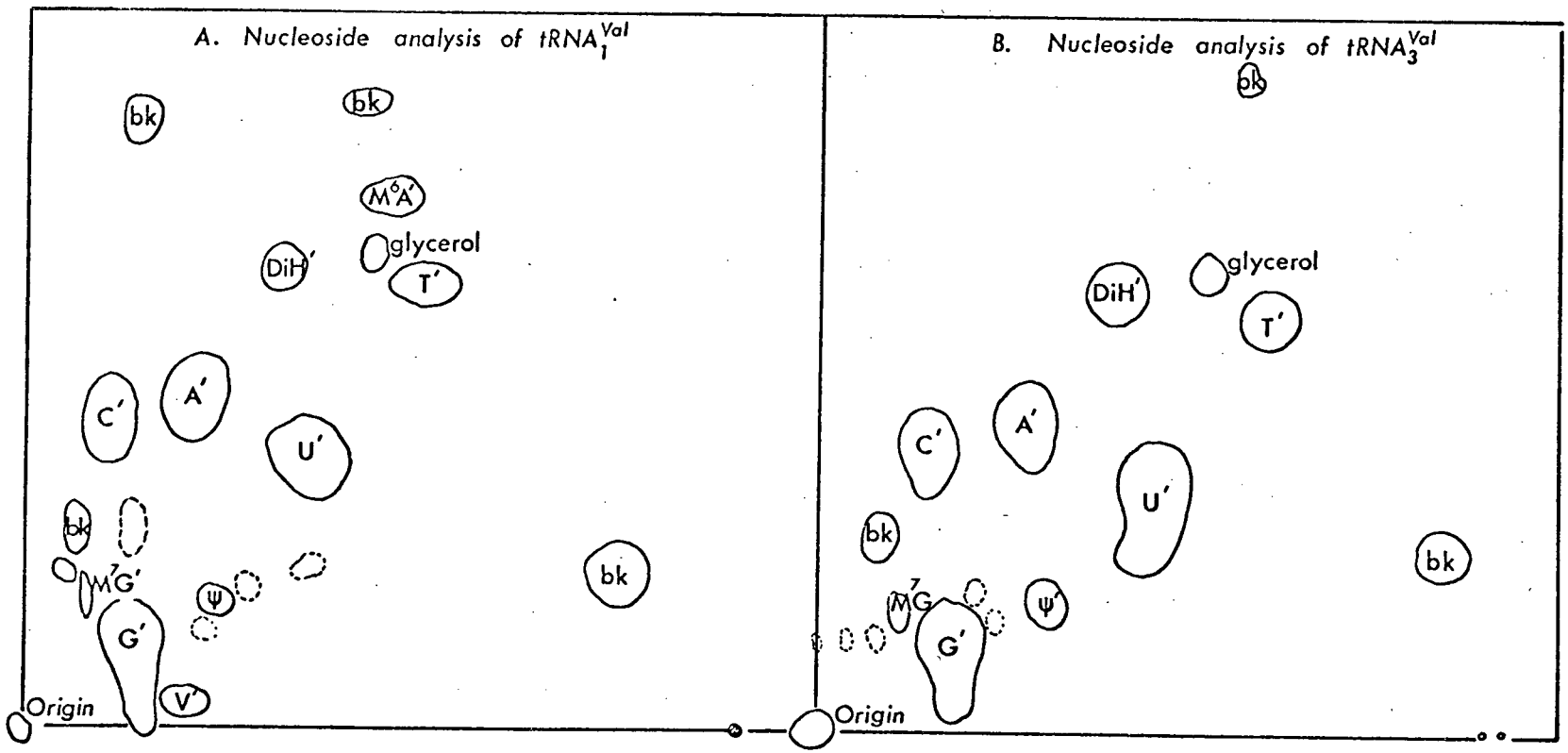


Table 6. Nucleoside Analysis of $tRNA^{Val}$

Nucleoside	1 predicted	2a predicted	2b predicted	3 predicted	3 observed	1 observed
A	14	13	16	15	13.8	14.0
C	23	21	18	23	23.0	23.1
G	23	25	22	23	22.8	22.1
U + s^4U	10	10	13	11	11.5	10.4
T	1	1	1	1	1.0	0.8
Ψ	1	1	1	1	0.9	0.9
hU	1	4	4	1	2.8	1.0
m^7G	1	1	1	1	0.6	0.6
m^6A	1	-	-	-	-	0.8
V	1	-	-	-	-	0.9
X	-	1	1	-	-	-

V - uridine-5-oxy-acetic acid
X - 1-(3-amino-3-carboxypropyl) uridine

predicted: means the # of nucleoside residues per tRNA molecule based upon the known or postulated sequence.

observed: means the # of nucleosides experimentally determined based upon the average of three measurements and calculated for a tRNA molecule of 76 nucleoside residues.

values reported are the average of three determinations. The m^7G spot has not been corrected for degradation to glycerol. The chemistry of the Randerath technique is such that s^4U is degraded to U during the course of the reaction. It has been shown previously, however, that there is little or no s^4U in $tRNA_3^{Val}$. The experimentally determined nucleoside analysis of $tRNA_1^{Val}$ is very close to that predicted based upon the known sequence. The only significant difference is that there is approximately one less G residue per tRNA than predicted. The accuracy of the determination of the nucleoside content of $tRNA_1^{Val}$ increases confidence in the experimental values obtained for $tRNA_3^{Val}$. A column in Table 6 has been called 3 predicted. This column refers to the anticipated nucleoside composition if $tRNA_3^{Val}$ is an undermodified form of $tRNA_1^{Val}$ lacking the modified nucleoside s^4U, m^6A and V (uridine 5-oxyacetic acid) and having instead U, A and U respectively at those positions. As can be seen from the table, the data for C, G, U, T, m^7G, m^6A and ψ of $tRNA_3^{Val}$ experimental is consistent with $tRNA_3^{Val}$ predicted as previously defined except that $tRNA_3^{Val}$ has approximately one too few A residues and nearly two extra hU residues (2.9, 2.7, 2.8 average of 2.8). The hU data would argue that $tRNA_3^{Val}$ is a precursor of either $tRNA_{2a}^{Val}$ or $tRNA_{2b}^{Val}$. However, the data for $tRNA_3^{Val}$ for A residues is very different from that for $tRNA_{2b}^{Val}$ predicted while the data for G residues is very different from that for $tRNA_{2a}^{Val}$ predicted. The nucleoside analysis of $tRNA_3^{Val}$ experimental seems most similar to that for $tRNA_3^{Val}$ predicted.

The RNase T_1 3'OH terminal oligonucleotide analysis indicated that $tRNA_3^{Val}$ had a terminal oligonucleotide of the same size and sequence as $tRNA_1^{Val}$. Subsequent nucleoside and nucleotide analysis showed that $tRNA_3^{Val}$ differs in total nucleoside composition from $tRNA_1^{Val}$ by the absence of the modified nucleosides s^4U, V and m^6A. It appears that $tRNA_3^{Val}$ could be an undermodified form of $tRNA_1^{Val}$ which accumulates under adverse growth conditions.

However, a possibility which is not eliminated by the present data is that $tRNA_3^{Val}$ is transcribed from a unique gene which is very similar to that for $tRNA_1^{Val}$ and which is derepressed during adverse growth conditions. This possibility should be disproved by further experimentation.

tRNA of E. coli B str^D

It has been reported in the literature (320) that streptomycin dependent E. coli have an altered amino acid metabolism such that they excrete up to 10% of their total carbon source as valine. Any theory which includes some form of amino acid control of tRNA biosynthesis would predict changes in the tRNA population of such an organism. If valine is part (e.g. Val-$tRNA^{Val}$) of a feedback repressor complex for $tRNA^{Val}$ then a decrease in the [^{14}C]valine to [^{14}C]threonine acceptance ratio is predicted for streptomycin dependent E. coli. The streptomycin dependent E. coli B strain was grown up in one liter flasks as described in methods and the tRNA analyzed. As shown in Table 7 very dramatic differences in the amino acid acceptance

Table 7. Amino acid acceptance of crude tRNA of *E. coli* B str^D

E. coli strain	Growth Rate dph	Acceptance $\frac{[^{14}C]Val}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Leu}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Ser}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Pro}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Tyr}{[^{14}C]Thr}$	Acceptance $\frac{[^{14}C]Arg}{[^{14}C]Thr}$	Picomoles of $[^{14}C]$threonine Acceptance per A_{260} unit
B	1.0	1.28	2.10	1.19	1.57	0.65	2.00	65
B str^D	0.40	0.63	2.65	1.33	2.10	0.65	3.10	54

ratios relative to the control parent strain were observed. Of all the acceptance ratios measured, only that for tyrosine remained unaltered. A 51% decrease in the [^{14}C]valine to [^{14}C] threonine acceptance ratio was observed. In contrast, a 55% increase in the [^{14}C]arginine to [^{14}C]threonine acceptance was noted. Since many acceptance ratios changed it is difficult to rationalize how any one of them was changed specifically. It is interesting that such a variety of differences are all the result of a single point mutation. Since such large changes in amino acid acceptance ratios were observed it was important to determine the tRNA isoacceptor distributions on RPC-5. Figs. 44-46 show the results of such experiments. A comparison of the RPC-5 elution profiles of [^{3}H]Val-tRNA^{Val}, [^{14}C]Leu-tRNA^{Leu} and [^{3}H]Ser-tRNA^{Ser} of E. coli B str^{D} with the corresponding profiles for E. coli B (figures 1, 16 and 11 respectively) suggests that, while the corresponding profiles are not identical, they are remarkably similar. The individual isoacceptor contribution to the total acceptance was in each instance determined by measuring the peak area of each isoacceptor. Although a 51% decrease in the [^{14}C]valine acceptance to [^{14}C]threonine acceptance ratio was noted earlier, on RPC-5 there are only small differences in the relative distribution of the major valine isoacceptors 1, 2a and 2b. None of the minor valine isoacceptor 3 is observed in [^{3}H]Val-tRNA^{Val} E. coli B str^{D} tRNA. Similarly, while a 26% increase in the [^{14}C]leucine acceptance to [^{14}C] threonine acceptance ratio is observed, only slight differences in the leucine isoacceptor distribution on RPC-5 are noted.

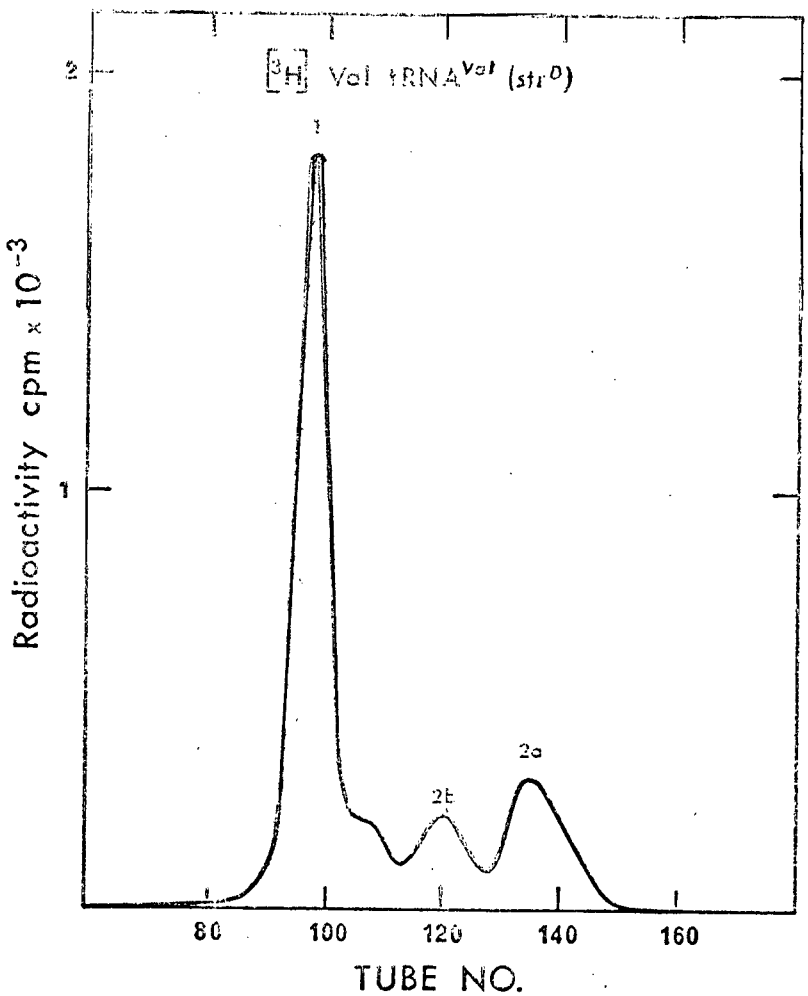


Figure 44. The RPC-5 elution profile of [^{3}H]Val-tRNA^{Val} E. coli B str^D. In this experiment 5.9 A_{260} units of crude E. coli B str^D tRNA containing 5.7 x 10^4 cpm [^{3}H]Val (10% counting efficiency) were applied to a standard analytical column and eluted with the standard buffers within a linear NaCl gradient of 0.50 to 0.65 M. In the gradient 2.5 x 10^4 cpm (5% counting efficiency) was recovered i.e. 88% of that applied. The [^{3}H]valine used in the aminoacylation reaction had a specific activity of 1300 µCi/µmole.

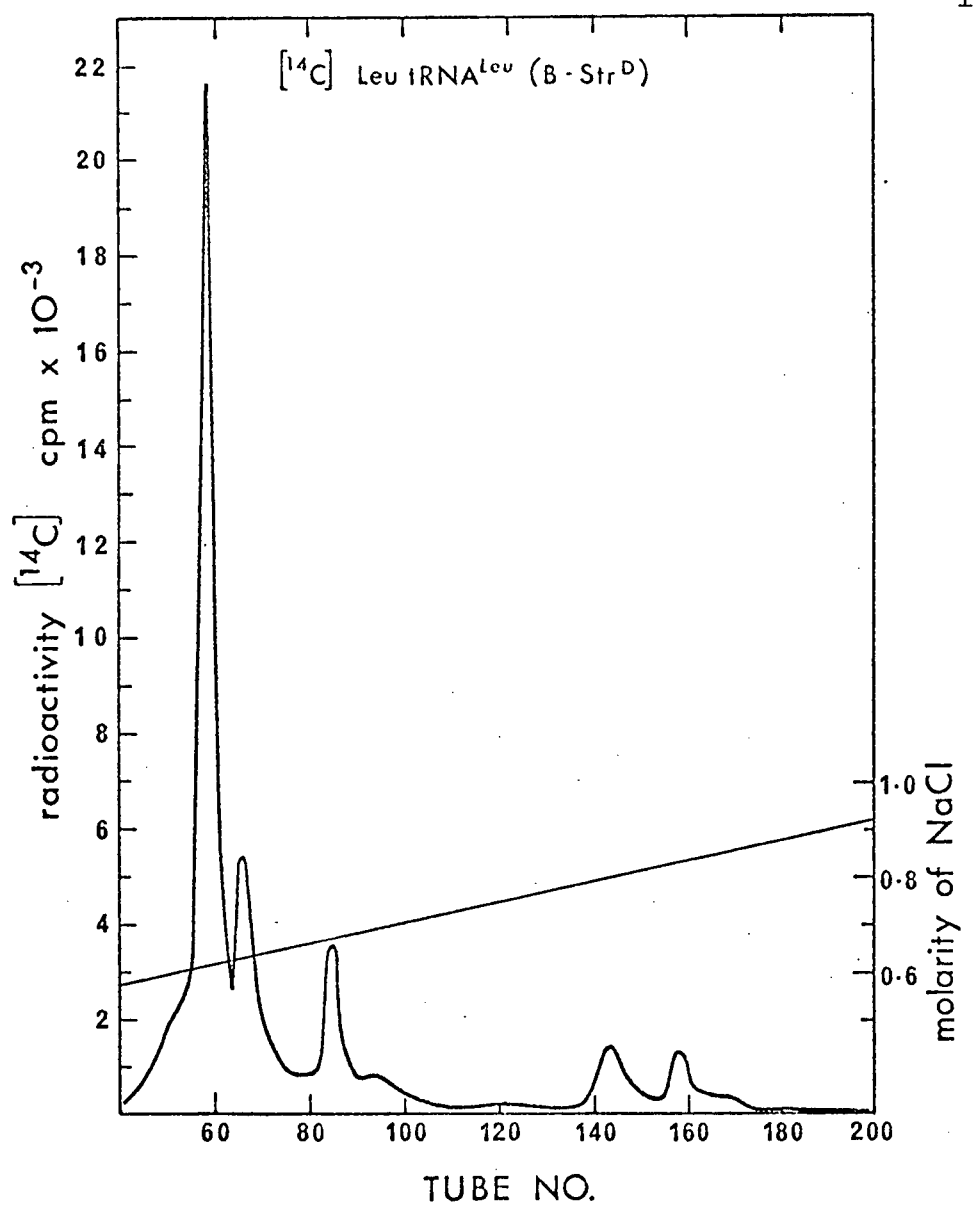


Figure 45. The RPC-5 elution profile of [^{14}C]Leu-tRNA^{Leu} E. coli B str^{D}. In this experiment 6.3 A_{260} units of crude E. coli B str^{D} tRNA containing 2.9 x 10^5 cpm [^{14}C]Leu. (76% counting efficiency) were applied to a standard analytical column and eluted with the standard buffers within a linear NaCl gradient of 0.50 to 0.95 M. Approximately 77% of the applied radioactivity was recovered in the gradient. The [^{14}C]leucine used in the aminoacylation reaction had a specific activity of 294 µCi/µmole.

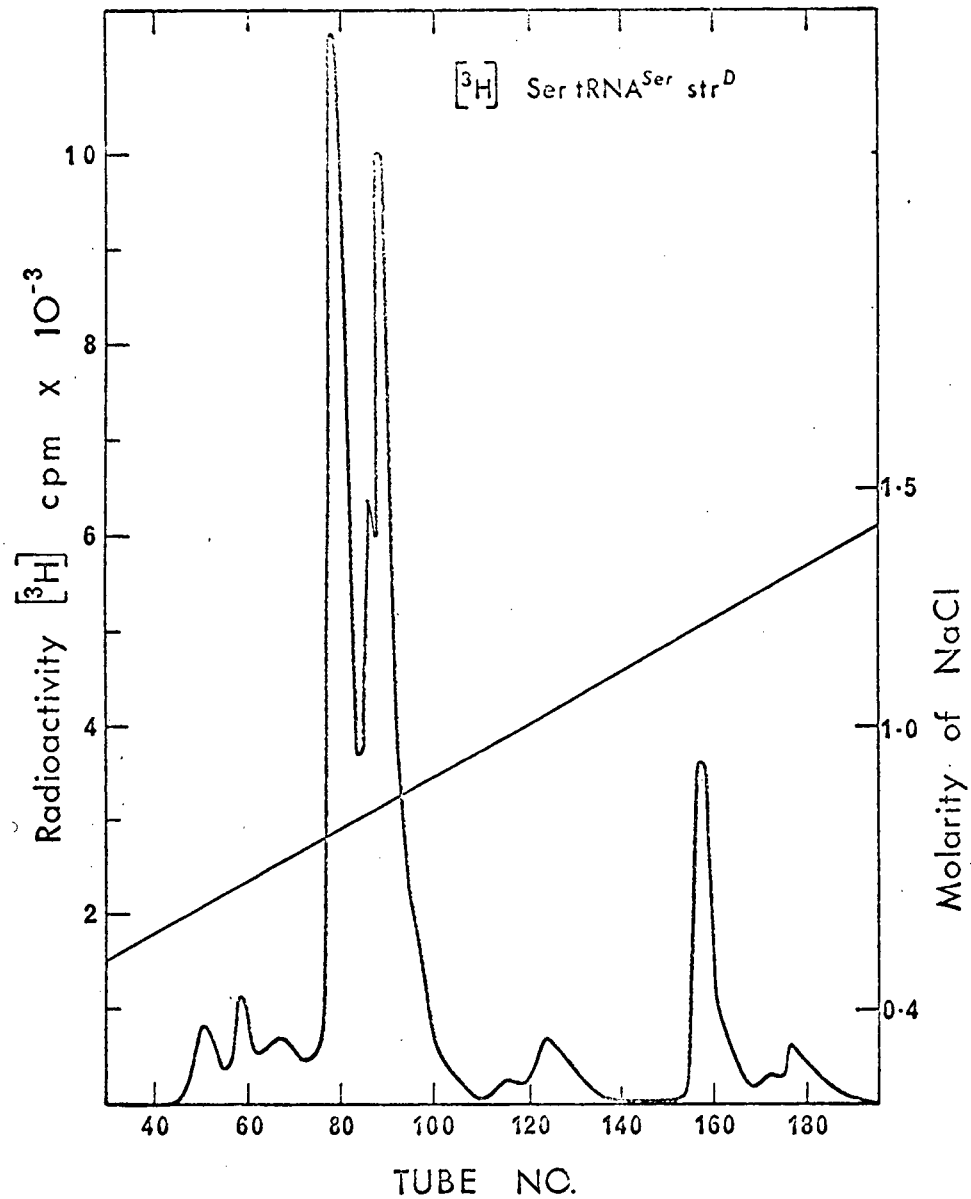


Figure 46. The RPC-5 elution profile of [^{3}H]Ser-tRNA^{Ser} E. coli B str^D. In this experiment 5.6 A_{260} units of crude E. coli B str^D tRNA containing 3.5 x 10^5 cpm [^{3}H]Ser (10% counting efficiency) were applied to a standard analytical column and eluted with the standard buffers within a linear NaCl gradient of 0.45 to 1.50 M. Approximately 76% of the applied radioactivity was recovered in the gradient. The [^{3}H]serine used in the aminoacylation reaction had a specific activity of 3370 µCi/µmole.

In neither instance can the overall change in the amino acid acceptance ratio be explained in terms of the altered synthesis and/or degradation of particular isoacceptors. It is particularly interesting that the relative isoacceptor distribution is so well maintained. This is an important concept that would have to be explained in any theory postulating specific control of tRNA biosynthesis. Some differences in the serine isoacceptor distributions are observed. In particular, the relative distribution between the two major serine isoaccepting peaks is different and the final eluting serine isoacceptor of [^{3}H]Ser-tRNA^{Ser} E. coli B str^{D} is considerably diminished. These changes however do not readily explain the 12% increase in the [^{14}C] serine acceptance to [^{14}C]threonine acceptance ratio which was observed.

Recent experiments in another lab have shown that amino acid excretion in streptomycin dependent E. coli K12 is very sensitive to the concentration of Fe^{++} in the medium (W.J. Polglase-personal communication). Valine is excreted in Fe^{++} containing media (0.1 micromolar) and glutamate is excreted in Fe^{++} deficient media. It has not been shown if such results apply to E. coli B. As the Fe^{++} of the growth media used in my experiments is not precisely known, interpretation of the results may be difficult. Further work is necessary to check on any dependence of the tRNA population in streptomycin dependent cells on the Fe^{++} concentration of the medium. It is also necessary to obtain information on tRNA^{Glu} under the various growth conditions. Experiments should be

done to check for any changes in the tRNA population as a function of the streptomycin levels of the media.

Gel Electrophoresis of Crude E. coli tRNA

Although the E. coli growth and subsequent tRNA extraction procedures used as described in Methods are not optimal for the isolation of precursor tRNAs having extra nucleotide sequences, the crude tRNAs isolated were analyzed by polyacrylamide gel electrophoresis to check for such precursors. In these studies electrophoresis in 15% polyacrylamide gels in 6 M urea gives good resolution of 4-6S RNAs. The major differences seen in such gels (Figures 47 to 49) between crude tRNA preparations from cells grown at the various temperatures under several growth conditions are seen in the 4.5S-5S RNA regions. In the 4S, or tRNA region, the background of unchanged tRNAs is so great that the changes in particular species observed by RPC-5 chromatography are not detectable.

Growth of E. coli B at low temperatures results in the accumulation of material in the 5S region. This is noticeable in Fig. 47 for the 17°C and 20°C slots, slots 2 to 5 of Fig. 48 and slot 4 of Fig. 49. The identity of this RNA has not yet been established. A significant amount of the minor nucleoside ψ is present in the RNA of this region (I.C. Gillam-personal communication), thus suggesting that some of the material is tRNA precursor. The RNA of this region is approximately the same size as the β group precursor tRNAs reported by Dijk and Singhal (38).

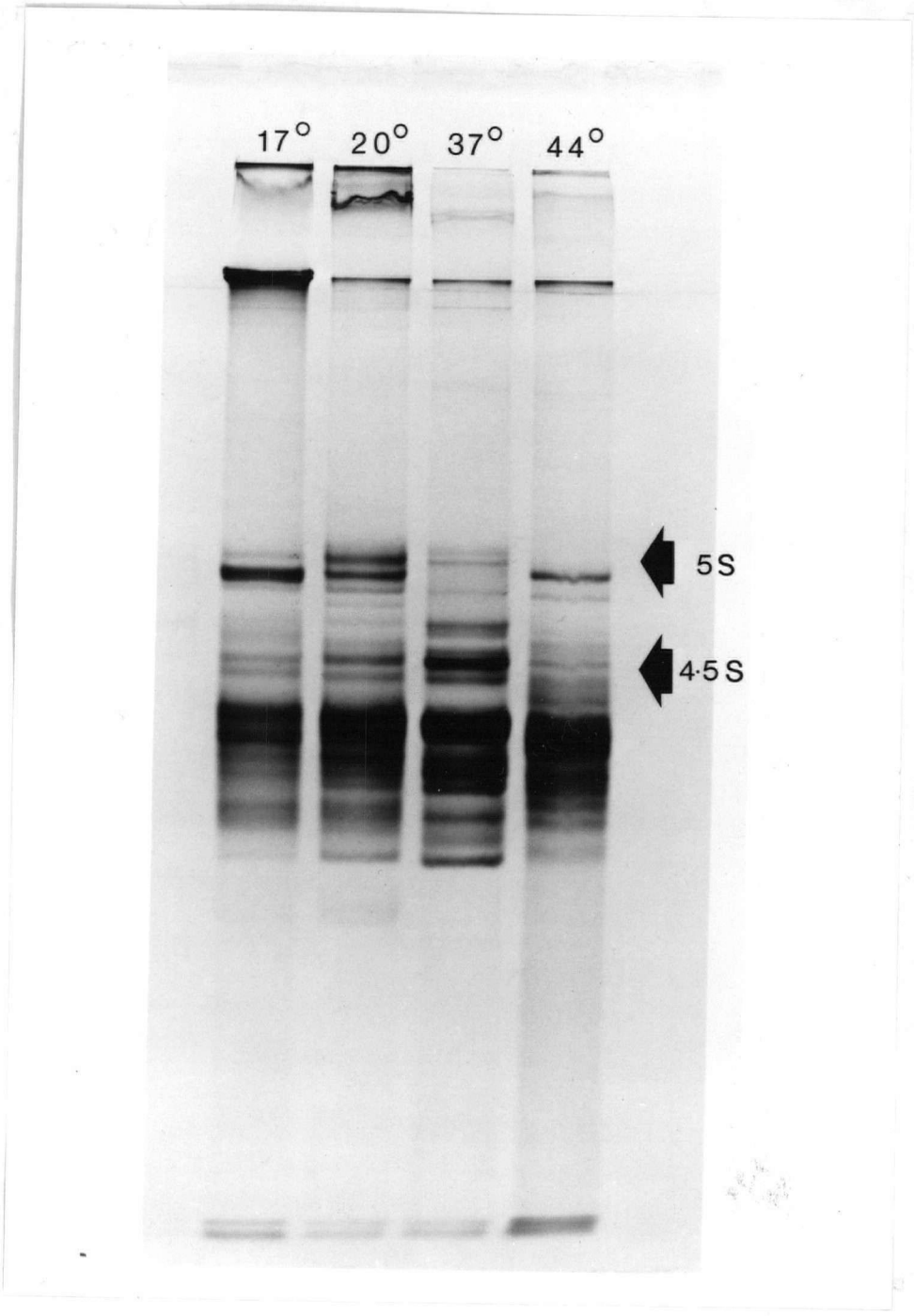


Figure 47. Acrylamide gel electrophoresis of crude tRNA preparations from E. coli B grown at 17°, 20°, 37° and 44°C. Gels were run and stained as described in Methods. Arrows indicate the positions to which markers migrated.

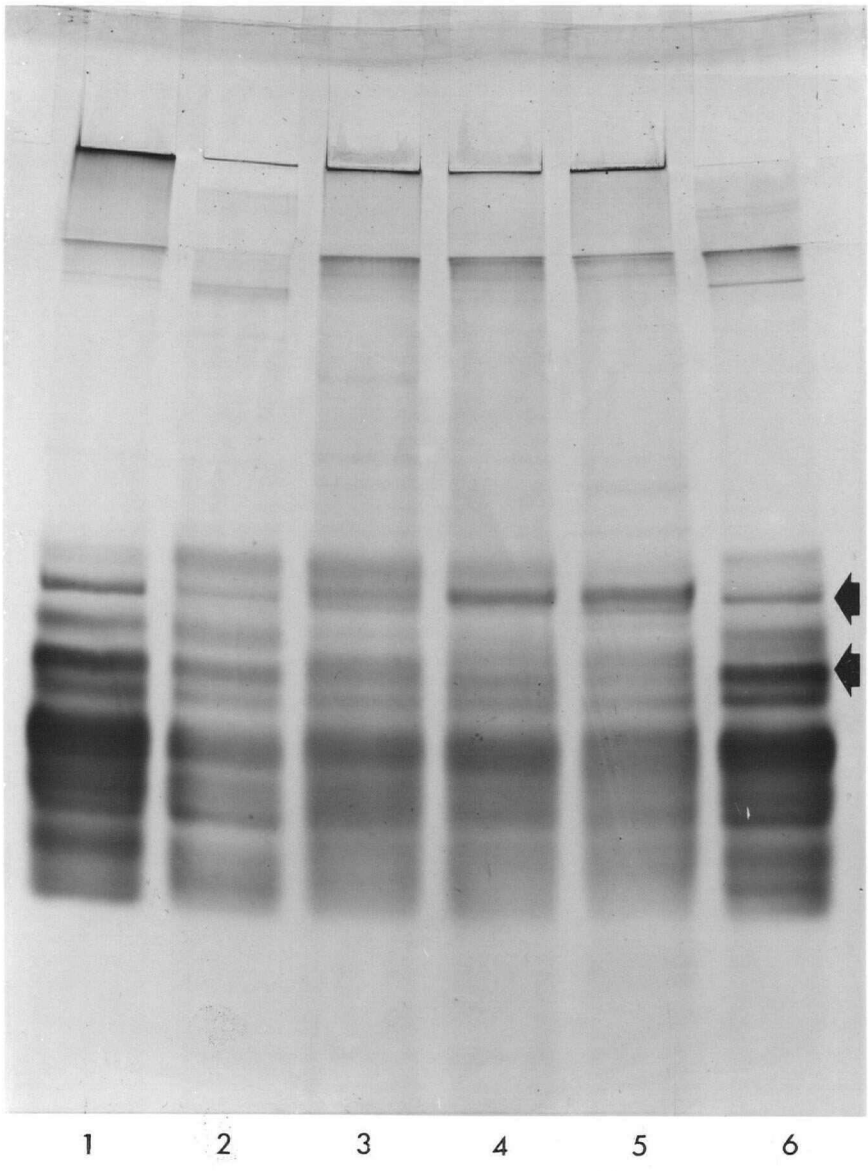


Figure 48. Acrylamide gel electrophoresis of crude tRNA preparations of E. coli. The 5S and 4.5S marker positions are indicated by the upper and lower arrows respectively. Slot 1. tRNA from E. coli B grown at 42°C in flasks on YT broth. Slot 2. tRNA from E. coli W grown at 21°C. Slot 3. tRNA from E. coli B grown at 21.5°C in YT broth. Slot 4. tRNA from E. coli B grown at 20.3°C. Slot 5. tRNA from E. coli B grown at 20°C. Slot 6. Commercial tRNA of E. coli B.

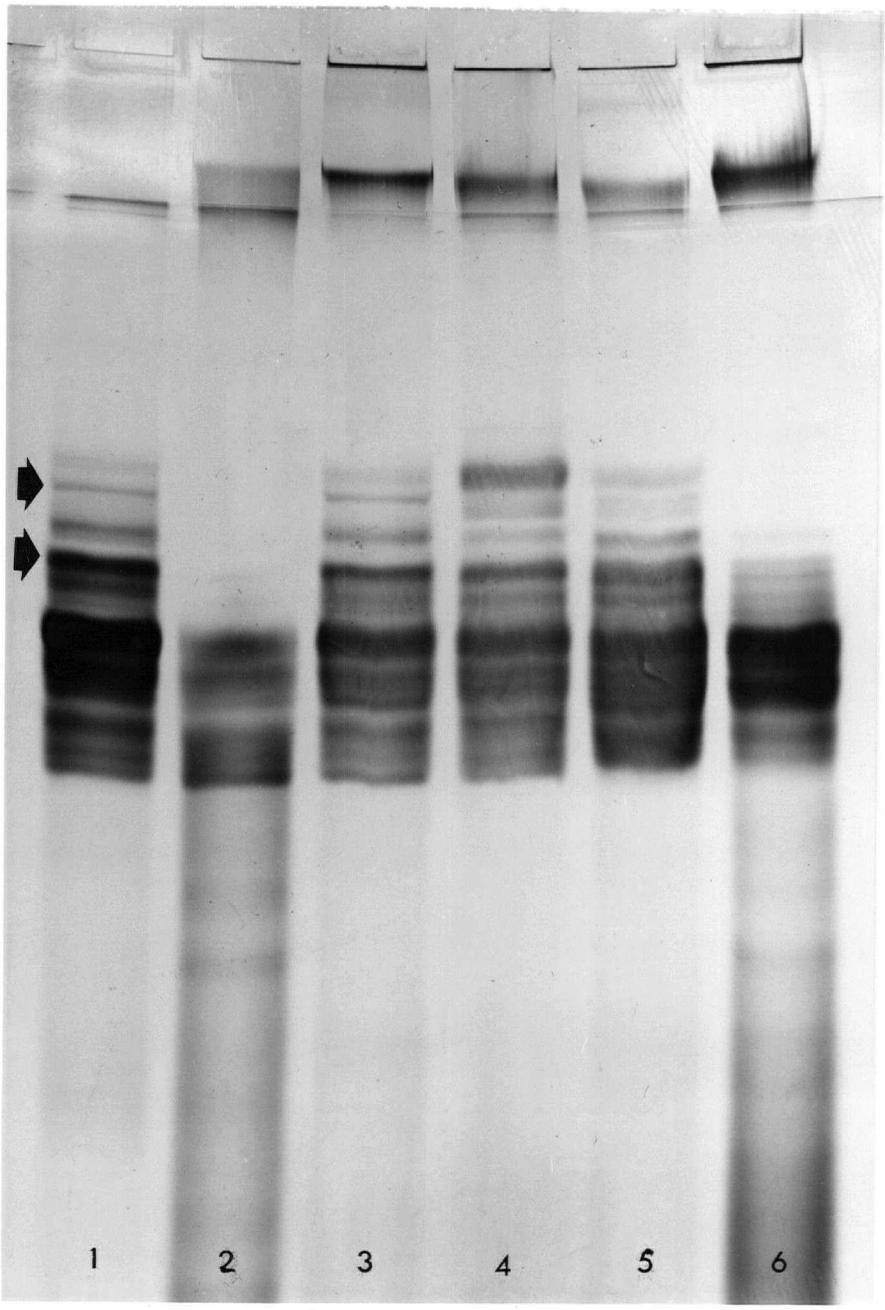


Figure 49. Acrylamide gel electrophoresis of crude tRNA preparations of E. coli B. The 5S and 4.5S marker positions are indicated by the upper and lower arrows respectively. Slot 1. Commercial tRNA of E. coli B. Slot 2. tRNA from E. coli B grown anaerobically at 37°C. Slot 3. tRNA from E. coli B grown at 41.5°C in a flask. Slot 4. tRNA from E. coli B grown at 21.5°C. Slot 5. tRNA from E. coli B grown at 44°C to the stationary state. Slot 6. tRNA from E. coli B grown at 43.5°C.

Growth at high temperature results in a diminished amount of material in the 5S region as is seen in Fig. 47 slot 44° and Fig. 49 slots 3 and 6. Our gel electrophoresis experiments detect negligible accumulation of RNAs running between 6 and 16S RNA in E. coli grown at various temperatures. These results are very preliminary. Work is in progress to quantitate the amount of material in the various regions and to characterize individual gel bands. It will also be necessary to isolate crude tRNA from the various growth conditions by other common extraction procedures designed to extract uncleaved precursor tRNAs in high yield.

Conclusions

The results obtained in the experiments described in this thesis argue against any simple role for aminoacyl-tRNA in the specific control of individual tRNA biosynthesis. tRNA populations as measured by amino acid acceptance were shown not to be static but rather to change as a function of growth conditions. It was not possible to correlate in any simple way the changes in tRNA populations observed with the type of growth condition causing them. It is concluded that there are two or more control mechanisms involved in regulating tRNA populations. The control mechanism observed most often brings about changes in total amino acid acceptance while maintaining a constant relative isoacceptor distribution. A second control mechanism can bring about changes in total amino acid acceptance by altering the amounts of particular isoacceptors thus changing the relative isoacceptor distribution. $tRNA^{Ser}$ levels appear to be subject to both control mechanisms. $tRNA^{Val}$ and $tRNA^{Leu}$ are regulated by the first control mechanism but the presence of large amounts of novel isoacceptors of unknown gene origin does not allow us to determine if particular growth conditions result in the second control mechanism becoming operative for these tRNAs. It may be that some tRNAs are controlled by both mechanisms while others are subject to control by only one of the two mechanisms.

Maintenance of relative isoacceptor distribution requires that all the genes are controlled or recognized by the same repressor. It is possible that tRNAs could be divided into groups

of transcription units each having its own repressor. It is very likely (P. Primakoff-personal communication) that several different tRNAs are transcribed together in single units. Maintenance of relative isoacceptor distribution requires that several transcription units having tRNAs for different amino acids must all be co-ordinately controlled. This would explain why groups of changes in amino acid acceptances could be observed (when only the first control mechanism described is operative). One must also ask what factor(s) determines relative isoacceptor distribution. Can gene number and differential rates of degradation explain the relative amounts of isoacceptors observed and/or is RNA polymerase affinity for different promoters for each isoacceptor coupled with interaction with a common repressor responsible for the relative levels of the isoacceptors observed? The results of this thesis give several useful starting points for further investigation of control mechanisms for tRNA biosynthesis.

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