STUDIES WITH MAMMALIAN TRANSFER RIBONUCLEIC ACID

I. THE REGULATION OF MAMMALIAN TRANSFER RNA BIOSYNTHESIS

II. CHARACTERIZATION OF A NEW TECHNIQUE FOR THE PURIFICATION OF ISOACCEPTING FAMILIES OF TRANSFER RNA

by

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A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Health
Sciences Center in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
June 1976

APPROVED:

[redacted]

(Professor in charge of Thesis)

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(Chairman, Graduate Council)

Acknowledgements

I am grateful to Dr. Michael Litt for his human and reasonable approach to scientific investigation, to Dr. David Kabat for helpful discussion of my work throughout the course of its development, to Chuck Lytle and Lavelle Jackson for their friendship and technical assistance, and to Priscilla Burk for the generous donation of her good will and valuable literary skills.

I was supported throughout the course of this work by USPHS grant GM01200.

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Abbreviations

FL cells	Friend leukemia cells
TCA	Trichloroacetic acid
Hepes	N'-2-hydroxyethylpiperazine-N'-ethane-sulfonic acid
PPO	2,5-diphenyloxazole
Me_2POPOP	1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene
Pn	Fraction of total ribosomal material sedimenting as polysomes.
GTP	Guanosine 5' triphosphate
SDS	Sodium dodecyl sulfate
ψ	psi factor or pseudouridine
A_{260}	Absorbance at 260 nm
A_{260} unit	That amount of material which in a volume of 1 ml gives an absorbance of 1 in a cuvette with a 1 cm light path.
EDTA	Ethylenediaminetetraacetate
DTT	Dithiothreitol

PART I

THE REGULATION OF MAMMALIAN TRANSFER RIBONUCLEIC ACID BIOSYNTHESIS

I. Introduction

The following dissertation will be presented in two independent parts, each dealing with a different problem in mammalian tRNA metabolism. The first and larger section will deal with the study of the regulation of total tRNA synthesis in cultured murine leukemia cells and in nuclei isolated from these same cells. The second and smaller section will describe experiments characterizing a technique designed to allow the purification of a single family of isoaccepting tRNAs. Superficial examination of these two sections would suggest that they are related only by their common interest in mammalian tRNA. The second part was undertaken to provide a technique for the study of the regulation of both synthesis and degradation of individual families of tRNA. Hence, this represents a primitive extension of the first part of the thesis and is therefore related to it in a more meaningful way. However for the purposes of clarity, these two parts will be dealt with as separate presentations, each containing its own literature citations and separate sections of text including introduction, materials and methods, results, discussion, and summary. The initial introduction,

of which this discussion is a part, includes background information on the discovery of tRNA and its role in protein synthesis, its physical, chemical, and biological properties. Hence it should serve as a common denominator for both parts of the presentation to follow.

A. Discovery of Transfer RNA and Its Role in Protein Synthesis

The concept of transfer RNA function in protein synthesis as we know it today was originally developed by Zamecnik and his colleagues as well as others in the decade from 1950 to 1960. A number of reviews covering the subject in detail and providing citations to the original literature are readily available (1-6). The bulk of the following discussion is taken from these accounts. Hence, individual references will not be cited.

These early investigators were primarily interested in elucidating the pathway leading from free amino acid to functional polypeptide chain and they approached the problem by attempting to fractionate whole cells and tissues into their component parts, thereby identifying the required molecular apparatus. The initial studies attempted to identify amino acids in novel forms which could serve as intermediates in the polymerization of protein. Such an intermediate was observed in experiments utilizing the soluble, non-particulate, "pH 5 fraction" of liver tissues in which the formation of an enzyme bound aminoacyl-AMP moiety could be detected. Since this fraction was also required for the incorporation

of [^{14}C] amino acid into protein, this activated form of amino acid seemed to fit the requirements for such an intermediate. Fortuitously, it was discovered very soon thereafter that this same "pH 5 fraction" contained a soluble RNA component which had the capacity to interact with amino acids in a highly specific fashion. Thus a stable isolable intermediate was found and could be shown to transfer the activated amino acid into the protein fraction of large ribonucleoprotein particles, later termed ribosomes, under the appropriate conditions. There are two aspects which must be considered essential in identifying the role played by the "transfer" RNA component: 1) the nature of its interaction with the 20 different amino acids, and 2) the nature of the donation of amino acid into protein.

Further investigation of the specificity of the RNA-amino acid linkage revealed that amino acids were additively and non-competitively attached by covalent bonds to the RNA. While this indicated that there were separate sites for each amino acid it did not establish that for each amino acid there was at least one unique group of RNA molecules. This concept required the work of several laboratories and culminated in the demonstration that periodate oxidation of the 2',3'-cis-diol structure of the 3' terminal ribose moiety of the RNA destroyed the amino acid acceptance of the RNA population, with the exception of those molecules to which specific amino acids were esterified at the time of treatment.

The second aspect of these early tRNA studies served to

establish some of the very basic events involved in protein biosynthesis and was directed towards the understanding of the transfer of aminoacyl-RNA into ribosome associated protein. These studies utilized both in vivo and in vitro experimental systems. The in vivo work led to the demonstration that the kinetics of labeling of various components of the system with both amino acids and nucleotide precursors were consistent with a role for this activated amino acid-RNA complex in polypeptide chain synthesis. Thus a pulse of [^{14}C] amino acid into cells could be followed initially into the tRNA fraction where it rapidly reached a plateau value, and secondarily in "particulate RNA", followed by "particulate protein", and finally soluble protein. The in vitro experiments demonstrated that tRNA with radioactive amino acid attached donated that amino acid into protein in an irreversible fashion and in a manner kinetically similar to the in vivo system. In addition, these early cell free systems could be made dependent upon the addition of aminoacyl-tRNA. While these early investigations did leave much unknown about the mechanism of protein synthesis, they set the stage for the wide variety of sophisticated experimentation completed in the last 15 years which has led to our current understanding of this subject. With regard to the understanding of the function of transfer RNA, however, they resulted in establishing the basic concept; that of an intermediate which could recognize the 20 different amino acids and transfer them to the site of polymerization in a specific fashion, resulting in

proteins with unique, non-random sequences.

B. Physical and Chemical Properties of Transfer RNA

The physical and chemical properties of tRNA have been intensely investigated since its discovery with an eye to elucidating those characteristics which confer upon this population of remarkably similar polynucleotides the specificity required for coordinate translation of both the protein code for amino acids and the DNA code consisting of the four deoxyribonucleotides. While a vast literature has appeared as a result of this study, the molecular basis of much of this specificity remains unclear. The following brief discussion is intended only to provide a very basic description of this aspect of tRNA research. Several excellent reviews are readily available to provide details which have been omitted here (7-12).

Initial studies on the size of the amino acid accepting tRNA using analytical ultracentrifugation and diffusion data suggested a molecular weight of 15,000 to 20,000 daltons (13). This value was modified to approximately 30,000 daltons on the basis of chain end analysis (14). Later studies on *E. coli* tRNA gave a sedimentation constant of 4S and a corresponding molecular weight of 27,000 daltons (15). This value is consistent with a chain length of 75 to 90 nucleotides. (This "4S" nomenclature will be referred to occasionally in the text to follow, and in this field in general, but does not necessarily imply the determination of the sedimentation constant

for the material being described.)

While tRNA may appear to be a homogeneous population, it is clear from the studies on amino acid acceptance (2-4) that it must necessarily be composed of a heterogeneous group of molecules. Indeed, it is likely that there are more than 60 chemically distinct species of tRNA. Evidence in support of this heterogeneity initially came from studies attempting to separate populations of tRNA into unique fractions with enhanced amino acid acceptance for a specific amino acid (16,17; see Introduction, Part II of this thesis).

This heterogeneity might arise from two sources: 1) heterogeneity of the primary nucleotide sequences, and 2) heterogeneity in the nature and extent of post-transcriptional modification of a unique primary transcript. Such modification includes methylation of the ribose moiety at the 2'-hydroxyl position, methylation of the purine or pyrimidine portion of various nucleotides, and other more complex modifications including formation of pseudouridine, ribothymidylic acid, and dihydrouridine (7,8,10). While other nucleic acids are also structurally modified following transcription, the extent and variety of such alteration is quite limited in comparison with that observed in tRNA (10). In fact, the variety of odd nucleotides which can be observed in alkaline hydrolysates of tRNA preparations serves as a characteristic of tRNA as a macromolecular class. In addition, the base compositions of tRNA from a wide variety of sources are quite similar, while

those of DNA or ribosomal RNA from the same sources are much less similar (4,8).

With the availability of techniques for the purification of individual species of tRNA and for determination of nucleotide sequences in low molecular RNAs, the primary structure of alanine tRNA ($tRNA^{Ala}$) from yeast was elucidated (18). Since that time many tRNA sequences have been determined and have provided the basis for speculation and experimentation on the secondary and tertiary structure of the molecule. The most widely accepted view of the secondary structure of the polynucleotide chain was first advanced by Holley et al. (18) on the basis of their original sequence for yeast $tRNA^{Ala}$. This model, known as the cloverleaf, can be formed with all tRNAs for which the sequence is known (19) and is derived by making the largest number of hydrogen bonded GC base pairs. The concept of a polynucleotide chain folding back on itself, as outlined by this model, is supported by data showing hyperchromicity in solution upon heating (20), chemical labeling of particular residues using a specific reactive probe (21), and by X-ray diffraction studies on mixed crystals and in solution (22). The ability to form crystals of mixed unfractionated tRNAs argues effectively that the secondary and tertiary structure of all tRNAs must be remarkably similar.

There have been a number of proposals advanced concerning the three-dimensional conformation of tRNA as reviewed by Cramer

(11). However until the recent crystallization of a number of purified tRNA species, no exceptional experimental evidence has been available (23-25). Kim et al. and Robertus et al. have succeeded in determining the three-dimensional structure of the yeast $tRNA^{Phe}$ at a resolution sufficient to identify the way in which the polynucleotide chain is folded in space (26-28). Essentially, the molecule can be viewed as having a flattened L-shape conformation with the anticodon loop and the amino acid acceptor stem held at right angles to each other and the TψC and dihydrouridine loops in the corner. Most of the tertiary interactions which appear to stabilize the structure involve nucleotides which are common to all tRNAs and thus may account for the remarkable similarity of all tRNA three-dimensional structures. The X-ray crystallographic data also confirm the hydrogen bonding interactions suggested with the cloverleaf configuration and can account for most of the chemical modification studies (28).

C. Biological Properties of Transfer RNA

The role which tRNA plays in both prokaryote and eukaryote biology can be considered as composed of two aspects: 1) the interactions directly involved in protein biosynthesis including only that activity required for template directed transfer of free amino acid into protein, and 2) interactions which affect the structure and function of previously synthesized protein. While the first role has been worked out in great detail, the understanding of the role that tRNAs play in the regulation of other cellular processes is still

in its infancy. The current understanding of both aspects of tRNA biology will be briefly discussed in the following section.

Investigation into the specific interactions of tRNA both with soluble proteins and with ribosomes and ribosome-associated proteins occupied the attention of many investigators throughout the decade from 1960 to 1970. These interactions may be most easily defined by describing a single cycle in polypeptide chain elongation. This information has been described in great detail in several review publications and an ample description may be found in any modern textbook of biochemistry (6, 29-32).

The interaction of amino acid with the specific aminoacyl-tRNA synthetase enzyme and the cognate tRNA results in the formation of the specific activated intermediate, aminoacyl-tRNA. This molecule now interacts with a soluble protein called elongation factor Tu (in *E. coli*) or elongation factor I (in eukaryotes). These proteins are highly specific for recognition and binding of aminoacyl-tRNA and will not form such complexes with uncharged tRNA. The formation of this complex depends upon the presence of GTP. The complexed aminoacyl-tRNA is now available for interaction with ribosomes in a highly specific manner. Only those aminoacyl-tRNAs with correct nucleotide sequences in the anticodon are able to bind to the ribosome-messenger RNA complex in response to the nucleotide sequence of the codon at which the ribosome is stopped, awaiting the incoming aminoacyl-tRNA. The complex of the ribosome with

aminoacyl-tRNA is stabilized by codon-anticodon base pairing, interactions with both the 3' CCA terminus and the 5' phosphate terminus of the tRNA as well as other potential interactions between the polynucleotide chains and the ribosomal proteins. The tRNA is bound at a specific location, termed the acceptor site, which is part of the large ribosomal subunit. Thus the transfer of aminoacyl-tRNA to the ribosome is specific, well regulated, and results in the correct reading of the linear code of the mRNA.

Once the aminoacyl-tRNA is bound at the acceptor site of the ribosome, it is ready to accept the growing peptide chain from the peptidyl-tRNA seated in the donor site on the large ribosomal subunit. The peptidyl transferase activity, also believed to be part of the large subunit, catalyzes the formation of the new peptide bond via nucleophilic attack by the α-amino group of the aminoacyl-tRNA on the carboxyl carbon atom of the peptidyl-tRNA. This converts the former peptidyl-tRNA to free tRNA, which leaves the donor site on the ribosome and returns to solution to pick up another amino acid. The former aminoacyl-tRNA, now the peptidyl-tRNA, still occupies the acceptor position on the ribosome, preventing incoming aminoacyl-tRNA from binding. Another soluble elongation factor termed EF-G (*E. coli*) or EF-II (eukaryotes) now interacts with the ribosome, and results in the hydrolysis of GTP, the shift of the peptidyl-tRNA to the donor site on the large subunit and the corresponding shift of the ribosome along the mRNA by one nucleotide triplet codon. This process is

termed translocation. The cycle of elongation continues as each new codon is read until reaching a triplet signaling for termination of the peptide chain.

The initiation of protein synthesis also involves tRNA but is regulated independently of elongation and utilizes an entirely separate group of soluble factors including a specific tRNA. Thus there is only one species of tRNA which recognizes the initiation codon on the mRNA and is thereby directly involved in the formation of the translational initiation complex. The result of initiation is the binding of the initiator tRNA in the donor position of the ribosome in a fashion ready for elongation of the protein chain.

As the function of tRNA in protein synthesis became clearly defined, a number of workers began to investigate other roles for this class of RNA. While a large number of mutations are now recognized in bacterial systems which result in the suppression of previous mutations, and these suppressor mutations involve specific tRNAs, it is clear that such suppression is primarily the result of base changes in the anticodon triplet of the tRNA allowing the recognition and translation of a nonsense codon which represented the original mutation. Thus such "abnormal" functions of tRNA are explained by the normal function of tRNA in protein synthesis. There are, however, a number of instances in which specific tRNAs play roles independent of protein synthesis and which result in altered physiological function (33).

Ames and his colleagues have examined the behavior of a series of mutants in the histidine operon of *S. typhimurium* (34). They have found that the synthesis of enzymes encoded by the His operon and catalyzing a number of steps in the synthesis of histidine are regulated by the absolute amount of histidinyl-$tRNA^{His}$ in the cell (35,36). The regulation depends upon the modification of two uridine residues in the anticodon loop to pseudouridine. The wild type tRNA represses transcription of the operon by interaction with the operator sequence of the gene. The role of aminoacyl-tRNA in regulation of other bacterial amino acid synthetic enzyme pathways is also indicated (33).

Jacobsen et al. have also observed the participation of tRNA in regulation of enzyme activity in eukaryote cells (37,38). They have shown that the suppression of a mutant in the vermillion locus of *Drosophila melanogaster* was due to the absence of one isoaccepting species of $tRNA^{Tyr}$. The enzymic defect responsible for the mutant eye color was a deficiency in tryptophan pyrrolase activity, an enzyme responsible for the synthesis of an intermediate in the metabolic pathway leading to the vermillion eye pigment. Jacobsen showed that the $tRNA^{Tyr}$ isoacceptor fraction 2 was missing in mutant flies, and, in a series of elegant experiments, that this particular tRNA molecule was an effective inhibitor of tryptophan pyrrolase and its presence was probably responsible for the physiological expression of the mutant phenotype.

A third function for tRNA exclusive of amino acid donation in messenger-RNA-ribosome directed protein synthesis involves donation of amino acids in reactions which are not template directed (33). Such messenger independent peptide bond formation includes synthesis of aminoacyl phosphatidyl glycerol (39), terminal addition of amino acids to existing proteins (40), and the synthesis of cell wall components in gram positive bacteria (41). No doubt as the search continues and the regulation of gene expression in eukaryotic cells is further elaborated, new independent functions for tRNA molecules will be observed.

D. Biosynthesis and Accumulation of Stable RNA Species: Coupling with Protein Synthesis

The previous discussion has centered on the physical, chemical, and biological properties of tRNA from both eukaryotic and prokaryotic sources. As the purpose of the experimental portion of this section of the thesis is to evaluate the regulatory aspects of tRNA synthesis, it is of interest now to consider the experimental evidence bearing on the biosynthesis and accumulation of both ribosomal and transfer RNA synthesis. These two classes are grouped together under the general heading of stable RNA species since they are both long lived molecules compared to the relatively short lived messenger RNA species. The following discussion will consider the synthesis of both rRNA and tRNA for several reasons. 1) Both species of RNA make up the non-specific translational machinery of the cell

having the specificity of their function dictated by the nucleotide sequence of messenger RNA. Hence, it is of interest to examine the biosynthesis of such functionally related molecular species together. 2) There is more information available concerning the synthesis of rRNA as it relates to protein synthesis and thus, such discussion should provide insight into the regulatory aspects of the synthesis of tRNA.

Early in the study of nucleic acid and protein biosynthesis it was observed that such processes are not independent events occurring within the cell but rather depend on each other for normal activity (42). Observation of this apparent synergistic relationship requires an experimental situation producing stimulation or depression of the synthesis of one species of macromolecule. In this manner alterations in the synthetic processes of other macromolecular components may be measured. The inhibition of protein synthesis by use of a variety of inhibitory substances provides an experimental system of this sort which allows the assessment of RNA synthesis as a function of both the extent of protein synthesis as well as the specific mechanism by which it is inhibited.

Bacterial cells were the first to be investigated concerning translational and transcriptional coupling and the study of such systems has yielded a considerable amount of information (43). The deprivation of a single amino acid from a bacterial strain auxotrophic for that amino acid results in a series of molecular changes

in the cell characterized primarily by a rapid and severe inhibition of the rate of accumulation of both rRNA and tRNA. This sequence of events has been termed the stringent response. The linkage of RNA synthesis to translational inhibition is controlled by the product of a single genetic locus (44). The signal for activation of the regulatory scheme is the presence of a deacylated tRNA bound to the ribosome in a codon specific fashion (45). This specific binding results in the enzymatic conversion of guanosine di- or tri-phosphate to guanosine tetra- or penta-phosphates (ppGpp, pppGpp), also known as magic spots I and II (46). These unusual nucleotides were originally observed by Cashel and Gallant (47) and have been shown to inhibit stable RNA synthesis in vitro by a number of routes. Gallant has shown that ppGpp inhibits the synthesis of guanine nucleosides by competitively inhibiting inosine monophosphate dehydrogenase (48). In addition, Cashel has shown that the nucleotide (ppGpp) inhibits initiation of transcription at guanine sites in the genome (49). The transcription of stable classes of RNA has been shown to initiate primarily at purine sites, in particular guanine (48,49). Travers et al. have also observed that magic spot compounds interfere with the function of the ψ (psi) factor, a regulatory component of bacterial transcription responsible for stimulation of stable RNA synthesis (50). It appears that the combined effects of these odd nucleotides may be sufficient to account for the stringent response reduction in stable RNA synthesis. While this

information is strictly applicable only to bacterial systems, it can serve as a sound platform upon which to base experimental work in higher organisms.

Transcription in eukaryotes is conducted in a fashion which appears to be much more complex than that of prokaryotes. In order to consider potential regulatory schemes, it is of interest to consider briefly what is known about the enzymology involved in RNA synthesis. It is now well documented that there are several species of DNA dependent RNA polymerases in eukaryotic nuclei (51). These different enzymes can be distinguished on the basis of chromatographic behavior on ion exchange columns, intranuclear localization, ionic strength requirements, metal cation optima, and sensitivity to the fungal toxin α-amanitin. They fall into three distinct classes differing primarily in their sensitivity to this toxin as well as their functional role in transcribing separate classes of RNA (52). Class I or A is an enzyme localized primarily in the nucleolus, is insensitive to even high levels of α-amanitin and is believed to transcribe ribosomal RNA genes (52,53). Type II or B is a nucleoplasmic enzyme which is highly sensitive to α-amanitin and synthesizes RNA with a base composition similar to DNA. Thus it is probably responsible for the synthesis of the putative mRNA precursor, heterogeneous nuclear RNA (52-54). The third class, termed III or C, is also a nucleoplasmic enzyme which is sensitive to the fungal toxin only at high concentrations and is responsible

for the synthesis of low molecular weight RNAs including 5S ribosomal RNA, transfer RNA and other low molecular weight RNAs of the cell (55-57).

Because of this heterogeneity in the enzymology of eukaryotic RNA synthesis, it is possible to have the transcription of separate classes of RNA differentially sensitive to the same set of conditions within the cell. Consequently one would predict that transcription of the three classes of RNA described above would be non-coordinately controlled. This appears to be the case in cultured cells when protein synthesis is inhibited in a variety of ways. Thus while mRNA transcription is not significantly affected during short term inhibition of protein synthesis by amino acid deprivation, both ribosomal RNA and transfer RNA are affected though the magnitude of the effect is different between these two classes.

The bulk of available information concerning the regulation of transcription in response to the inhibition of protein synthesis has been directed toward the effect on ribosomal RNA synthesis. Hence, it is of value to consider this data briefly in terms of its potential contribution to the understanding of the transcription of tRNA genes as well. rRNA is known to be synthesized as a large 45S precursor which matures to the 28S and 18S species via a scheme involving cleavage of the chain and base modification (58). When protein synthesis is inhibited via amino acid deprivation or administration of inhibitory substances, the rate of transcription of the

precursor as well as the rate of its maturation are greatly decreased and result in the appearance of completed chains at a level roughly 30-40% of normal values (59-61). On the basis of a variety of studies which indicated that the amount of active polymerase I present in inhibited nuclei was not significantly different from controls, it was suggested that rRNA synthesis depended upon a rapidly turning-over factor which was depleted in the absence of continuing protein synthesis (62-63). Recently it has been reported that the deprivation of histidine from Erlich's ascites tumor cells results in a drop in the cellular pool of ATP and GTP to 60% of control values and that this change is kinetically consistent as an explanation for the inhibition of rRNA synthesis by amino acid deprivation (64). In addition, restoration of the GTP and ATP pools by inclusion of the appropriate nucleoside in the incubation medium restored rRNA synthesis without restoring protein synthesis. The use of cycloheximide to inhibit protein synthesis did not depress the cellular ATP or GTP levels but did inhibit rRNA synthesis in a manner resistant to restoration by extracellular nucleoside. Hence, the regulation of rRNA synthesis is not simply dependent upon the extent of protein synthesis as originally suggested but appears to be a more complex process.

The available information concerning the regulation of the synthesis of eukaryote tRNA is relatively small. In many of the early studies concerning the synthesis of ribosomal RNA, little or no effect

of inhibiting protein synthesis could be observed on the rate of synthesis of other RNA classes (59,65). More recent studies have clearly demonstrated that the synthesis of tRNA is not simply dependent upon continuous protein synthesis (66-69). Bernhardt and Darnell (66) and Bölcsföldi et al. (60) both showed that the deprivation of an essential amino acid resulted in a 35-50% decrease in the rate of synthesis of tRNA. However, Willis et al. observed that the administration of cycloheximide, diptheria toxin, or puromycin all resulted in a significant stimulation of tRNA synthesis relative to that in control cells (68). Bölcsfoldi extended these studies by measuring the effect of these various inhibitors on tRNA synthesis as well as the polyribosome pattern (69). What he observed was an apparent correlation between these two parameters, such that the aggregation of ribosomes on the mRNA (resulting from inhibitors affecting primarily translational elongation) occurred with concomitant stimulation of tRNA synthesis. Alternatively, the use of inhibitors causing polyribosomal disaggregation (by inhibiting translational initiation) seemed to correlate with the inhibition of tRNA synthesis. On the basis of this data it was suggested that tRNA synthesis is controlled in a negative fashion by a factor which reversibly associates with polysomes. Hence when the number of ribosomes bound to messenger RNA increases, this factor is removed from solution and visa versa. Bölcsföldi proposed tRNA itself as such a factor and considered the possibility that each individual kind

of tRNA could regulate its own rate of synthesis via such a mechanism.

E. Statement of Thesis Objectives

The experimental portion of this section of the thesis is concerned with evaluating this proposed model for the regulation of tRNA synthesis. I have attempted to confirm the original findings in a line of Friend virus infected murine erythroleukemia cells (FL cells) by the use of the protein synthesis inhibitors cycloheximide, pactamycin, puromycin, o-methylthreonine, and histidinol. The latter two drugs are amino acid analogues which inhibit the aminoacylation of $tRNA^{Ile}$ and $tRNA^{His}$, respectively, and thereby mimic that aspect of amino acid deprivation directly relevant to protein synthesis (i.e., decreasing available aminoacyl-tRNA). The results of this work suggested that the correlation proposed by Bölcsföldi is invalid. Instead, the data argue in favor of a role for deacylated tRNA in regulating its own rate of synthesis. These studies have been further extended to include assessment of the role of tRNA in in vitro RNA synthesis by isolated nuclei from FL cells. While data from these latter experiments do not provide support for the concept of tRNA itself regulating its own transcription directly at the gene level, they are consistent with a proposal for the regulation of the synthesis of tRNA involving uncharged tRNA as a primary signal.

II. Materials and Methods

A. Buffers

Buffer A: 50 mM sodium acetate, 100 mM NaCl, 10 mM EDTA, pH 5.0; buffer B: 50 mM Tris Cl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.5; buffer C: 10 mM Tris Cl, 100 mM KCl, 1 mM EDTA, pH 7.5; buffer D: 10 mM Tris Cl, 2 mM $Mg(Acetate)_2$, 3 mM $CaCl_2$, 0.3 M sucrose and 0.5 mM DTT, pH 8.0; buffer E: 25% glycerol, 5 mM $Mg(Acetate)_2$, 50 mM Tris Cl, 0.1 mM EDTA, and 5 mM DTT, pH 8.0.

B. Cell Culture

The FSD-1 clone of Friend-virus infected erythroleukemia cells derived by Ostertag et al. (70) was used in all experiments. This cell line is grown in suspension in Eagle's medium with Earle's salts, 25 mM Hepes, 3x glutamine, 2x amino acids, 2x vitamins, and 10% fetal calf serum (Grand Island Biological Co.). The doubling time for this line is about 20-24 hr and the cells reach a maximum density of $3\text{-}4 \cdot 10^6$ cells/ml. Cultures used for experiments were innoculated at approximately $2\text{-}3 \cdot 10^5$ cells/ml and following growth to $2 \cdot 10^6$ cells/ml were maintained in log phase growth by daily 1:1 dilution in fresh medium. Cultures to be used with histidinol or O-methylthreonine were first washed and then resuspended in medium similar to the above except that dialyzed fetal calf serum replaced normal serum and either histidine or isoleucine was reduced to 1/10 the normal amino acid concentration (reduced values are 10 μM for histidine and 40 μM for isoleucine). The cells were grown for 24 hr in this medium before use and the growth rate was unaffected by

the lower amino acid concentrations.

C. Assay of Protein Synthesis

Cultures containing 1-2·10^6 cells/ml were divided into 2.5 ml aliquots which were incubated at 37°C in the presence or absence of various drugs. One µCi of [^{14}C]leucine (Amersham-Searle, 348 Ci/mole) was added to each culture and 0.25 ml aliquots removed at 30 min intervals for 2 hr. The samples were placed in 2 volumes of 10% TCA, heated to 90°C for 20 min to hydrolyze labeled aminoacyl-tRNA and filtered on glass fiber filters (Reeve Angel, grade 934AH). The filters were washed with 15 ml cold 5% TCA, dried at 100°C and counted by liquid scintillation in a toluene based cocktail containing 0.3% PPO and 0.3% Me_2POPOP. The incorporated radioactivity was plotted vs time and the slope of each plot was taken as the rate of protein synthesis.

D. Labeling and Extraction of RNA

Five ml cultures were labeled with 25 µCi [5-^{3}H]uridine (0.33 Ci/mmole, Nuclear Dynamics) or 1.7 µCi [2-^{14}C]uridine (56 Ci/mole, Nuclear Dynamics) for 2 hr at 37°C. The cultures were cooled and collected by centrifugation at 1000 x g for 5 min. The procedure for RNA extraction was modified after Nienhuis et al. (71). Briefly, the cell pellet was resuspended in cold fresh medium at about 2·10^7 cells/ml and extracted at room temperature for 10 min in a mixture of 2 volumes H_2O saturated redistilled phenol and 1 volume buffer A including 0.5% sodium dodecyl sulfate. To this

mixture was added an additional 2 volumes of buffer A and the extraction continued for a second 10 min. The phases were separated by centrifugation and the aqueous phase re-extracted at room temperature with an equal volume of a 1:1 mixture of H_2O saturated redistilled phenol and chloroform:isoamyl alcohol (99:1 V/V) for an additional 10 min. After separating the phases by centrifugation, the aqueous phase was precipitated with ethanol. The precipitate was collected by centrifugation at 12,000 x g, washed three times in ethanol and dried under vacuum.

E. Electrophoretic Analysis of RNA

Electrophoresis followed the method of Loening (72). The RNA precipitate was dissolved in deionized water and was diluted with an equal volume of electrophoretic buffer (25 mM Tris acetate, 1 mM Mg^{++} acetate, pH 8.0). The sample was then applied to 0.6 x 8 cm 10% polyacrylamide gels and electrophoresed at 5 mA/gel for 1.5-2 hr. The RNA bands were localized by UV detection ($\sim$ 254 nM) (73) and the gels were sliced into thirty 1 mm sections. Slices were combined in groups of two and counted by liquid scintillation in 10 ml of toluene containing 3% Protosol (New England Nuclear), 0.3% PPO, and 0.03% Me_2POPOP following at least 12 hr in a 37°C shaker bath.

F. Sucrose Gradient Analysis of RNA

Sucrose gradient analysis of RNA isolated from whole cells or nuclei was carried out essentially as described under the appropriate sections. Linear sucrose gradients were constructed

by mixing equal volumes of low and high sucrose concentrations in a 30 ml plexiglass gradient mixer. The solutions were mixed as they were pumped into 16 ml nitrocellulose centrifuge tubes (Beckman-Spinco). Isokinetic gradients (used in polyribosome analysis) were constructed using 8.6 ml of heavy sucrose and 7.4 ml of light sucrose for each 16 ml gradient. The level in the light side of the gradient mixer was made equal to that in the heavy side by means of including a short glass rod in the light chamber. All sucrose solutions were made using RNAse-free sucrose (Schwarz/Mann, special density gradient grade) and were boiled before use. The gradient mixer was routinely cleaned with a solution of approximately 1% SDS followed by a copious rinsing with deionized H_2O. Occasionally it was rinsed with a 0.2% solution of diethylpyrocarbonate.

G. Analysis of Polyribosomes on Sucrose Gradients

Cell cultures were labeled following dilution into fresh medium with 0.3 μCi/ml [^{14}C]uridine for 20-24 hr. Following the experimental treatment, the cultures were harvested by centrifugation at 1000 x g for 5 min at 4°C. The pellet was resuspended in 0.5 ml buffer B including 0.3% Triton X-100. The lysate was centrifuged at 12,000 x g for 10 min and the supernate layered on 15-40% isokinetic sucrose gradients made with buffer B. Centrifugation was at 2°C in a Spinco SW27 rotor for 3 hr at 27,000 rpm. Gradients were fractionated into 0.5 ml fractions and counted

directly by liquid scintillation in 5 ml Hydromix (Yorktown). In some instances, 50 µl of each fraction was spotted on 2.4 cm Whatman 3 MM filter paper circles and cold acid precipitable radioactivity determined (74).

H. Analysis of tRNA per Culture

Twenty ml cultures were grown for 12 hr in the presence or absence of inhibitors. RNA was extracted as described above following the addition of a small amount of very high specific activity [^{3}H]uridine labeled FL cell total RNA in the presence of SDS and phenol. This served as an internal standard controlling for the yield of RNA in each extraction. Following the ethanol precipitation, the samples were layered on linear 5-15% sucrose gradients made in buffer B and centrifuged in a SW27 rotor at 25,000 rpm for 20 hr at 2°C. Following collection of the 4S fraction from each gradient, the absorbance at 260 nM and the ^{3}H radioactivity were measured. The ratio of absorbance to radioactivity was used to compare the tRNA content of the various cultures. In these experiments, cell viability, assayed by trypan blue exclusion, was always greater than 95%.

I. Analysis of radioactive Nucleotides in tRNA

Ten ml cultures were labeled for 2 hr with 5 uCi/ml [5-^{3}H]uridine in the presence or absence of inhibitors. RNA was extracted as described above and following ethanol precipitation each sample was dissolved in 0.5 ml buffer C. Dissolved samples

were layered on 16 ml 15-25% isokinetic sucrose gradients made in buffer C and sedimented at 22,500 rpm at 2°C in the SW27 rotor for 17 hr. The 4S fraction from each gradient was precipitated with ethanol, washed and dried as described previously. Each sample was subjected to alkaline hydrolysis in 0.3 N KOH for 12 hr at 37°C, and uridine separated from cytidine by one-dimensional thin layer chromatography (75). The solvent composition was isopropanol:concentrated HCl:H_2O, 70:15:15. The location of uridine and cytidine nucleotides was determined by UV detection and comparison with standards run on the same thin layer plate. Each spot was cut out, eluted with 0.5 ml deionized H_2O for 30 min at room temperature and counted by liquid scintillation in 5 ml Hydromix.

J. Preparation of Nuclei from FL Cells

Nuclei were prepared from logarithmically growing cultures of FL cells exactly as described by Marzluff et al. (76). Briefly, cells were pelleted by centrifugation at 1000 x g for 5 min at 4°C. The supernatant medium was discarded and the cell pellet was resuspended at approximately 10^7 cells/ml in buffer D. This cell suspension was homogenized in a glass Dounce type homogenizer with 20 strokes of the tight fitting pestle and then mixed with a equal volume of 10 mM Tris Cl, 2 mM Mg(Acetate)$_2$, 2 M sucrose and 0.5 mM DTT, pH 8.0. This mixture was layered over a 5 ml cushion of this same buffer and centrifuged at 17,000 rpm for 90 min in the SW27 rotor at 4°C. The supernatant was discarded and the nuclei were

gently resuspended in buffer E. Nuclei were either used directly or stored at -70°C. Cell counts and nuclear counts were done using a standard hemocytometer and at least 500 particles were counted in each determination.

K. Assay of Nuclear RNA Synthesis

Nuclei were assayed essentially as described by Reeder and Roeder (53) and by Marzluff et al. (76). Incubation was routinely performed in a reaction volume of 0.8 ml containing 2 x 10^7 nuclei, 12.5% glycerol, 5 mM $Mg(Acetate)_2$, 1 mM $MnCl_2$, 25 mM Tris Cl, 0.05 mM EDTA, 2.5 mM DTT, 0.4 mM of three ribonucleoside triphosphates, and 0.05 mM of the fourth ribonucleoside triphosphate. In all cases [^{14}C] or [^{3}H] GTP (New England Nuclear) was the fourth triphosphate and was included at a specific activity of 100 - 1000 dpm/picomole. KCl was also included in the reaction at a concentration of 0.15 M except as indicated. In analysis of total RNA synthesis the sample was dropped into 2 volumes of 10% TCA and after 10 min on ice filtered on glass fiber filters. Filters were dried under a heat lamp or in an oven at 100°C for 15 min and counted by liquid scintillation. In cases where the RNA was to be isolated for further analysis, the reaction was terminated on ice by the addition of an equal volume of buffer A containing 0.5% SDS. The RNA was extracted as described above.

Before analysis on sucrose gradients or polyacrylamide gels, samples were taken up in the appropriate buffers and incubated

with 10 µgm/ml RNAse free DNAse I (Worthington) at 37°C for 15 min. If samples were to be run on gradients, the reaction was terminated by addition of 10% SDS to a final concentration of 1% and the gradients (in 50 mM Tris Cl, 25 mM Na Cl, 5 mM $MgCl_2$, pH 7.5) were run at 21°C. If polyacrylamide gel electrophoresis was to be done, the DNAse I digested sample was diluted with an equal volume of 1 M Na Acetate, pH 4.5 and extracted with two original volumes of H_2O saturated phenol by vigorous vortexing for 2-3 min at room temperature. The phases were separated by centrifugation at 5000 x g for 10 min at 4°C and the organic phase was washed with one-half its own volume of 1 M Na Acetate pH 4.5. The aqueous phases were pooled and precipitated by addition of 2 volumes of 95% ethanol. The precipitates were collected by centrifugation at 5000 x g for 10 min at 4°C, washed two times with 70% ethanol containing 2% K Acetate, pH 5.3 and one time with 95% ethanol. The washed RNA sample was then dried under vacuum. The samples were electrophoresed as described in section II-E above.

L. Preparation of Rat Liver tRNA

Rat liver tRNA was prepared as described under the appropriate heading of the Materials and Methods section of Part II of this thesis.

M. Periodate Oxidation of Rat Liver tRNA

Rat liver tRNA was deacylated according to Sarin and Zamecnik (77). The stripped tRNA was treated with periodate according to Vaughan and Hansen (78). The tRNA sample was dissolved in

0.1 M Na Acetate, pH 4.5. To this solution was added 0.2 vol of 10 mM Na Periodate in 0.1 M Na Acetate, pH 4.5. This mixture was maintained at room temperature for 30 min in a light tight container. After this time period 0.1 vol of a solution containing 1 M ethylene glycol and 0.1 M Na Acetate, pH 4.5 was added and maintained at room temperature for a second 30 min period. Following this the RNA was precipitated from solution by addition of 0.1 vol of 20% K Acetate, pH 5.3 and 2 vol of 95% ethanol.

N. Preparation of Cytoplasmic Extract from FL Cells

FL cells in log phase growth were centrifuged at 1000 x g for 5 min at 4°C. The pelleted cells were resuspended at 10^8 cells/ml in buffer E and homogenized in a glass Dounce type homogenizer with 20-30 strokes of the tight fitting pestle. The homogenate was spun at 30,000 x g for 30 min at 2°C. The supernatant was saved and the pellet discarded. The supernatant constituted the cytoplasmic extract. Protein was measured by reading the absorbance difference between 228.5 nm and 234.5 nm according to Ehresmann (79). This gave a value of roughly 1-2 mg protein/ml. This preparation was either used directly or stored frozen at -70°C. The amount of cytoplasmic extract used in nuclei incubations was 100 µl per 10^7 nuclei. This corresponded to the amount of extract obtained from 10^7 cells.

O. Inhibitors of Protein Synthesis

Cycloheximide (Actidione) was obtained from Calbiochem.

Puromycin was a product of Nutritional Biochemical Co. Histidinol and O-methylthreonine were purchased from Sigma Chemical Company. Pactamycin was a generous gift of Dr. Gary Neal of the Upjohn Company. The inhibitors were dissolved in deionized H_2O and appropriate volumes added to 5 ml cultures. Equal volumes of deionized H_2O were added to control cultures.

P. Reagents

All chemicals used were reagent grade except as specified otherwise in the text.

III. Results

A. Measurement of Protein Synthesis, Polyribosome Structure, and RNA Synthesis

In order to evaluate the relationship between tRNA synthesis and polysome patterns, it is necessary to measure these parameters in cells treated with one or more inhibitory drugs over a wide effective dose range. Accordingly, protein synthesis was assayed in the presence or absence of each drug by measuring the incorporation of radiolabeled leucine into hot TCA precipitable material. The results are given in Table I-1. It can be seen that drug concentrations were used which span a range from mild to severe inhibition of protein synthesis. The range of inhibition observed with cycloheximide and pactamycin is more limited than that with the amino acid analogues, but this was required to obtain the desired effect on polyribosome structure. The degree of inhibition resulting from the use of histidinol and O-methylthreonine is roughly equivalent to that obtained in cells deprived of essential amino acids (60,66).

Figure I-1 (a-c) shows typical polysome profiles from normal, O-methylthreonine treated, and cycloheximide treated cells. The distribution of ribosomes was quantitated according to Willems and Penman (80) as the percent of total ribosomal material sedimenting in the polyribosome fraction. In normal logarithmically growing FL cells the average fraction which is polysomal (Pn) is about 50%.

Table I-1. Rates of Protein Synthesis

Drug	Concentration µg/ml	[^{14}C]Leucine Incorporation (% of Control)[a]
Histidinol	33	75
	66	25
	132	15
O-methylthreonine	$2.6 \cdot 10^3$	83
	$5.3 \cdot 10^3$	30
	$10.6 \cdot 10^3$	20
Cycloheximide	0.5	18
	5.0	9
	50.0	5
Pactamycin	0.03	23
	0.06	7
	0.3-60	2
Puromycin	1	42
	100	9

[a]Each value represents a single determination.

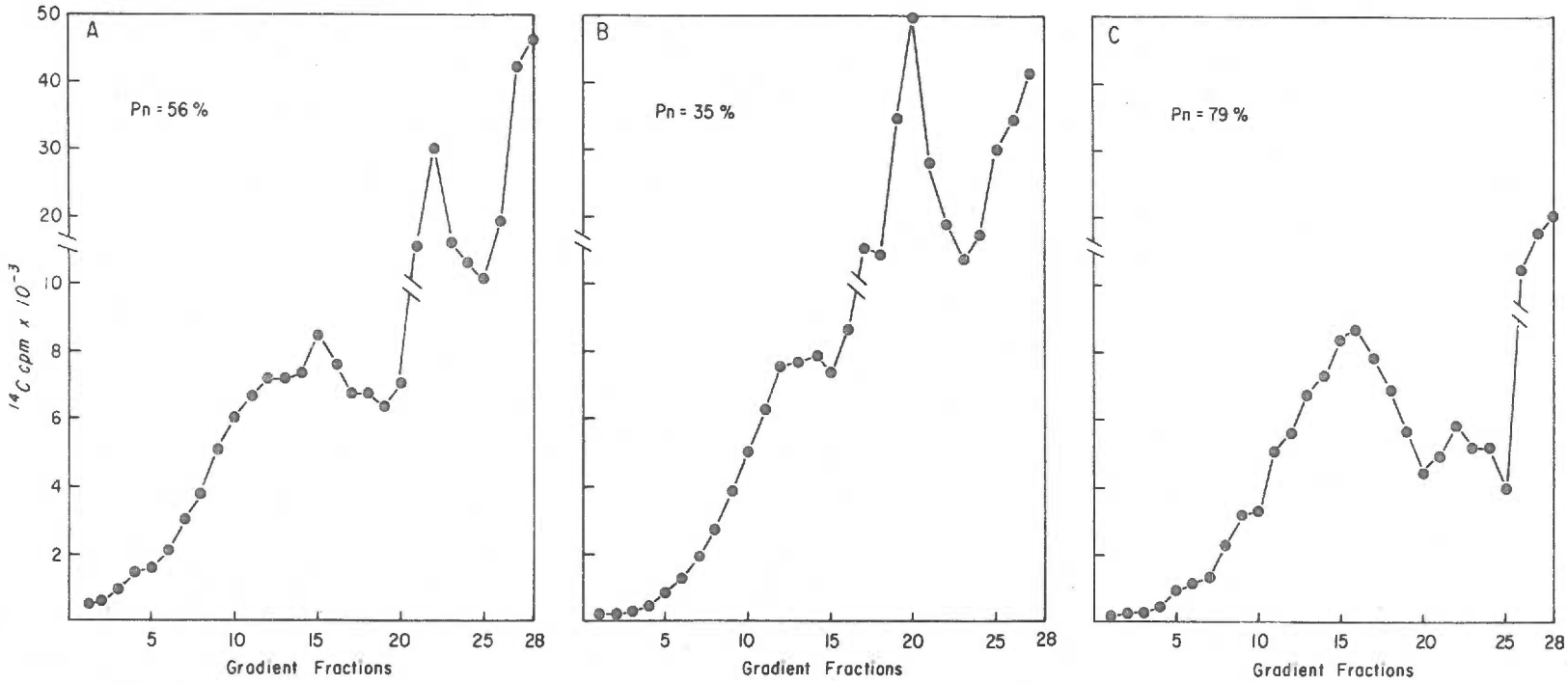

A
Pn = 56 %
B
Pn = 35 %
C
Pn = 79 %
^{14}C cpm x 10^{-3}
Gradient Fractions
Gradient Fractions
Gradient Fractions

Figure I-1

Treatment with O-methylthreonine, which inhibits initiation primarily (78), reduced the Pn to about 30% while treatment with cycloheximide increased the value to about 80%. In addition to the quantitative changes, one can also observe qualitative rearrangement within the polysome region. For example, O-methylthreonine resulted in a decrease of the average polysome size, whereas cycloheximide caused an increase.

tRNA synthesis was measured simultaneously in cell cultures identical to those used to measure polysome patterns. One untreated culture was labeled for 2 hr with [^{14}C]uridine while another untreated culture and several cultures treated with varying concentrations of drugs were labeled with [^{3}H]uridine. Following the labeling period, equal volumes of ^{14}C and ^{3}H labeled cultures were combined, and the RNA was extracted and electrophoresed on 10% polyacrylamide gels. The ^{3}H and ^{14}C radioactivity in the region of 4S RNA was determined. Figure I-2 shows a typical electrophoretic pattern from untreated cultures. The first small peak represents 5S ribosomal RNA, the major peak, 4S RNA, and the small shoulder preceding the 4S peak represents pre-4S RNA (66). The radioactivity in the 4S plus pre-4S region was summed and the ^{3}H to ^{14}C ratio calculated. This ratio was used to compare relative rates of 4S RNA synthesis. As the extraction method minimizes the extraction of nuclear RNA and the 5S RNA peak is not included in the calculation, it is presumed that the calculated rates of synthesis pertain

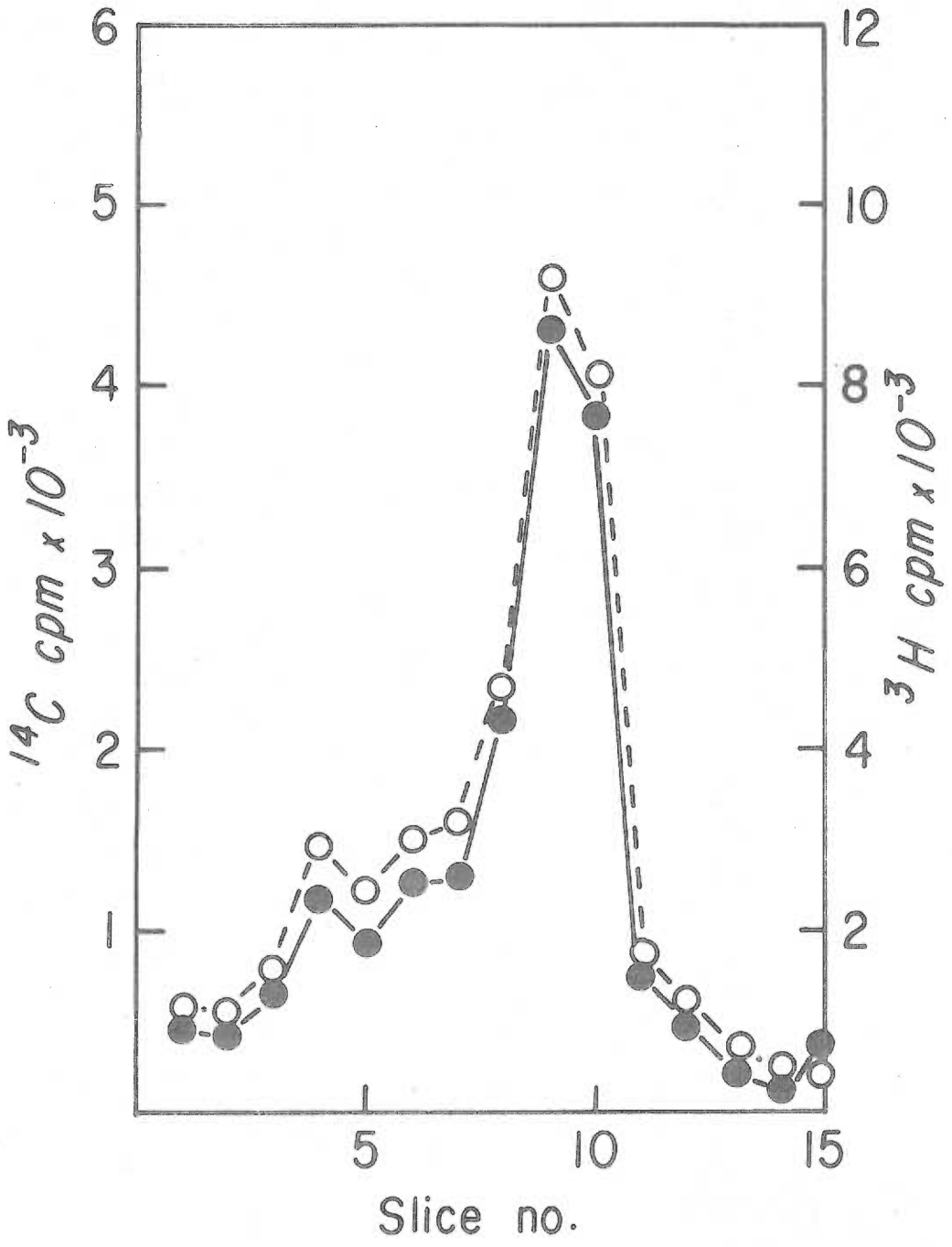


Figure I-2

primarily to tRNA. In some experiments the RNA was separated by sucrose density gradient centrifugation and the 4S peak analyzed as above. In this case the 5S contaminant represented less than 5% of the preparation.

B. Effect of Histidinol and O-methylthreonine on tRNA Synthesis and Polyribosome Structure

The inhibitory effect of amino acid deprivation on protein synthesis derives from limiting the availability of the immediate precursor to peptide synthesis, aminoacyl tRNA (78). Rather than deprive cells of the amino acids themselves, we have utilized two amino acid analogues which result in competitive inhibition of specific aminoacyl tRNA formation: histidinol, which inhibits the activation of histidine (81) and O-methylthreonine, which blocks isoleucine activation (82). The inhibition of protein synthesis produced by these drugs occurs primarily at the site of initiation of protein synthesis and results in the disaggregation of polysomes. The molecular mechanism is believed to involve deacylated tRNA (78, 83). Both O-methylthreonine and histidinol were used in dose ranges which inhibited protein synthesis from 20 to 80% (Table I-1). Table I-2 shows the relationship between the inhibitor concentration, the Pn and the rate of tRNA synthesis during a 2 hr labeling period. The data are expressed as a percentage of control cell values and demonstrate clearly a correlation between polysome breakdown and the decrease in

Table I-2. Relative Pn and Rates of tRNA Synthesis in Histidinol and O-methylthreonine Treated FL Cells.

Drug	Concentration µg/ml	Pn (% of Control)[a]	tRNA Synthesis (% of Control)[a]
Histidinol	33	69	82
	66	65	71
	132	59	69
O-methylthreonine	$2.6 \cdot 10^3$	79	85
	$5.3 \cdot 10^3$	71	81
	$10.6 \cdot 10^3$	63	48

[a]Values are from a single experiment.

tRNA synthesis. Each value represents a single experiment and it can be seen that the data for the two drugs agree well, indicating that the inhibition of tRNA synthesis is probably dependent upon a common effect of both inhibitors. The use of amino acid analogues as opposed to the deprivation of individual amino acids suggests that the absence of the amino acid itself is not responsible for the observed effect on tRNA synthesis. Rather, the limited availability of aminoacyl tRNA or perhaps the presence of abnormally high levels of deacylated tRNA are more directly responsible for the observed changes in both polysome structure and tRNA synthesis. It should be pointed out that the measurements of polysome patterns were made following 2 hr in the presence of the drugs and thus represent the state of the polysomes at the end of that time period. The measurement of RNA synthesis averages the rates of synthesis occurring throughout the labeling period.

C. Effect of Cycloheximide on tRNA Synthesis and Polyribosome Structure

Cycloheximide inhibits protein synthesis primarily by interferring with the process of elongation (84). Thus ribosomes queue up along the messenger RNA as their rate of movement along it is suppressed. The effect of this drug is also dose dependent and the dose range used in these experiments inhibited protein synthesis by 80-95% (Table I-1). Table I-3 demonstrates the effect of cycloheximide on the Pn and the rate of tRNA synthesis. The degree of

Table I-3. Relative Pn and Rates of Protein Synthesis in Cycloheximide, Puromycin, or Pactamycin Treated FL Cells.

Drug	Concentration µg/ml	Pn (% of Control)[a]	tRNA Synthesis (% of Control)[a]
Cycloheximide	0.5	147	(152) 125,176,158
	5.0	149	(142) 116,167
	50.0	128	(159) 105,173,198
Puromycin	1.0	54	103
	10.0	63	(102) 103,100
	100.0	85	(129) 119,138
Pactamycin	0.3	(100) 100,100	(122) 136,123,108
	3.0	(73), 66, 79	(128) 103,150,130
	60.0	(105) 102,108	(140) 110,139,172

[a]Each value represents a single experiment. Numbers in parentheses represent the mean where applicable.

polysome aggregation increases to 150% of control and then is moderately decreased at the highest dose. The synthesis of tRNA is also stimulated an average of 50% beyond control values, thus clearly exhibiting a correlation between tRNA synthesis and the change in polyribosome structure.

The data presented here concerning the effect of amino acid analogues and cycloheximide on both ribosome distribution and the rate of tRNA synthesis confirm the findings of Bölcsföldi (69). The rate of synthesis of this class of RNA is not directly dependent on the rate of protein synthesis. Rather, there is an apparent correlation between the ribosome distribution and the rate of tRNA synthesis such that polysome breakdown occurs with an inhibition of tRNA synthesis and conversely, polysome aggregation accompanies stimulation of tRNA synthesis.

D. Effect of Pactamycin and Puromycin on tRNA Synthesis and Polyribosome Structure

If the rate of tRNA synthesis is dependent upon the state of polyribosomes, a drug which causes polysome breakdown should inhibit tRNA synthesis. Alternatively, a drug which causes, in a dose dependent fashion, either breakdown or aggregation of polysomes should result in a differential effect on tRNA synthesis. Puromycin has been used routinely as an inhibitor of protein synthesis and acts as an analogue for the aminoacyl adenosine terminus of charged tRNA, accepting the nascent peptide chain from

the peptidyl tRNA molecule on the ribosome (84). Pactamycin has been shown to inhibit initiation in low concentrations and both initiation and elongation in higher concentrations (85,86). Consequently, low doses result in polysome breakdown while higher doses give mild aggregation. Using these two drugs, one can test the hypothesis developed above. Table I-3 shows the results of experiments done using puromycin. The Pn is most severely affected at the lowest puromycin concentration used (1 µg/ml) and nearly returns to control values as the dose is increased to 100 µg/ml. However, tRNA synthesis is not inhibited but rather is stimulated with the highest degree of stimulation being observed at the highest dose. These results, in agreement with observations made by Willis et al. (68), contradict those obtained by Bölcsföldi (69), and suggest that tRNA synthesis may be independent of the polyribosome state. This possibility is further supported by experiments involving pactamycin as seen in Table I-3. Here, as the pactamycin concentration is increased, the Pn value initially decreases and then increases slightly above control values in a fashion consistent with its specific mode of action on protein synthesis. Again tRNA synthesis is stimulated independently of the ribosome distribution. The numerical variation observed in these experiments is large, yet in a total of 23 separate experiments involving these three drugs

the rate of tRNA synthesis was always elevated over control values. The qualitative consistency of these observations suggests that these changes are significant. It seems apparent from this data that the proposed correlation between tRNA synthesis and polysome structure does not exist. While a role for tRNA in regulating its own rate of synthesis is not ruled out by these findings, a need for re-evaluation of available evidence is indicated.

E. Effect of Cycloheximide and Histidinol on the Kinetics of tRNA Synthesis

All the experiments so far presented deal with 2 hr drug treatment and radiolabeling periods. In order to assess the short and long term effects of these treatments, tRNA synthesis was assayed at times ranging from 30 min to 8 hr in cell cultures treated with either cycloheximide or histidinol. The results of these experiments are shown in Table I-4. In the presence of 0.5 µg/ml cycloheximide, the rate of tRNA synthesis is stimulated throughout the initial 90 min period, with a maximum rate being observed between 30 and 60 min after the addition of the inhibitor. Beyond 2 hr the rate of synthesis decreases rapidly and by 6-8 hr after drug administration, is inhibited by nearly 70%. While this observation is in contrast to that of Willis et al. (68), it seems reasonable to predict that such severe inhibition of protein synthesis for long periods of time might result in an inhibition of much cellular metabolism, including transcription.

Table I-4. Kinetics of tRNA Synthesis.

Time	tRNA Synthesis (% of Control)[a]	
	Cycloheximide (0.5 µg/ml)	Histidinol (66 µg/ml)
0-30 min	128	100
30-60 min	145	84
60-90 min	123	77
2-4 hr	55	73
4-6 hr	34	67
6-8 hr	31	65

[a]Each value represents a single experiment.

Histidinol at 66 µg/ml shows no effect on tRNA synthesis until 30-60 min after administration of the drug. This is consistent with results reported by Vaughan et al. (78) who routinely preincubated cells for 40 min in the presence of histidinol before beginning assays of protein synthesis. The maximum degree of inhibition (about 30%) is observed by 90 min and the rate remains depressed to this degree throughout the 8 hr period being examined. The difference in the magnitude of inhibition at late time periods (4-8 hr) between cycloheximide and histidinol treated cells can probably be accounted for by the difference in severity of the protein synthesis inhibition observed at the drug concentrations being used (Table I-1).

F. Effect of Histidinol and Cycloheximide on the 12 Hour Accumulation of tRNA in FL Cells

The total amount of tRNA per culture was measured after 12 hr in the presence or absence of either histidinol or cycloheximide at doses equivalent to those used in the previous section. Table I-5 shows that by 12 hr after administration of the drugs the total amount of tRNA per culture is reduced to approximately 30-40% below control values regardless of the drug being used. This reduction in tRNA content does not represent a decline in the total amount of tRNA originally present per culture but rather a failure to accumulate tRNA at a rate equal to control cells. Since cell viability was always greater than 95%, cell death in

Table I-5. Twelve Hour Accumulation of tRNA per Culture.

Drug	tRNA/Culture (% Control)[a]	Cells/Culture (% Control)[a]	tRNA/Cell (% Control)[a]
Cycloheximide 0.5 µg/ml	73±9 (4)	64±9 (4)	116±13
Histidinol 66 µg/ml	63±5 (2)	76±8 (2)	84±3

[a]Each value represents the mean ± 1/2 range. The number in parenthesis is the number of experiments from which the data was obtained.

inhibited cultures cannot account for this decline.

It is possible to estimate the rate of tRNA synthesis necessary to maintain a steady state concentration in cells which are growing exponentially. If the average number of tRNA molecules per cell = 2.5×10^7 and the cell doubling time is 24 hr, then every 12 hr the number of tRNA molecules per original cell must have increased to $\approx 3.5 \times 10^7$. Since the half life for tRNA in exponentially growing FL cells is 72 hr (88) then approximately 0.25×10^7 molecules of the initial 2.5×10^7 will have been degraded at the end of 12 hr. Hence, the total synthesis must provide 1.25×10^7 new tRNA molecules per original cell every 12 hr. If the amount of tRNA per culture is reduced by 30% in inhibited cells, then the number of tRNA molecules per original cell after 12 hr would be 2.45×10^7. Now since 0.25×10^7 molecules of the original 2.5×10^7 have been degraded (assuming no change in the $t_{1/2}$ for tRNA), then the total amount of tRNA synthesized would be only $2.45 \times 10^7 - 2.25 \times 10^7 = 0.20 \times 10^7$ molecules, or about 16% of control. Thus if effects on the rate of synthesis of tRNA by both cycloheximide and histidinol are to account for the reduction in the amount of tRNA per culture, they would have to inhibit the synthesis of tRNA throughout the 12 hr period by an average of 85%. However, measurement of tRNA synthesis in the presence of either drug, as shown in Table I-4, demonstrates that the average inhibition is only 30-40%. Thus in order to account for the

reduction in tRNA per culture, it is necessary to propose that the half life for tRNA has been reduced by a factor of 2-3. Table I-5 also demonstrates that the number of cells per culture has failed to increase in inhibited samples to roughly the same extent as observed with tRNA. Thus, in comparing inhibited cells to control cells, I am also comparing a non-growing population to a growing population. It is interesting to note in this regard that the measured half life of tRNA in non-growing cell populations is 36 hr compared to 72 hr in growing cells (87,88). Therefore, it is reasonable to conlude that an effect of either inhibitor on the 12 hr accumulation of tRNA is to result in an increased rate of degradation of tRNA. Since the total amount of tRNA per cell also remains fairly constant (Table I-5, differences shown may not be significant), this suggests that the rate of tRNA accumulation is limited at least in part by the cellular growth rate.

G. Effect of Inhibitors on Radiolabeling of Cellular Nucleotide Pools

The methods used for measuring the rate of RNA synthesis assumes that the specific radioactivity of the precursor pool remains unaffected by the experimental treatment. If, for example, the treatment of cells with an inhibitor resulted in an increased or decreased specific activity of this pool, the measurement of incorporated radioactivity would not accurately reflect the actual rate of synthesis of the molecule in question. In spite of this

problem, the specific activity of the pool has not been measured for several reasons. 1) There is evidence available suggesting the existence of multiple uridine phosphate pools, only one of which would represent the immediate precursor for transcription of tRNA (89-92). Hence, any measurement of specific activity of the acid soluble cellular uridine pool would not provide meaningful information. 2) Willis et al. (68) have measured the specific radioactivity in the UTP pool in chick embryo fibroblasts treated with cycloheximide and though they observed a variation, the change was in a direction which would result in underestimating the degree of stimulation of RNA synthesis in our experiments. 3) Bölcsföldi and Eliasson (60) measured both [^{14}C]uridine and [^{14}C]methylmethionine incorporation into tRNA in Chang's liver cells deprived of one essential amino acid and found identical results regardless of the nature of the label, indicating that the uridine pools are not affected under these conditions. 4) If the inhibition of protein synthesis per se were to affect the specific activity of the uridine pools, the effect should be identical regardless of the method used to achieve this inhibition. Since the results reported here and elsewhere clearly demonstrate opposite effects on tRNA synthesis by various drugs, it is concluded that the observed variations in tRNA synthesis are not simply a function of variation in the uridine specific activity.

Cells grown with radiolabeled uridine convert a significant

percentage of label into cytidine. Since the 3' C-C-A terminus of tRNA turns over independently of transcription, it is possible that in short term labeling experiments there may be preferential labeling of tRNA. If any of the inhibitors used affected either the extent of uridine to cytidine conversion or the rate of C-C-A turnover, then any differential labeling patterns observed might potentially be explained through such a mechanism. In order to control for this possibility, the tRNA from cells labeled with [^{3}H]uridine for 2 hr, both drug treated and control, was subjected to alkaline hydrolysis, the nucleotides separated by thin-layer chromatography and the percentage of total radioactivity in uridine and cytidine determined. The results are expressed in Table I-6. The degree of conversion and labeling appears to be the same within the limits of experimental error, indicating that C-C-A turnover probably does not contribute to any differences observed in the radioactive labeling pattern of tRNA. In light of the above considerations it is presumed that the measured incorporation of radioactive label into tRNA adequately represents the actual rate of synthesis of this class of RNA.

H. Transcription of tRNA in Isolated FL Cell Nuclei: Preparation and Properties of the Cell-free System

In order to elucidate the regulation of tRNA synthesis, it is necessary to have an experimental system which is free of problems such as those outlined in the previous section. Hence,

Table I-6. Radioactivity in Cytidine from tRNA

Drug (μg/ml)	% Radioactivity in Cytidine[a]
Control	37
Cycloheximide (5)	33
Pactamycin (3.0)	31
O-methylthreonine ($5.3 \cdot 10^3$)	35
Histidinol (66)	30

[a]Each value represents a single experiment.

we need a system in which the chemical environment can be more closely controlled while normal structure and function of the transcription apparatus remains for the most part intact. Such a system would allow for testing the effects of substances normally excluded from the system and would eliminate non-specific effects on the nucleotide precursor pools. Marzluff et al. (56,76) have recently published several reports using isolated nuclei from mouse myeloma cells and their results suggest that the properties of this system satisfy these criteria at all levels including the in vivo like specificity of the in vitro product.

Before applying it to problems concerning the regulation of tRNA biosynthesis, the system was characterized. The nuclei were prepared as described in Materials and Methods and the final nuclear pellet was resuspended at a concentration of approximately 10^8 nuclei per ml. Examination of these preparations by light microscopy indicated that they contained primarily intact nuclei with little or no visible cellular debris or intact cells. In all experiments radiolabeled GTP was used to assay RNA synthesis. Figure I-3 shows the time course of total incorporation of GTP at two different temperatures. At 37°C the initial rate of synthesis is very rapid but by 10 min after the start of the reaction, incorporation of label ceases. However, when the incubation temperature is decreased to 25°C the initial rate of incorporation is mildly decreased but the synthesis of RNA continues for up to 60

Figure I-3

Effect of Temperature on the Kinetics of RNA Synthesis by Isolated FL Cell Nuclei. Duplicate 1.0 ml reactions containing 2.5 x 10^7 nuclei were incubated at either 25°C or 37°C with [^{14}C]-GTP (48.4 Ci/mole, 100 µCi/ml) at a specific activity of 100 dpm/pmole. At 2,5,10,20,30, and 50 min, duplicate 50 µl samples were taken and processed as described under Materials and Methods. (O--O, 25°C; ●--●, 37°C)

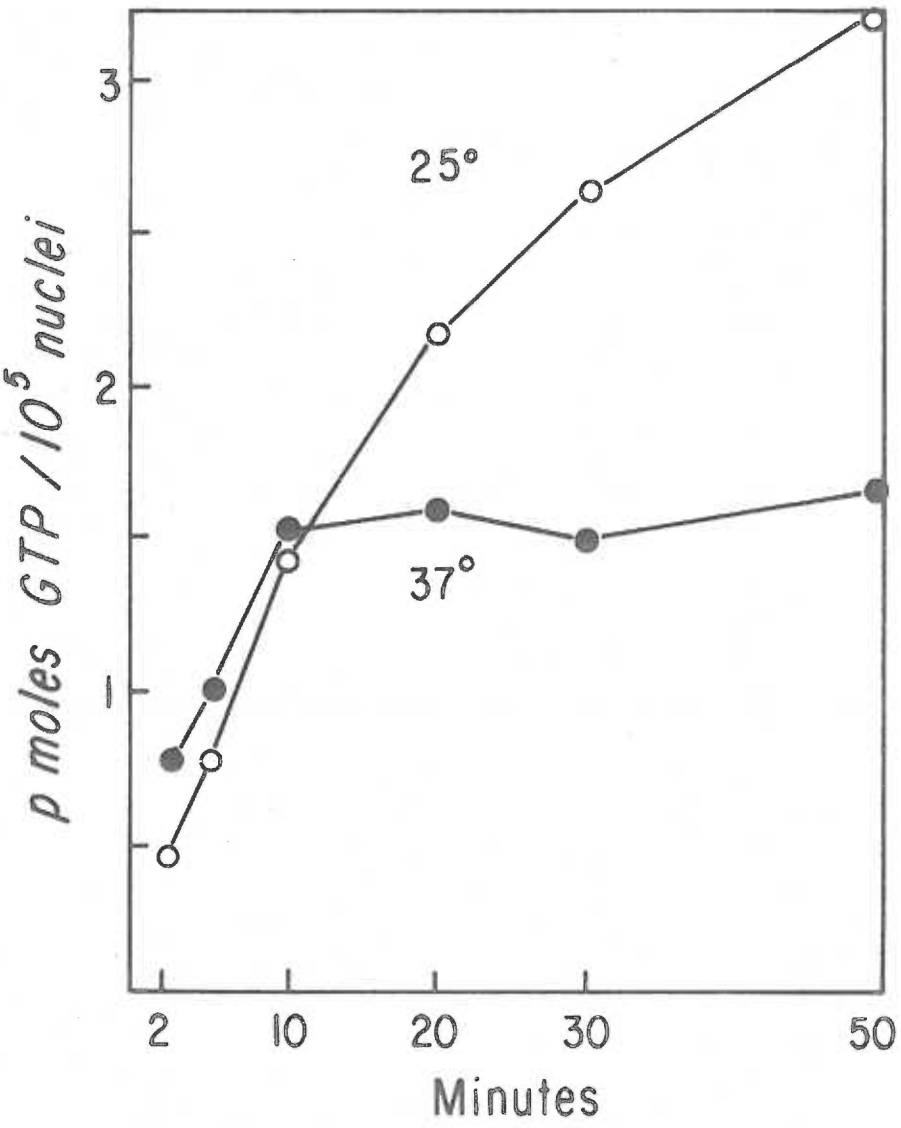


Fig. I-3

min in a nearly linear fashion. Thus, while initial early rates of synthesis at the two temperatures may be different, at 25°C the total incorporation is about 2-3 times that observed at 37°C. This effect of temperature on the kinetics and extent of incorporation was also observed by Marzluff et al. (76). At 25°C the average rate of synthesis presented in this experiment is 0.85 picomoles of GTP per μgm DNA per 10 min. This value agrees very well with that given by Marzluff et al. (56,76) of 0.6-1.0 picomoles of GTP per μgm per 10 min.

Figure I-4 shows that the incorporation of labeled precursor is linearly dependent on the amount of nuclei included in the incubation buffer. This dependence is seen whether the incubation period is 15 or 30 min and demonstrates that the measurements being made are not dependent upon non-specific adsorption of label to surfaces or to non-specific components of the reaction mixture. In addition, the incorporation of GTP was largely dependent upon all four ribonucleotides being included in the reaction mix. Roughly 30% of the total incorporation in 15 min occurred in the absence of ATP and/or UTP but this could probably be accounted for by nucleotides which are isolated with the nuclei as no step was included in the preparation to exclude such precursors.

The eukaryotic DNA dependent RNA polymerases exhibit different ionic strength preferences (51). While both enzymes I (A) and II (B) exhibit clear optima, the enzyme of interest to the

Figure I-4

Effect of Nuclei Concentrations on RNA Synthesis by FL Cell Nuclei. Duplicate 100 µl reactions containing nuclei as indicated and [^{14}C]-GTP were incubated at 25°C for 15 or 30 min. At the appropriate time samples were removed and processed as outlined under Materials and Methods. (●--●, 15 min; O--O, 30 min)

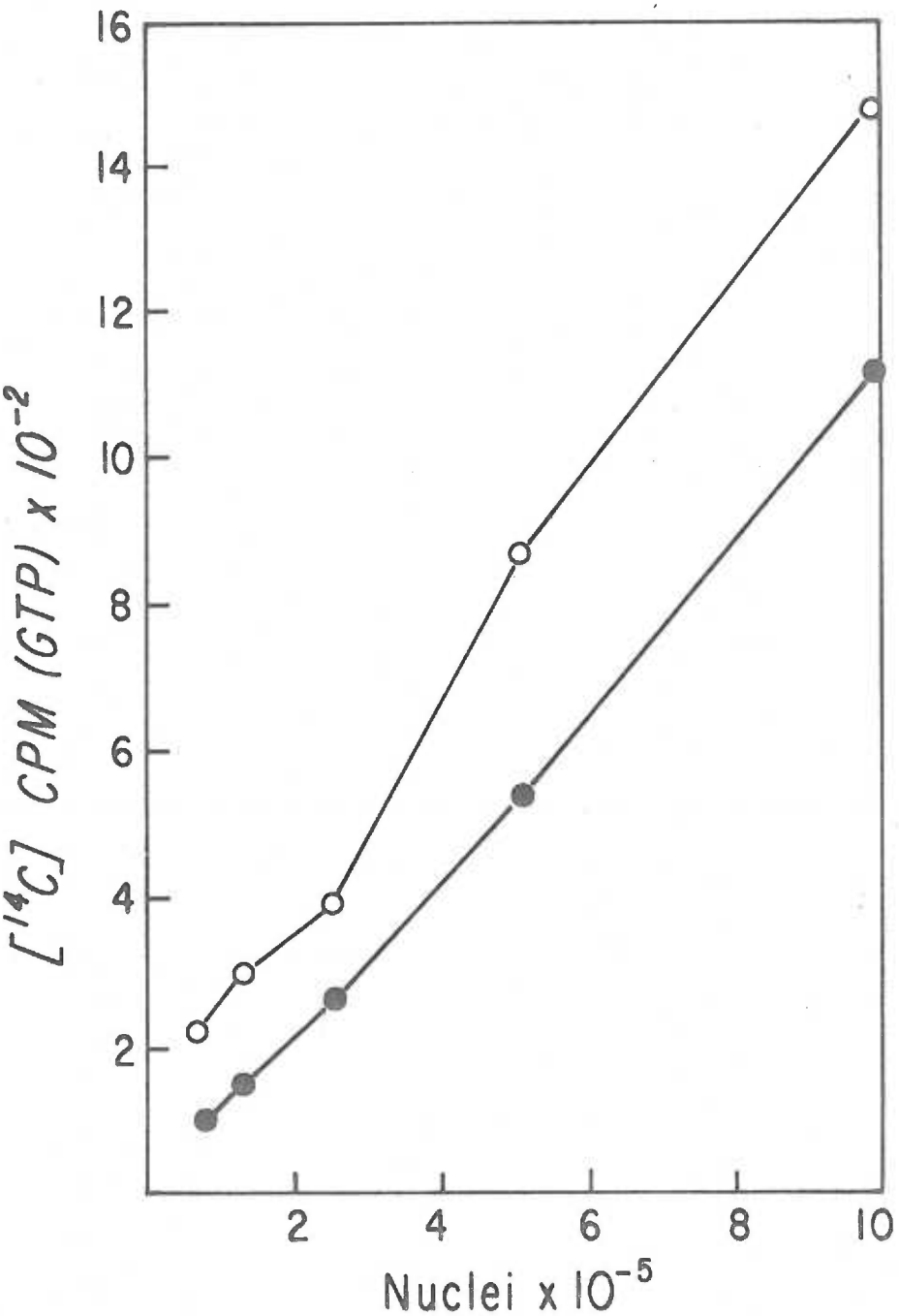


Fig. I-4

present discussion [III (C)] shows constant activity over a wide ionic strength range (57). The data presented in Figure I-5 show the results of a study of the ionic strength dependence of total RNA synthesis in the system employed here and indicate an optimum value of 0.1 M KCl. This value is consistent with data presented by Marzluff et al. (76). In all experiments to follow an ionic strength of 0.15 M KCl has been employed in order to duplicate the technique of Marzluff et al. (56).

Figure I-6 shows the distribution of RNA synthesized in vitro on 10-70% sucrose density gradients. Samples were taken at either 15 or 30 min after the start of the incubation. A significant proportion of the product is of high molecular weight, sedimenting faster than 28S rRNA. This product probably represents both 45S precursor rRNA and heterogenous nuclear RNA. However there is also a significant proportion of the product which is of low molecular weight and which sediments in a distinct peak close to the 4S RNA marker. This low molecular weight product represents about 10-15% of the total transcript both at 15 and 30 min. In addition, the distribution is remarkably similar between the two time points. This similarity is consistent with the concept of initiation of new chains in vitro as discussed in more detail below.

I. Effect of Aminoacyl tRNA, Deacylated tRNA and Cytoplasmic Extract on the Synthesis of Low Molecular Weight RNA by FL Cell Nuclei

Figure I-5

Effect of KCl Concentration on RNA Synthesis by FL Cell Nuclei. Duplicate 50 µl reactions containing 10^6 nuclei, [^{14}C]-GTP, and KCl as indicated, were incubated at 25°C for 15 min. Each sample was then processed as outlined under Materials and Methods.

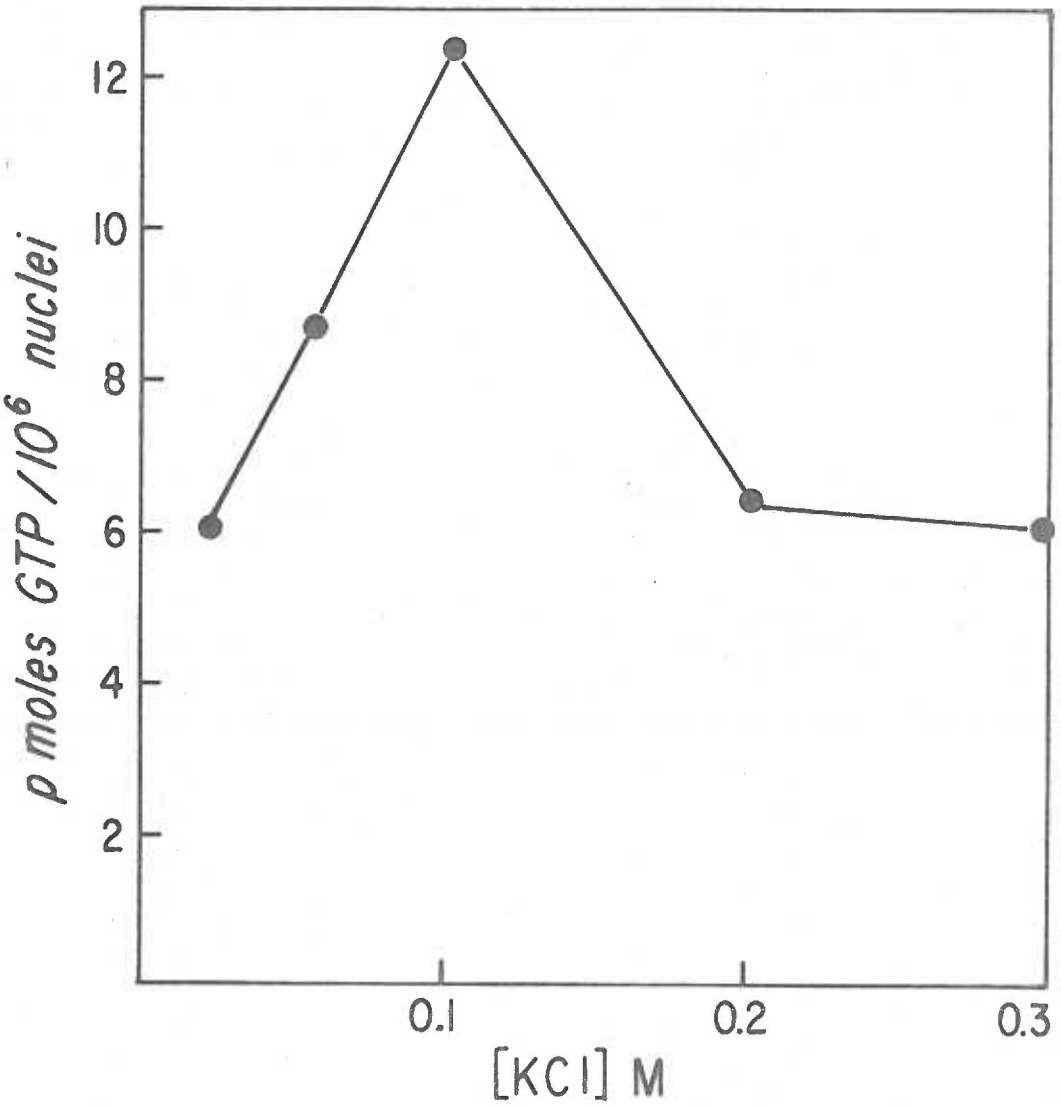


Fig. I-5

Figure I-6

Sucrose Gradient Analysis of RNA Synthesized by FL Cell Nuclei at 15 and 30 min. Two 1.6 ml samples containing 4×10^7 nuclei and [^{14}C]-GTP were incubated at 25°C for either 15 or 30 min. At the end of the incubation period, each sample was extracted and prepared for sucrose gradient analysis as described under Materials and Methods. Before the extraction ribosomal RNA and tRNA carrier were added to act as markers in the gradients. Each sample was sedimented on 16 ml 10-70% linear sucrose gradients made in TNM (50 mM Tris Cl, 25 mM Na Cl, and 5 mM $MgCl_2$, pH 7.5) for 24 hr at 25,000 rpm at 21°C in the Spinco SW27 rotor. 0.5 ml fractions were collected, the A_{260} of each fraction was read in a Gilford spectrophotometer and each fraction was precipitated with 10% TCA and 1.2 A_{260} units of yeast carrier tRNA. The precipitated fractions were collected on glass fiber filters (Reeve Angel) and, after drying, counted by liquid scintillation.

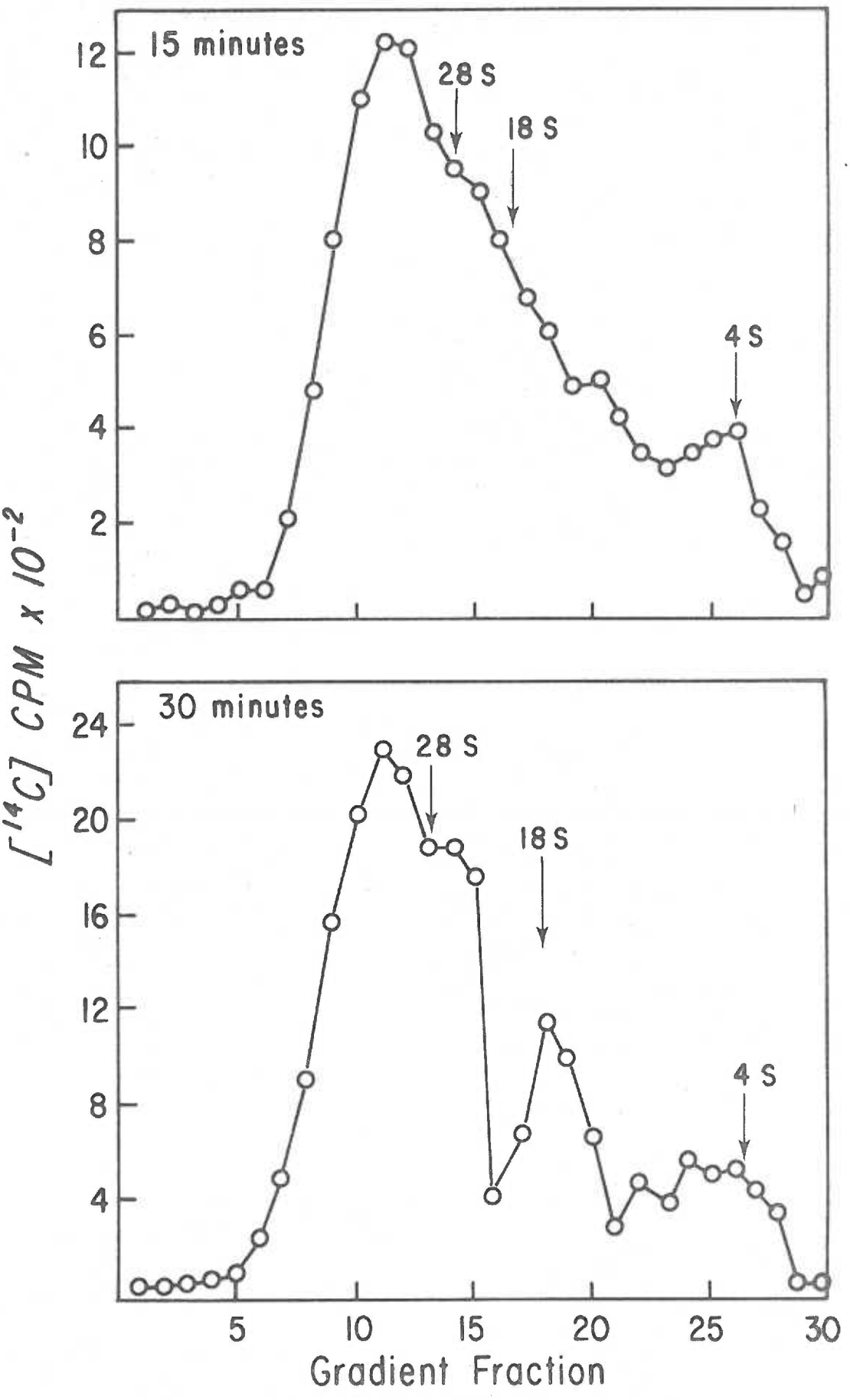

15 minutes
28 S
18 S
4 S
30 minutes
28 S
18 S
4 S
[14C] CPM x 10-2
Gradient Fraction

Fig. I-6

The experiments to be described in the following pages employ two independent variables. The first variable involves the addition of exogenous tRNA in various states of aminoacylation to the cell free RNA synthesis system. This experiment is prompted on the thought that perhaps acylated or deacylated tRNA might play a role in regulating its own transcription. The second variable involves the inclusion in the incubation mix of an extract of cytoplasm from FL cells. This latter variable was included because it seemed reasonable to suspect that a regulatory scheme involving tRNA might require the action of a cytoplasmic factor. The use of these two variables is more thoroughly described below.

In a preliminary experiment it was determined that the nuclear preparations contained significant aminoacyl tRNA synthetase activity as they were able to cause the incorporation of [^{14}C] leucine into acid precipitable RNA. Because of this activity, it was necessary to treat tRNA with periodate prior to use in the cell free system in order to destroy the amino acid acceptance. Thus deacylated tRNA used in the experiments to be described was first oxidized at the 3' terminus. Aminoacyl-tRNA was prepared in vitro with 15 amino acids present according to directions for aminoacylation of tRNA as described in the Materials and Methods section of Part II of this thesis. This procedure was shown to result in aminoacylation of approximately 50-60% of the theoretical maximum amino acid acceptance. The assumption was made that the remaining

tRNA would be acylated during the in vitro incubation with isolated nuclei. While this assumption is very likely invalid, no method for preparing quantitatively (100%) acylated tRNA is available.

The cytoplasmic extract, which actually represented a 30,000 x g supernatant of a cell lysate, had no detectable effect on the total amount of RNA being synthesized by the nuclei. However it did have a significant effect on the size distribution of the low molecular weight RNA product. Figure I-7 (A,B) shows the polyacrylamide gel electrophoretic pattern of RNA being made by the nuclei. In both experiments an aliquot of a preparation of [^{14}C] total FL cell RNA was added to each sample after extraction to serve as a marker on the gels. Panel A of Figure I-7 shows the size distribution of RNA made in the absence of the cytoplasmic extract. In this case the majority of the material migrates between 4S and 5S RNA, a size which is characteristic of the precursor to tRNA (56,66, 93). Panel B shows the effect of including the cytoplasmic extract in the incubation reaction. In this case, a significant percentage of the material now migrates with the 4S band, indicating that cleavage and modification of the primary transcript have occurred.

These results provide two tentative conclusions. 1) A significant proportion of the in vitro low molecular weight product probably represents primary transcripts of tRNA because of its initial size in the absence of cytoplasmic extract and because it can be converted to a smaller molecule which co-migrates with in

Figure I-7

Effect of Cytoplasmic Extract on the Size Distribution of RNA Synthesized by FL Cell Nuclei. 1.0 ml reactions containing 2.5×10^7 nuclei and [^{3}H]-GTP (5.6 Ci/mmole, 0.5 mCi/ml) at a specific activity of approximately 1000 dpm/pmole were incubated in the presence or absence of a cytoplasmic extract of FL cells at 25°C for 30 min. The reaction was terminated on ice and approximately 20,000 cpm of [^{14}C] labeled total FL cell RNA was added. Each sample was then processed and electrophoresed on 10% polyacrylamide gels as described under Materials and Methods. (^{3}H, ●--●; ^{14}C, O--O)

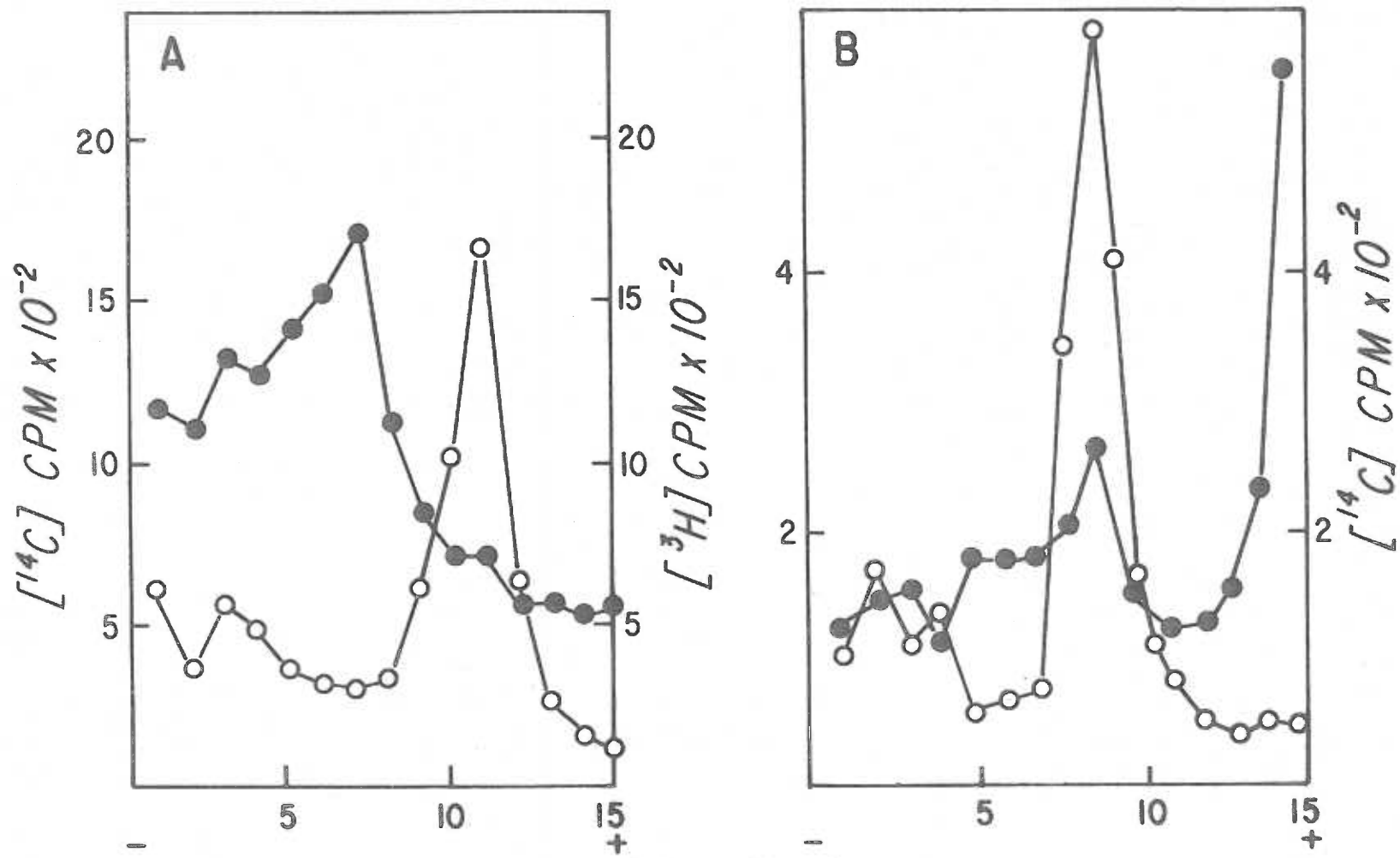


Fig. I-7

vivo synthesized 4S RNA. Such a size shift is one criterion for identification of pre-tRNA (56,66,93). This shift to a smaller size is not a result of nuclease contamination of the extract since incubation of the RNA product with the extract at 37°C for 30 min does not alter its size distribution or acid precipitability (data not shown). 2) While the ability to cause apparently specific changes in the product's characteristics argues that the extract was present in the reactions at a sufficient concentration, it cannot be ruled out that factors which might participate in the regulation of tRNA transcription might be highly labile under the experimental conditions employed or present in undetectable quantities.

The experiments were conducted in a fashion similar to those described in whole cells. Nuclei were labeled with [^{3}H]-GTP for 30 min in the presence or absence of aminoacyl tRNA or periodate oxidized tRNA and plus or minus cytoplasmic extract. The RNA was extracted from each sample in the presence of equal sized aliquots of [^{14}C] labeled total FL cell RNA which were included to control for yield within each sample. The RNA was sedimented on 5-15% linear sucrose gradients and the radioactivity in the 4S region was determined after TCA precipitation. Figure I-8 shows a typical sedimentation pattern. Only the top half of the gradient is shown in the figure. It is clear that, on gradients of this nature, which have better resolution in the low molecular weight range than those presented in Figure I-6, the product of the isolated

Figure I-8

Sucrose Gradient Analysis of Low Molecular Weight RNA Synthesized by FL Cell Nuclei. A 1.0 ml reaction containing 2.5×10^7 nuclei and [^{3}H]-GTP was incubated at 25°C for 30 min. The reaction was terminated on ice and 20,000 cpm of [^{14}C] labeled total FL cell RNA was added. The sample was then processed for sucrose gradient analysis as described under Materials and Methods. The processed sample was sedimented on a 16 ml 5-15% linear sucrose gradient made with TNM at 25,000 rpm and 21°C for 24 hr in the SW27 rotor. The top half of the gradient was fractionated into 0.5 ml fractions which were precipitated with the addition of 50 µgm DNA and 1.0 ml 10% TCA. Precipitated fractions were collected on HAWP, 24 mm, filters (Millipore), dried briefly and counted by liquid scintillation as follows. To each filter was added 0.3 ml 1.5 N NH_4OH and the vials were capped at room temperature for 30 min. 0.2 ml of 2 N HCl was then added and the samples were counted in Hydromix. Spillover from the [^{3}H] channel into the [^{14}C] channel was reduced to negligible levels and [^{14}C] spillover into the [^{3}H] channel was approximately 20%. (^{3}H, ●--●; ^{14}C, O--O)

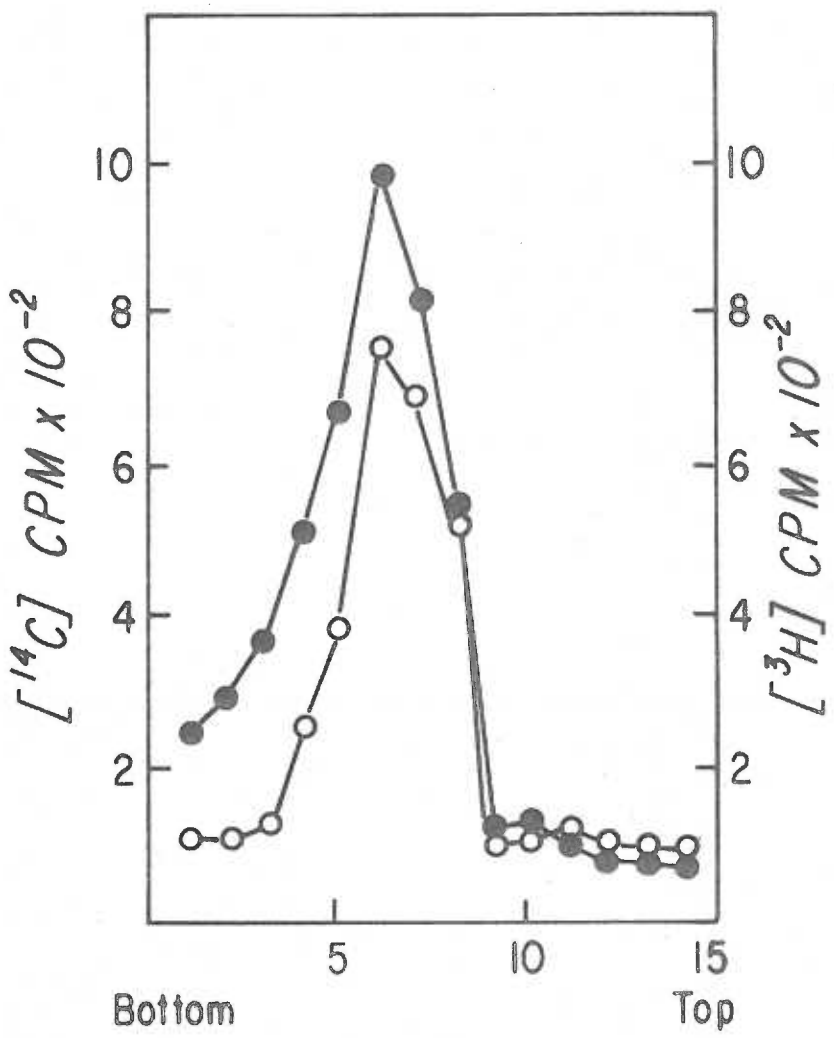

10
8
6
4
2
[14C] CPM x 10-2
[3H] CPM x 10-2
5
10
15
Bottom
Top
Gradient Fractions

Fig. I - 8

nuclei system contains a component which cosediments with cellular tRNA. While this product is shifted slightly to the heavy side of the 4S peak, this sedimentation behavior is consistent with the size distribution of this low molecular weight material as visualized by 10% polyacrylamide gel electrophoresis (see Figure I-7). The $^{3}H/^{14}C$ ratio of the peak was calculated for each sample and used for comparison of the relative rates of tRNA synthesis. While this peak is clearly heterogeneous in size, it is assumed that the majority of the material included represents tRNA and pre-tRNA.

The results of the experiment to test the effects of exogenous tRNA on tRNA synthesis are shown in Table I-7. All values are given as percent of control samples (i.e., samples which were not treated with tRNA). It is clear that there is no significant difference between any of the experimental samples or their appropriate controls. Thus tRNA, added to nuclei either in a partially aminoacylated or deacylated periodate oxidized state, causes no effect on the transcription of low molecular weight RNA or total RNA regardless of whether or not an extract of normal cytoplasm is present.

Table I-7. Effect of Periodate Oxidized tRNA or Aminoacyl tRNA on tRNA Synthesis by Isolated Nuclei in the Presence or Absence of Cytoplasmic Extract.

	PERCENT OF CONTROL	
TREATMENT	4S RNA[a]	TOTAL RNA[a]
+Extract, +P.O.[b] tRNA	99	96
-Extract, +P.O.[b] tRNA	92	92
+Extract, +A.A.[c] tRNA	90	119
-Extract, +A.A.[c] tRNA	99	108

[a]Each value represents a single determination.

[b]P.O. tRNA = periodate oxidized tRNA.

[c]A.A. tRNA = aminoacyl-tRNA.

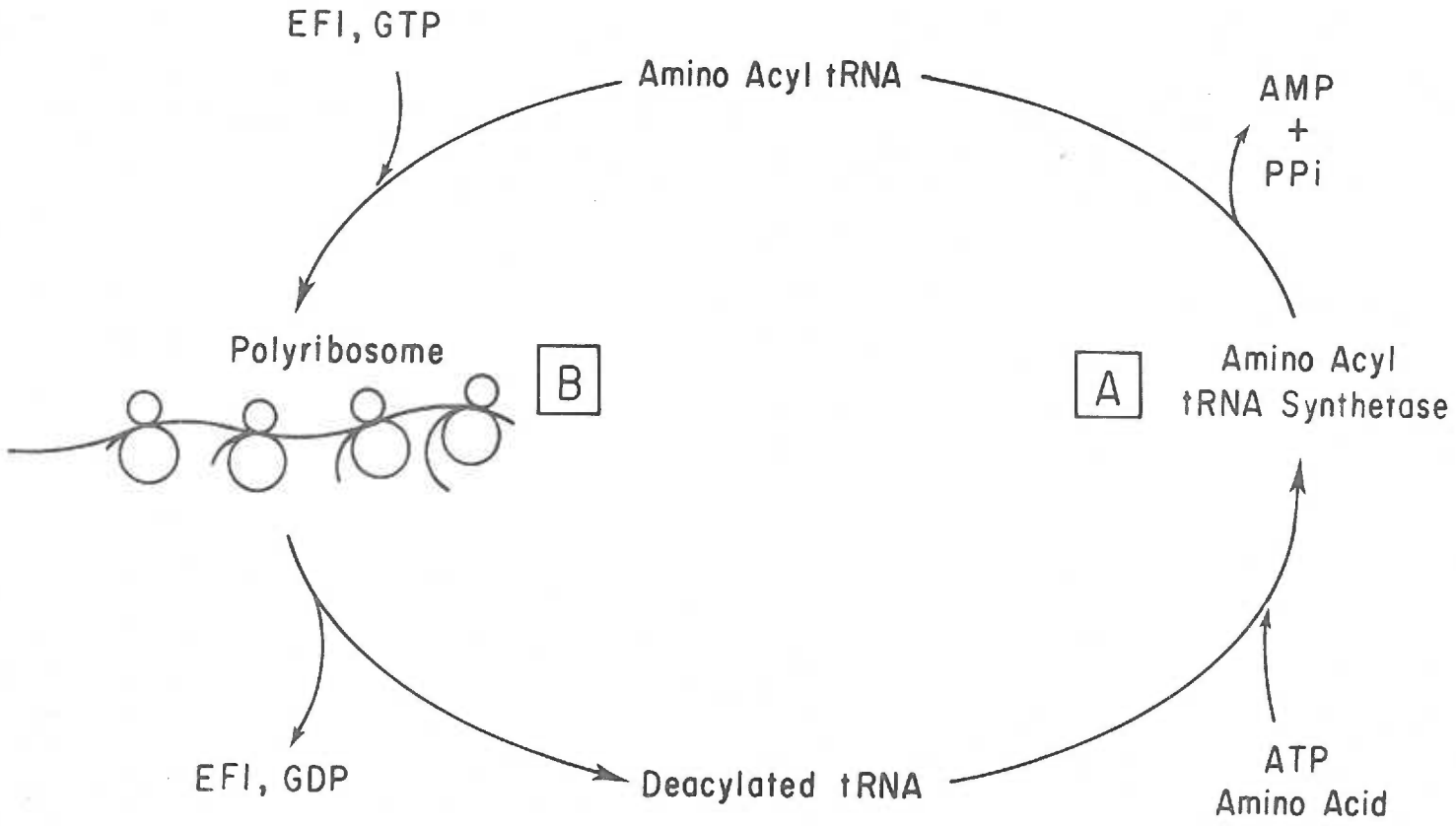
IV. Discussion

The experiments reported in the present communication represent an attempt to confirm a correlation between the fraction of total ribosomes involved in polysomes and the rate of tRNA synthesis, as reported by Bolcsfoldi (69). By using the amino acid analogues histidinol and O-methylthreonine or the antibiotic cycloheximide to inhibit protein synthesis, an apparent dependence of tRNA synthesis on polyribosome structure was observed. However when puromycin or pactamycin were used, at concentrations which cause either polysome breakdown or polysome aggregation, the rate of tRNA synthesis demonstrated no such dependence.

A reasonable explanation of the available data involves the aforementioned consideration that the effect of amino acid deprivation, or the administration of an amino acid analogue, limits the rate of protein synthesis by limiting the availability of the immediate precursor to peptide synthesis, aminoacyl tRNA. As shown schematically in Figure I-9, this treatment results in a block at the level of aminoacylation of a tRNA (point A) and will subsequently result in accumulation of that tRNA in a deacylated state. Alternatively, the use of antibiotic inhibitors of protein synthesis will block the tRNA cycle at the point of entry of charged tRNA into polysomes (point B) and thus should result in the accumulation of aminoacyl-tRNA at the expense of deacylated tRNA.

Figure I-9

Cycle of tRNA in Protein Synthesis.



CYCLE OF tRNA in PROTEIN SYNTHESIS

Figure I-9

This idea is supported by the work of Neidhardt (94) on a mutant of *E. coli* with a temperature sensitive valyl-tRNA synthetase. When the mutant is exposed to the restrictive temperature, protein synthesis is severely inhibited and the in vivo extent of $tRNA^{Val}$ aminoacylation is reduced to less than 25%. Simultaneously, the in vivo extent of $tRNA^{Leu}$ aminoacylation (and presumably other tRNA species) is increased to nearly 100%. Similar results were reported by Lewis and Ames with a series of histidine regulatory mutants (36). While histidinol or O-methylthreonine would also be expected to raise the level of aminoacylation for non cognate tRNA species, it is presumed that the total amount of deacylated tRNA will actually increase under such conditions.

Vaughn and Hansen (78) showed that histidinol and O-methylthreonine resulted in a reduction of the relative degree of charging of histidine and isoleucine tRNA, respectively, by 40% or more, thus demonstrating the accumulation of uncharged tRNA in cells grown under these conditions. Allen et al. (95) measured the actual levels of charging of tRNA in livers from rats starved for one or more essential amino acids and found that under normal conditions most tRNAs were 80-90% acylated, while rats maintained on diets deficient in one essential amino acid exhibited average charging levels for the tRNA in question of 56%. Smith and McNamara (96) found, for different amino acids, that the fraction of tRNA that is charged in rabbit reticulocytes varied between 71

and 97%. Therefore, it is reasonable to predict changes in the charging levels of specific tRNAs on the order of 30%. This degree of variation is clearly in a range which could be significant if the relative level of charging of tRNAs was a regulatory signal involved in tRNA synthesis. Thus the relative level of deacylated tRNA could be the molecular signal stimulating the observed changes in tRNA synthesis; inhibitors which may cause an accumulation of charged tRNA (cycloheximide or pactamycin) and thereby reduce the level of uncharged tRNA would result in an increased rate of synthesis, whereas inhibitors causing an accumulation of deacylated tRNA (histidinol or O-methylthreonine) would result in decreasing that rate.

The application of inhibitors such as those used in the current study is often open to criticism since they tend to have many toxic side effects. However, the fact that the rate of tRNA synthesis is stimulated in a characteristic, uniform manner by three drugs of highly different chemical and biological characteristics is convincing evidence against such a possibility.

In agreement with Willis et al. (68), I observed that a 2 hr incubation with puromycin caused a lesser degree of stimulation than observed with cycloheximide even when protein synthesis was most severely inhibited. While cycloheximide and pactamycin both inhibit the movement of ribosomes either onto or along the mRNA, puromycin acts primarily to inhibit the completion of functional

proteins (84). Thus, I am unable to predict the effect of puromycin on the entry or exit of tRNA from polysomes and consequently upon the extent of aminoacylation of tRNA.

The rate of synthesis of tRNA observed in cells treated with histidinol or O-methylthreonine is reduced to as little as 50% of control values, yet the average individual class of tRNA represents only 5% of the total tRNA pool. Hence, the inhibition must be expressed on the synthesis of most if not all classes of tRNA. Since a single amino acid analogue inhibits specifically the acylation of a single class of tRNA, it is proposed that a single kind of deacylated tRNA has the capacity to inhibit the rate of synthesis of most or all classes of tRNA.

The concept of a regulatory role for deacylated tRNA is not new and has precedent in both prokaryotic and eukaryotic systems. Of special interest is the involvement in the bacterial stringent response where accumulation of stable RNA (rRNA and tRNA) is severely limited in response to amino acid deprivation (43). The signal for activation of this regulatory scheme requires an uncharged tRNA bound to ribosomes in a codon dependent fashion (45). More recently Aboud and Pastan (97) have demonstrated a requirement for uncharged tRNA in the activation of transcription of lac messenger RNA in *E. coli*. Other investigators have demonstrated that uncharged tRNAs have the capacity to specifically interrupt the initiation of protein synthesis in eukaryotic cells and cell-free

systems (78,83). Hence, the involvement of deacylated tRNA in the regulation of transcription and translation may have been conserved in some fashion during the transition from prokaryote to eukaryote.

The work reported here assessing tRNA synthesis in isolated nuclei does not provide experimental support for a role of deacylated tRNA acting by inhibiting its own synthesis directly at the gene level. However, the validity of this data and the conclusions derived from it depend heavily on a number of assumptions involving the properties of the experimental system.

A primary consideration is whether or not new tRNA chains are being initiated in vitro. This is of paramount importance since the rate of initiation is a prime candidate as a site for regulatory phenomena. Thus, if tRNA chains are not reinitiating in vitro, incorporation is due solely to elongation and accordingly, only regulation which affects the rate of elongation of previously initiated chains would be observed. There is now a considerable body of evidence, compiled in several laboratories and involving at least four independent criteria, which suggests that at least low molecular weight transcripts are initiated in vitro under the conditions employed in the present study. 1) Synthesis of 4S and 5S RNA sequences are synthesized continuously throughout a 30 min period (56). If the incorporation of label into this population were solely due to elongation, then synthesis of these sequences, being very small relative to pre-rRNA and Hn RNA should

cease within several minutes at the rate at which transcription has been measured to occur in this system (52). In addition, the amount of 4S RNA made corresponds to about 10,000-20,000 copies per haploid genome. The mouse haploid genome has been estimated to contain approximately 4500 copies of tRNA genes (56,98). Hence, every gene would have to be copied 2-4 times during the incubation. However the size of the tRNA genes are such that a limit of two polymerase molecules could be bound at any one time [assuming a binding site occupying 40 nucleotides (99,100)]. Hence it seems reasonable to suggest that some initiation of chains must be occurring for these low molecular species of RNA. 2) Synthesis of 5S RNA is believed to be initiated with GTP at the 5' terminus (102). Labeling of RNA in vitro with γ-[$^{32}PO_4$]-GTP results in the incorporation of a significant amount of radioactivity into pppGp residues from alkaline hydrolysates of 5S RNA. However, a large proportion (≈80%) of both in vitro and in vivo synthesized 5S RNA has pGp as the 5' residue. Hence detection of initiation using this technique is difficult. Nevertheless, several laboratories have reported successful demonstration of initiation in the isolated nucleus system (54,56,102). 3) Incubation of nuclei in the presence of heparin, which inhibits initiation of transcription but allows elongation (102,103), abolishes incorporation of radiolabeled precursor into 4S and 5S RNA, indicating that in the absence of initiation very few 4S and 5S sequences are produced. 4) The sequential addition of two

different radioactive isotopes of the same precursor at different times to a system that is only elongating will necessarily result in differential incorporation of the two isotopes. Knowing the time of addition of the second isotope, it is possible to predict the ratio of the two isotopes in the RNA product. If reinitiation is occurring in vitro then a diminution in the predicted ratio will be observed. Using this system Udvardy and Seifart (52) demonstrated that initiation of low molecular weight RNAs occurred with a relatively high frequency, whereas ribosomal RNA precursor chains were initiated only to a limited extent.

In addition to the consideration of initiation frequency in vitro, the experimental system employed here has been shown to synthesize RNA with size characteristics very similar to that made in vivo during very short pulses and to transcribe only the correct strand for 5S rRNA (56). Hence, it has been concluded that the system provides a reasonable model for the in vitro study of transcriptional processes in eukaryotes. Since the nuclei I have used perform identically to those described in the literature with respect to the molecular weight distribution of the product, the ionic strength optimum, the total amount of incorporation per nucleus, and the temperature sensitivity, I have assumed that this system possesses those characteristics described above which have not been measured in the present study. On the basis of this reasonable assumption, the conclusions derived from utilizing this

system can be considered to be valid.

The observation that the addition of acylated or deacylated tRNA to isolated nuclei in the presence or absence of a cytoplasmic fraction has no significant effect on the amount of tRNA being synthesized does not rule out the possibility that the in vivo degree of aminoacylation of tRNA acts as a regulatory signal indirectly. In this regard, Grummt and Speckbacher have recently observed the in vitro degradation of GTP to guanosine in the presence of ribosomes, uncharged tRNA and two factors isolated from the microsomal wash fraction (105). This observation, together with the demonstration that rRNA synthesis appears to be regulated, during amino acid deprivation, by decreases in the pool size of the initiating nucleotides ATP and GTP (64,106), suggests a possible role for deacylated tRNA in regulating the synthesis of rRNA. While there is no direct experimental evidence to support the notion that such a scheme might apply to the regulation of tRNA biosynthesis, it would appear to be consistent with the available data in both whole cells and in isolated nuclei.

The physiological role of a mechanism such as that considered above is, of course, still to be determined. It is possible that the observation represents a eukaryotic version of the stringent response, such that total tRNA synthesis is linked to the amino acid supply for protein synthesis, thereby conserving energy under conditions of limited availability of one component of the system. A

natural question arising out of the current study is how the inhibitors employed here might affect individual classes of tRNA. Since no assessment has been made of the functional characteristics of the RNA examined here, additional experiments will be required to investigate this area.

V. Summary

The rate of tRNA synthesis in cultured Friend leukemia cells has been examined as a function of the variation in polyribosome structure produced by treatment with a variety of inhibitors of protein synthesis. The results indicate, in contrast to the conclusions of Bolcsfoldi (69), that no necessary relationship exists between the ribosome distribution and the rate of tRNA synthesis.

Alternatively, it is observed that inhibitors of tRNA aminoacylation cause, in all cases, a decrease in the rate of tRNA synthesis, whereas drugs which may stimulate the aminoacylation of tRNA cause, in all cases, an elevation of the rate of tRNA synthesis. It is concluded that tRNA synthesis in mammalian cells may be regulated by the relative levels of acylated and deacylated tRNA.

Further studies using isolated nuclei from FL cells suggest that tRNA synthesis is not regulated by deacylated tRNA directly at the gene level.

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Abbreviations

isoaccepting tRNAs	A series of chemically different RNA molecules with the capacity to accept the same amino acid.
RPC	Reversed Phase Chromatography
BD-cellulose	Benzoylated diethylaminoethylcellulose
DBAE-cellulose	Dihydroxyborylaminoethyl-cellulose
GTP	Guanosine 5' triphosphate
GDP	Guanosine 5' diphosphate
FL cells	FSD-1 line of Friend virus infected murine erythroleukemia.
A_{260}	Absorbance of 260 nm wavelength light
A_{260} unit	The amount of a material which has an absorbance of one when dissolved in 1 ml of solution and measured with a pathlength of 1 cm.
TCA	Trichloroacetic acid
PPO	2,5-diphenyloxazole
Me_2 POPOP	1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene
S	Sedimentation constant at 20°C in H_2O
isoleucyl-$tRNA^{Ile}$	That group of structurally heterogeneous RNA molecules which accept isoleucine and are esterified to isoleucine. This kind of designation is the same for any amino acid specific tRNA acylated with its cognate amino acid.

PART II

CHARACTERIZATION OF A NEW TECHNIQUE FOR THE PURIFICATION OF ISOACCEPTING FAMILIES OF TRANSFER RNA

I. Introduction

A. Statement of the Thesis Objective

In recent years a number of laboratories have examined the relationship between the relative concentration of isoaccepting families of transfer RNA and the requirement for the cognate amino acid in protein synthesis (1-3). This interest was initially motivated by the speculation that limiting amounts of specific isoaccepting tRNAs might be involved in the regulation of specific protein synthesis in highly differentiated cell types (4-8). While there is some indirect experimental evidence to support this contention (6,7) it is far from conclusive. However, in the course of such investigation it has become quite clear that the relative concentrations of specific tRNA families accurately reflect the amino acid composition of the proteins being synthesized by the cell type under examination (1-3). This phenomenon is not strictly limited to cells involved in the synthesis of primarily a single type of protein but can be observed in cells which make a wide variety of protein products such as liver and even some bacterial species (3). Hence the expression of the various tRNA genes within a cell, while appearing superficially constitutive, must be

regulated via some kind of fine tuning mechanism.

On the basis of this concept it becomes interesting to consider the regulation of the accumulation of specific tRNAs as well as their potential role in regulating other cellular processes. This problem can be considered as composed of two related aspects. 1) What is the intracellular signal which initiates the sequence of events leading to an alteration in the relative rate of accumulation of a specific tRNA? 2) What is the mechanistic route through which this alteration is achieved? In order to investigate either aspect effectively, it is essential to have at one's disposal a technique which will allow the quantitative examination of the accumulation of a single family of tRNA.

The second part of this thesis, then, involves the examination and characterization of a technique for the isolation of a family of isoaccepting tRNAs from an unfractionated preparation of tRNA. The interest in and need for such a technique stems from this interest in the metabolism of amino acid specific families of tRNA. Thus, if we wish to study the mechanisms by which the relative cellular concentration of such a family is varied in accordance with changes in the use of the cognate amino acid in protein synthesis, it is necessary to measure both rates of synthesis and of degradation for that family of tRNA as a whole. Determining rates of synthesis and of degradation in growing cells requires the measurement of both the incorporation of radiolabeled precursor and the disappearance of

previously labeled radioactive tRNA. The introduction of radioactive labeling into the experimental design, as a means of identifying a portion of the total tRNA population which is of specific interest, compounds the technical difficulty of such experiments. In order to examine the synthesis or degradation of single families of tRNAs, it is now necessary to utilize an experimental method which has the capacity to distinguish, within this selected population of radiolabeled molecules, a more select group which codes only for one particular amino acid. Thus conventional techniques, which measure the specific in vitro aminoacylation of a preparation of unfractionated tRNA, are insufficient. This is so because the fraction of the total population and the fraction of the radiolabeled population which accept the same amino acid may be significantly different numbers since each represents a distinct entity. Hence, in order to achieve the desired goal it is necessary to isolate, in a pure state, that proportion of the total population which accepts the given amino acid. In so doing, the proportion of radioactivity which copurifies represents the desired information.

B. Currently Available Techniques

There have been a wide variety of methods published in the literature designed to separate and purify isoaccepting tRNAs (9-22). Perhaps the most powerful technique to achieve this end is that developed at Oak Ridge National Laboratories and termed

reversed phase chromatography (11,12). Unfortunately, despite its rather remarkable resolving powers, this technique is of very little value for the isolation of families of tRNA because the method separates each family into many of its component species. The net result is to spread out the family as subspecies over a large part of the elution volume. Thus, while certain isoacceptors may be partially purified, there is little, if any, purification of the group of isoacceptors as a whole.

The most successful approach to this problem is to take advantage of the inherent specificity of the aminoacyl tRNA synthetase enzymes, each of which has an affinity for its own family of isoaccepting tRNAs. Thus, in order to purify a family one reacts an unfractionated population of tRNA with the enzyme preparation and the single amino acid of interest. The desired family of tRNAs can then be isolated by subjecting this specifically charged population to a technique which can distinguish between aminoacylated and deacylated tRNA.

There are several such techniques which have been developed and presented in the literature (10, 13-22). These methods distinguish on a chemical or biological basis between aminoacyl or free tRNA. The following treatment will discuss the specific nature of currently available examples and outline in detail the characteristics of the method we felt was best suited to our needs.

The most thoroughly established method involves the use

of benzoylated DEAE cellulose (BD-cellulose) first developed by Gillam et al. (13-15,23,24). This chromatographic material has a very strong affinity for aromatic moieties on macromolecules and it can be used to fractionate uncharged tRNA preparations, giving much the same result as seen with reversed phase systems. However, by taking advantage of the reactivity of the amino group on the aminoacyl portion of aminoacylated tRNA, one can attach aromatic residues to the molecule. While most tRNAs require only a strong salt solution to elute from the column, aromatically derivatized aminoacyl-tRNA requires strong salt plus 15-20% ethanol. By use of these properties, the single family of tRNA can be quickly and easily isolated.

However for quantitative analysis the problem becomes more complex. There are several potential problems. 1) A certain fraction of the deacylated tRNA contains sites which may react with the aromatic derivatizing reagent during the modification reaction (23,25). Hence, this non-specific fraction will coelute with the desired aminoacylated species. In order to circumvent this problem it is necessary, prior to specific aminoacylation, to derivatize the deacylated tRNA preparation and chromatograph it on BD-cellulose under conditions identical to those to be used in the actual purification step. The material eluting with the salt gradient alone is recovered and that requiring ethanol for elution is discarded. This process, termed "sham acylation" by Powers and Peterkofsky (23),

thus removes that material which would otherwise contaminate the final product. 2) Because of incomplete derivatization of the aminoacyl-tRNA the yield of purified tRNA will not be quantitative (15). This problem will, of course, be potentially involved in any technique which utilizes chemical modification of charged tRNA as a means of distinguishing it from free tRNA. 3) A third potential problem involves the possibility of incomplete charging of a tRNA preparation during the in vitro aminoacylation. Since conditions may vary for optimum charging from family to family, conditions which give complete or nearly complete charging for one amino acid may not for others. Thus, tRNA charged under such conditions and then purified by BD-cellulose chromatography would result in an incomplete yield of the desired family of tRNA. This problem is, of course, potentially involved in any purification scheme utilizing specific in vitro aminoacylation as outlined above. A portion of the experimental work to be presented is directly involved with the examination of this problem.

A second method which distinguishes charged and uncharged tRNA on a chemical basis is that utilizing a variety of dihydroxyboryl substituted chromatographic supports including cellulose, polyacrylamide, and glass (16-19). The technique depends on the ability of cis-diol structures to form cyclic complexes with the immobilized dihydroxyboryl moiety. Thus deacylated tRNA, which possesses the intact cis-diol structure at its 3' terminus, can form this complex under pH and ionic conditions where the anionic form of the borate

group is predominant (16). Alternatively, acylated tRNA, in which the vicinal hydroxyl structure is blocked by the aminoacyl bond, is unable to form the complex. Hence, theoretically, such a technique should allow a rapid separation of the two species by use of a stepwise chromatographic elution from the borate substituted resin. The uncharged tRNA which binds to the resin can be recovered by eluting the column with a buffer of lower pH. Again however, the method is difficult to use for the quantitative analysis of tRNA preparations for a number of reasons. The major problems stem from the unreacted ionic groups attached to the solid matrix (20). For example, if aminoethyl cellulose is used as the solid support, the unsubstituted amino groups tend to bind RNA samples to the resin irreversibly at a pH and ionic strength compatible with maintenance of a stable aminoacyl bond. To correct for this McCutcheon et al. acetylated the unreacted aminoethyl groups on the cellulose resin (20). The extent of acetylation governs the capacity of the resin for tRNA and the ease with which it can be recovered in the elution buffer. Preparation of the support is thus technically difficult. Even with a correctly acetylated cellulose there is a certain amount of nonspecific binding of charged tRNA. Thus while a considerable purification may be achieved, there is not a quantitative yield of the desired family of tRNA. In addition, because of the chemical nature of the method, there is a certain amount of material which, while non-acylated, would behave in a manner similar to

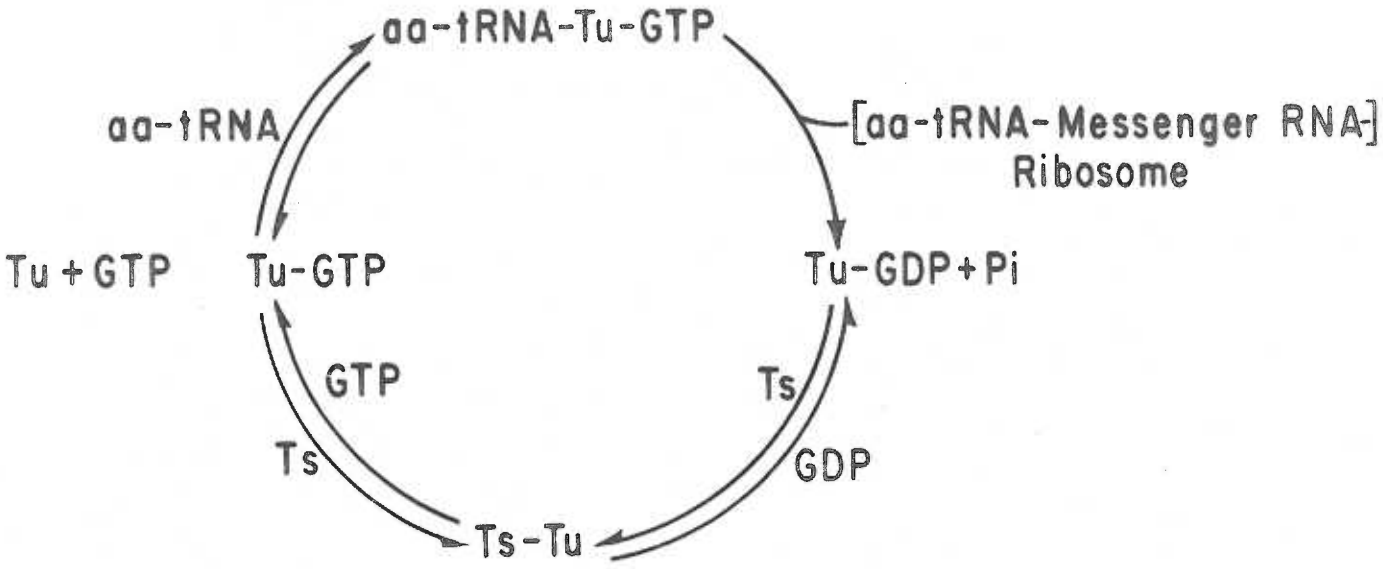
charged tRNA. This material would include RNA fragments with a 3' phosphate and, of course, acylated tRNA which may have survived the deacylating procedure. This material must be initially removed by a pre-run on DBAE prior to in vitro aminoacylation in a manner similar to the sham acylation procedure required with BD-cellulose.

The technique which we chose to examine and develop for use in studying specific tRNA metabolism is one recently reported by Klyde and Bernfield (21,22). Of all the published techniques for separating aminoacyl-tRNA from free tRNA, this is the only one which relies entirely upon biological specificity. For this reason the problems resulting from the non-specific nature of many of the previously described chemically oriented techniques should be minimized.

This technique takes advantage of the inherent specificity of the bacterial protein synthesis elongation factor EF-Tu to recognize and distinguish between aminoacyl tRNA and free tRNA (26,27). EF-Tu and other related bacterial elongation factors, when activated by the binding of GTP, are able to form a ternary complex with all unmodified aminoacyl tRNAs except initiator f-met-$tRNA^{met}_f$ which does not participate in polypeptide elongation (28). The biochemical properties of this ternary complex indicate that it plays a role in protein synthesis as a link between aminoacyl tRNA and the ribosome-messenger RNA complex as outlined in Figure II-1 (29-31). While EF-Tu has a molecular weight of 42,000 daltons (32)

Figure II-1

Taken from reference 31.



Cycle of Tu and Ts in the
Ribosomal Binding of aa-tRNA

Fig. II-1

and tRNA an average molecular weight of 25,000 daltons, the ternary complex formed between aminoacyl tRNA and EF-Tu-GTP has a molecular weight of roughly 65,000 daltons (21). By a combination of specific aminoacylation followed by ternary complex formation between the acylated tRNA and the activated elongation factor, the charged tRNA should be separable from free tRNA by use of any technique which can resolve particles with molecular weights in this range (21,29).

In the course of attempting to develop this technique for routine use, I have been able to demonstrate the formation of ternary complex by two independent criteria. I have, however, made observations which suggest that the value of the technique for the purification of mammalian isoaccepting tRNA families may be quite limited. The experimental section of this presentation will describe the use and characteristics of the method and outline a number of problems which were encountered in the course of the work.

II. Materials and Methods

A. Cell Culture

The FSD-1 line of Friend leukemia cells was maintained according to details given in the Materials and Methods section of Part I of this thesis.

B. Preparation of tRNA

1. Rat Liver tRNA

tRNA was prepared from rat liver by the method of Brunngraber (33). Briefly, rats were fasted for 24 hr and then killed by decapitation and the livers removed and rinsed in ice cold homogenization buffer (1.0 M NaCl, 0.005 M EDTA, and 0.1 M Tris Cl, pH 7.5). The livers were then minced and homogenized in a Waring blender for 5 min with approximately 1.5 vol (1 vol in ml = original weight of liver in grams) of homogenization buffer and 1.5 vol of redistilled H_2O saturated phenol. The homogenate was centrifuged at 10,000 x g for 10 min. The aqueous phase was precipated with 2.5 vol of 95% ethanol and stored at -20°C for more than 1 hr. The precipitate was collected by centrifugation at 5,000 x g. The alcohol supernatant was discarded and the precipitate redissolved in 1 vol of 0.1 M Tris Cl, pH 7.5 containing 10 µg/ml RNAse free DNAse I (2500 U/mg, Worthington) and incubated for 15 min at 37°C. Following the incubation the sample was diluted with two original volumes of 0.1 M Tris Cl, pH 7.5 and applied to a DEAE cellulose (DE-52) column equilibrated with 0.1 M Tris Cl at pH 7.5.

After the column with the RNA had been washed with approximately 10-15 vol of 0.1 M Tris Cl, pH 7.5, the tRNA was eluted with 1.0 M NaCl and 0.1 M Tris Cl, pH 7.5. The absorbance of the effluent was monitored at 260 nm and the peak fractions pooled and precipitated with ethanol. The precipitated RNA was collected by centrifugation at 5,000 x g for 10 min and the precipitate taken up in 1.8 M Tris Cl, pH 8.0 and incubated at 37°C for 90 min to deacylate the tRNA (34). Following this stripping incubation the tRNA was again precipitated with 2 vol of 95% ethanol in the presence of 0.2 M NaCl as above and following centrifugation and washing of the precipitate with 70% ethanol containing 2% K Acetate at pH 5.3 and 95% ethanol, the sample was dried, taken up in deionized H_2O and stored at -20°C at a concentration of approximately 200 A_{260} units/ml.

2. FL Cell tRNA

RNA was radioactively labeled in FL cells by addition of [^{14}C]-uridine (New England Nuclear, 55 mCi/mmole) to a final concentration of 6.0 μM. Cells were incubated at 37°C for 18 to 24 hr and harvested by centrifugation at 1,000 x g for 5 min in the cold. The total RNA was extracted from the cells by a modification of the method of Nienhuis et al. (35) as outlined in detail under the Materials and Methods section of Part I of this thesis. Following the isolation of total cytoplasmic RNA, the tRNA was purified by electrophoresis on 10% polyacrylamide gels. The RNA was dissolved

in deionized H_2O at approximately 250 A_{260} units/ml and diluted with an equal volume of electrophoresis buffer (25 mM Tris Acetate, pH 8.0, 1 mM Mg Acetate) made to 40% sucrose (special density gradient grade, Schwarz-Mann). To this solution was added a small volume of bromphenol blue as a tracking dye and the samples were layered carefully on 8 x 0.6 cm 10% polyacrylamide gels. The samples were electrophoresed at 5 mA per gel for 1.5 to 2 hr, the tRNA band was visualized by UV absorbance (254 nm) (36), cut from the gel with a razor blade and reinserted in the original acrylic tubes. A small dialysis bag filled with the electrophoretic buffer was slipped over the anodic end of the tube, care being taken to avoid entrapment of bubbles between the acrylamide and the buffer. The samples were then subjected to electrophoresis again at 5 mA per gel for an additional 20 to 30 min. The buffer within the dialysis sack was removed, each sack was washed with 0.5 ml of deionized H_2O and the pooled samples were precipitated with 2.5 vol of 95% ethanol as described above. The RNA precipitate was collected on nitrocellulose filters (Millipore, 24 mm, HAWP) and eluted in a small volume of 1.8 M Tris Cl, pH 8.0 at 37°C for 90 min (34). In some cases the tRNA was subjected to an additional stripping procedure according to Scofield and Zamecnik (37). Finally, the tRNA was precipitated with 95% ethanol, collected by centrifugation, washed with 70% ethanol containing 2% K Acetate, pH 5.3 and with 95% ethanol, dried under vacuum and stored at -20°C. The specific radioactivity of

such tRNA preparations was routinely about 3×10^5 cpm per A_{260} unit. Unlabeled FL cell 4S RNA was prepared by velocity sedimentation on sucrose density gradients as described in detail in the Materials and Methods section of Part I of this thesis.

C. Preparation of Elongation Factors

1. Preparation of EF-Tu from *Escherichia coli*

EF-Tu was prepared by M. Litt and C. Lytle from *E. coli* B (Miles Laboratories) harvested in mid log phase growth. The purification scheme was modified after Miller and Weissbach (32) and included centrifugation at 100,000 x g for 60 min, $(NH_4)_2SO_4$ fractionation, DEAE Sephadex chromatography, and Sephadex G-100 gel filtration. Protein was measured according to Lowry (38). The final product was stored in the presence of GDP under liquid nitrogen. The activity of this preparation is described under Results.

2. Preparation of EF-S_3 from *Bacillus stearothermophilus*

Elongation factor EF-S_3, purified from *B. stearothermophilus* was a generous gift of Jan Dijk and David Novelli from the Biology Division of the Oak Ridge National Laboratory. The protein was stored in 50 µl aliquots in sealed glass vials either under liquid nitrogen or at -70°C. The activity of the preparation is described under Results.

D. Preparation of Rat Liver Aminoacyl tRNA Synthetases

Male Sprague-Dawley rats weighing 300 to 400 g were fasted overnight and killed by decapitation. The livers were removed and

rinsed immediately in iced homogenizing buffer (100 mM Tris Cl, pH 7.5, 10 mM $MgCl_2$, 0.35 M sucrose). The livers were minced with scissors and homogenized in 2 vol of homogenizing buffer in a glass Dounce type homogenizer using 10 strokes with a loose fitting pestle and 10 strokes with a tight fitting pestle. The homogenate was centrifuged at 30,000 x g for 10 min and again at 100,000 x g for 60 min. The supernatant was then passed through glass wool to remove fatty material and dialyzed overnight against DE-52 loading buffer (10 mM Na phosphate, pH 7.4, 5 mM KCl, and 10 mM β-mercaptoethanol). The dialyzed sample was applied to a DEAE-cellulose (Whatman DE-52) column (5 ml) equilibrated with DE-52 loading buffer. The column was washed thoroughly (approximately 10-15 vol) with loading buffer and then eluted with DE-52 stripping buffer (10 mM Na phosphate, pH 7.5, 250 mM KCl, 1 mM DTT). The difference in absorbance between 228.5 nm and 234.5 nm in the column effluent was monitored according to Ehresmann (39). This value, referred to as ΔA (228-234), is linear with protein concentration and is unaffected by nucleic acid contamination. In comparison with protein values obtained with the Lowry method on a variety of tissue samples, 1 mg of protein equals approximately 4-5 ΔA (228-234) units. The fractions containing peak ΔA (228-234) values were pooled and passed through a column of Sephadex G-50 equilibrated with DE-52 stripping buffer. Glycerol, previously filtered through glass fiber filter to remove traces of charcoal, was added to a concentration of 40% (V/V) and the product was stored at -20°C.

Activity was retained in these preparations for over six months.

E. In Vitro Aminoacylation of tRNA

In vitro aminoacylation of tRNA was conducted in a reaction mix containing 50 mM Tris Cl, pH 7.5, 10 mM ATP, 0.25 mM CTP, 20 mM $MgCl_2$, 10 mM β-MSH, 50 μM amino acid(s), and [^{3}H] or [^{14}C] amino acid(s) to give specific activities of approximately 50 to 100 dpm per picomole. (In some preparative aminoacylations no radioactive amino acid was added.) In addition the reaction contained, per 0.1 ml vol, approximately 0.1-1 A_{260} units of tRNA and 0.25 mg of aminoacyl tRNA synthetase enzyme. The reaction was started by the addition of enzyme and was carried out at 37°C for 20 or 30 min. The reaction was terminated by the addition of 0.5 ml of 10% TCA and 1 A_{260} unit of yeast carrier RNA. After several minutes on ice, the samples were filtered on glass fiber filters (Reeve Angel, grade 934AH), washed with cold 5% TCA and dried at 100°C for 15 min. Each filter was counted by liquid scintillation in 5 ml of toluene containing 0.3% PPO and 0.03% Me_2POPOP. The efficiency of counting with [^{14}C] was 75%. Conditions of maximum aminoacylation were determined by assaying a constant amount of tRNA with increasing amounts of enzyme. The point where additional enzyme did not increase the incorporation of acid precipitable radioactivity was taken as the amount of enzyme required for maximum amino acid acceptance.

When samples of RNA were to be aminoacylated for use in ternary complex formation the reaction was terminated by the addition

of an equal volume of 1 M Na Acetate, pH 4.5 and 2 vol of H_2O saturated redistilled phenol. The mixture was vortexed vigorously at room temperature for 1-2 min and the phases were separated by centrifugation at 5,000 x g for 10 min. The aqueous phase was recovered and the organic phase washed with one original volume of 1 M Na Acetate, pH 4.5 and treated as before. The final aqueous phases were pooled and precipitated with 2 vol of 95% ethanol. The precipitated RNA was recovered by centrifugation at 5,000 x g for 10 min, washed two times with 70% ethanol containing 2% K Acetate, pH 5.3 and once with 95% ethanol, dried under vacuum and stored at -70°C.

F. Filter Binding Assay of EF-Tu or EF-S_3 for GTP Exchange

The activity of elongation factor preparations was assayed by measurement of radiolabeled GTP binding to millipore filters. Two separate sets of assay conditions were employed and their effect on factor activity is described under Results. They are listed here as conditions A and B.

1. Conditions A

Conditions A are taken from Jan Dijk (personal communication) and were designed for the assay of EF-S_3 from *B. stearothermophilus*. The assay is conducted in a final volume of 1 ml containing 50 mM Tris Cl, pH 7.5, 10 mM Mg $(Acetate)_2$, 160 mM KCl, 1 mM DTT, 1.5 mM phosphoenolpyruvate, 4 µg/ml pyruvate kinase (Sigma, 400 U/mg protein) and 5 µM GTP labeled with [^{3}H] or [^{14}C] to a specific activity of 10

to 500 dpm per picomole. This mixture was incubated at 37°C for 10 min to convert GDP to GTP and a sample of elongation factor containing between 20 and 200 units of activity was added. This mixture is incubated for an additional 10 min at 37°C. The solution is cooled on ice, diluted with approximately 2-3 ml of wash buffer [10 mM Tris Cl, pH 7.5, 10 mM Mg $(Acetate)_2$] and filtered on millipore filters (24 mm, HAMK) which have been presoaked overnight in the wash buffer. The filters are washed two times with 5 ml of wash buffer and dried under a heat lamp. Each filter is counted in 5 ml of toluene containing 0.3% PPO and 0.03% Me_2POPOP.

2. Conditions B

Conditions B are taken from Shelton and Bernfield (22) and represent a modification of the technique of Weissbach et al. (31). The reaction volume was routinely 0.1 ml containing 50 mM Tris Cl, pH 7.5, 10 mM $MgCl_2$, 50 mM NH_4Cl, 1 mM phosphoenolpyruvate, 5 mM DTT, 40 µg/ml pyruvate kinase (Sigma, 400 U/mg protein) and 2.5 µM GTP labeled with [^{3}H] or [^{14}C] to a specific activity from 100 to 500 dpm per picomole. To this solution was added a sample of elongation factor containing 2-25 units of GTP binding activity. This was incubated at 37°C for 5 min, cooled on ice for 5 min and diluted with 1-2 ml of wash buffer (50 mM Tris Cl, pH 7.5, 50 mM NH_4Cl, and 10 mM $MgCl_2$). This solution was filtered on millipore filters (24 mm, HAMK, presoaked overnight in wash buffer), washed twice with 5 ml of wash buffer and dried under a heat lamp. Each

filter was counted as above.

G. Filter Binding Assay of Ternary Complex Formation

1. Conditions A

After forming the EF-Tu-GTP or EF-S_3-GTP complex according to conditions outlined above, the ternary complex with aminoacyl-tRNA was formed as follows: a) For EF-S_3 the aminoacyl-tRNA [dissolved in 10 mM Na Acetate, pH 4.5, 10 mM Mg $(Acetate)_2$] was added to the EF-S_3-GTP solution and incubated for an additional 10 min at 37°C. Following the last 10 min incubation the samples were processed in exactly the same fashion as outlined previously (J. Dijk, personal communication).

2. Conditions B

For EF-Tu the aminoacyl-tRNA (dissolved in 0.5 mM Na Cacodylate, pH 4.5) was added to the sample on ice and kept either on ice or at room temperature for an additional 5 min period, after which it was processed exactly as above under conditions B. The amount of ternary complex formed was taken as the difference between samples of Tu or S_3 to which no aminoacyl-tRNA had been added and those to which tRNA charged with amino acid had been added (22).

H. Polyacrylamide Gel Electrophoretic Analysis of Ternary Complex Formation

The ternary complex, containing [^{14}C] tRNA, formed under conditions B as outlined above, was not diluted with wash buffer but rather with an equal volume of S_3 gel buffer (5 mM Maleic acid, 10

mM NH_4 Acetate, 10 mM Mg Acetate, pH 6.5) containing 40% sucrose (w/v) (Schwarz-Mann, special density gradient grade) and a small volume of bromphenol blue. The samples were layered on 10% polyacrylamide gels made with S_3 gel buffer. These gels had been pre-electrophoresed (5 mA/gel, 45 min) with 5 mM mercaptosuccinic acid, pH 6.5, in the upper buffer chamber. This was done to equilibrate the gels with a sulfhydryl reagent as they cannot be polymerized with such agents in the buffer. The samples were electrophoresed at 5 mA/gel for 7-8 hr in the cold. The gels were sliced into 1 mm sections, combined in groups of two and counted by liquid scintillation according to Petri (40). In double label experiments the spillover of [^{3}H] radioactivity into the [^{14}C] channel was reduced to less than 0.5% and the [^{3}H] radioactivity corrected for the 12% spillover of [^{14}C] radioactivity into the [^{3}H] channel.

I. Fluorescamine Assay of Primary Amine Generation

Primary amine generation by the aminoacyl-tRNA synthetase preparations was followed by the fluorescamine technique of Udenfriend et al. (41). The sample, in a volume of 50 µl, was precipitated with 20 µl of 25% TCA, kept on ice for 10 min and centrifuged at 5,000 x g for 10 min. The supernatant was recovered and added to 1.5 ml of a 0.2 M Na Borate, pH 9.0 solution. To this mixture was added 0.5 ml of a solution containing 20 mg/ml of fluorescamine (Fluram, Roche Laboratories) in acetone while vortexing. The samples were allowed to sit for several minutes at room temperature and then the relative

fluorescence intensity was measured at an excitation maximum of 390 nm and an emission maximum of 485 nm in an Aminco-Bowman spectrophotofluorimeter.

A standard curve was constructed using sample buffer plus amounts of isoleucine varying from 100 picomoles to 10 namomoles. The nanomoles of primary amine detected were determined from the sample fluorescence intensity compared with values derived from the standards.

J. Amino Acid Analysis

Amino acid analysis was done on the Beckman Model 120C automatic amino acid analyzer by Jim Joyce and/or Bert Hansen. Samples were prepared for amino acid analysis by precipitation of the reaction mix with 0.4 volumes of 25% TCA on ice for 10 min followed by centrifugation at 5,000 x g for 10 min. The supernatant was used for amino acid analysis.

K. Reagents

[^{14}C] GTP (48 mC/mmole, 100 µCi/ml) was purchased from New England Nuclear. [^{3}H] GTP (2 Ci/mmole, 1 mCi/ml) was purchased from Amersham/Searle. [^{3}H] isoleucine (65 Ci/mmole, 1 mCi/ml) and [^{14}C] leucine (308 mCi/mmole, 100 µCi/ml) were purchased from New England Nuclear. [^{14}C] isoleucine (342 mCi/mmole, 50 µCi/ml) and the equimolar mixture of 14 [^{14}C] amino acids containing L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-leucine, L-isoleucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine (each at 10 mCi/mmole) were purchased from Amersham/Searle. All other chemicals were reagent grade or as outlined in the text.

III. Results

A. Properties of Elongation Factors from *Escherichia coli* and *Bacillus stearothermophilus*

The approach to this problem can be viewed best as a series of projects, each of which serves as the basis for the one to follow. These stepwise projects involve demonstration of the several biological properties of the bacterial elongation factor (i.e., GTP binding, ternary complex formation with aminoacyl-tRNA), the development of a polyacrylamide gel electrophoretic separation of ternary complex from unbound tRNA, and finally the characterization of this technique with respect to its utility as a general method for purifying eukaryotic tRNA. Throughout these experiments, elongation factor isolated from *B. stearothermophilus* was used and is termed here EF-S_3. In some experiments the elongation factor from *E. coli* was used and is termed here EF-Tu. There appeared to be no significant difference between these two proteins with respect to their biological activities.

The initial experiments involve the demonstration of GTP binding by the S_3 factor. This assay depends upon the ability of nitrocellulose filters to bind the protein (42). Thus, when incubated in a reaction mix containing radiolabeled GTP and an energy source to convert GDP to GTP, the activity is assessed as the amount of radioactive GTP which is retained on millipore filters. The conversion of GDP to GTP is a necessary part of the assay because the elongation factors exhibit much stronger binding affinity for GDP then for GTP.

Table II-1 shows the results of an experiment of this type using assay conditions described by Jan Dijk as outlined under Materials and Methods. These values demonstrate that the retention of radiolabeled GTP by millipore filters depends in a linear fashion on the amount of elongation factor added. The peak activity under these assay conditions for this preparation was 14 units of EF-S_3 per microgram of protein. One unit of activity equals one picomole of GTP retained on a filter. This preparation of elongation factor was used throughout the work to be described.

The assay conditions used to obtain the data presented in Table II-1 were those described as conditions A in the Materials and Methods section of this chapter. These conditions differ somewhat from those used for the assay of *E. coli* EF-Tu as described by Shelton and Bernfield (22). Table II-2 shows a comparison of GTP binding activity under both assay conditions. The data clearly show that the specific activity of either factor is two-fold higher when assayed under conditions A. The difference between the two sets of conditions appears to be essentially a function of reaction volume. The results of an experiment done to test the effect of volume on GTP binding activity are presented in Table II-3. Here it can be seen that as reaction volumes are reduced, and the concentration of EF-S_3 correspondingly increased, the specific GTP binding activity falls to nearly 50% of maximum values. This data then coincides very well with that presented in Table II-2. It would seem on the basis of these two

Table II-1. Filter Binding Assay of EF-S_3-GTP Complex Formation

EF-S_3 (µg)	GTP bound (picomoles)
1.4	27
3.5	53
7.1	105

The assay was conducted according to filter binding assay conditions A as outlined under Materials and Methods. Each value represents a single experiment.

Table II-2. Effect of Assay Conditions A and B on GTP Binding by EF-S_3 or EF-Tu

Factor	Assay Conditions	Units/µg Protein
EF-S_3	A	13.8
EF-Tu	A	16
EF-S_3	B	6.5
EF-Tu	B	8.5

Assays were conducted under the appropriate conditions as detailed under Materials and Methods.

Table II-3. Effect of EF-S_3 Concentration on GTP Binding Activity

[EF-S_3] µg/ml	Specific Activity Units/µg
7.1	14
14.2	12.8
35.5	11.4
71	10
142	8.0

In each assay the amount of EF-S_3 relative to GTP, PEP, and pyruvate kinase remained identical. The reaction volume was merely decreased to result in an increased concentration of EF-S_3 per assay.

experiments, that at high concentration of factor, the ability to bind GTP is significantly reduced. The molecular mechanism involved in this phenomenon is not known. In all further experiments assays were conducted using the conditions B as these gave better results with ternary complex formation despite the lowered GTP binding activity.

The second objective was to demonstrate the formation of ternary complex using EF-S_3-GTP and aminoacyl-tRNA. The easiest but least accurate method for assessing EF-S_3-GTP-aminoacyl-tRNA complex formation again makes use of the millipore filter retention of EF-S_3-GTP. If the activated factor now reacts with aminoacyl-tRNA, its affinity for the filter is remarkably reduced (42). Thus, complex formation is measured as the loss of bound radioactive GTP upon additional incubation of S_3-GTP with aminoacyl-tRNA. Figure II-2 shows data from an experiment designed to demonstrate complex formation in this manner. As one adds charged [^{14}C] isoleucyl-tRNA^{Ile} it is clear that the amount of radiolabeled GTP which is retained by nitrocellulose filters is successively reduced. The magnitude of reduction correlates well with the amount of charged tRNA added. Even adding a large excess of uncharged tRNA failed to reduce the amount of GTP bound to filters to the large extent observed with much smaller amounts of charged tRNA. The accuracy and reproducibility of this particular assay is questionable and appears to depend, at least to a certain extent, on the particular batch of nitrocellulose filters

Figure II-2

Filter binding assay of EF-S_3-GTP-aminoacyl-tRNA formation. The assay was conducted as described under Materials and Methods. 3.5 μg of EF-S_3 was used in each incubation. Formation of EF-S_3-GTP followed conditions B. Isoleucyl-$tRNA^{Ile}$ was prepared according to Materials and Methods; rat liver tRNA was aminoacylated to approximately 60 picomoles per A_{260} unit of RNA. Uncharged rat liver tRNA was from the same RNA preparation which had not been exposed to the aminoacyl tRNA synthetase preparation. (O---O, Ile-$tRNA^{Ile}$; ●---●, uncharged tRNA)

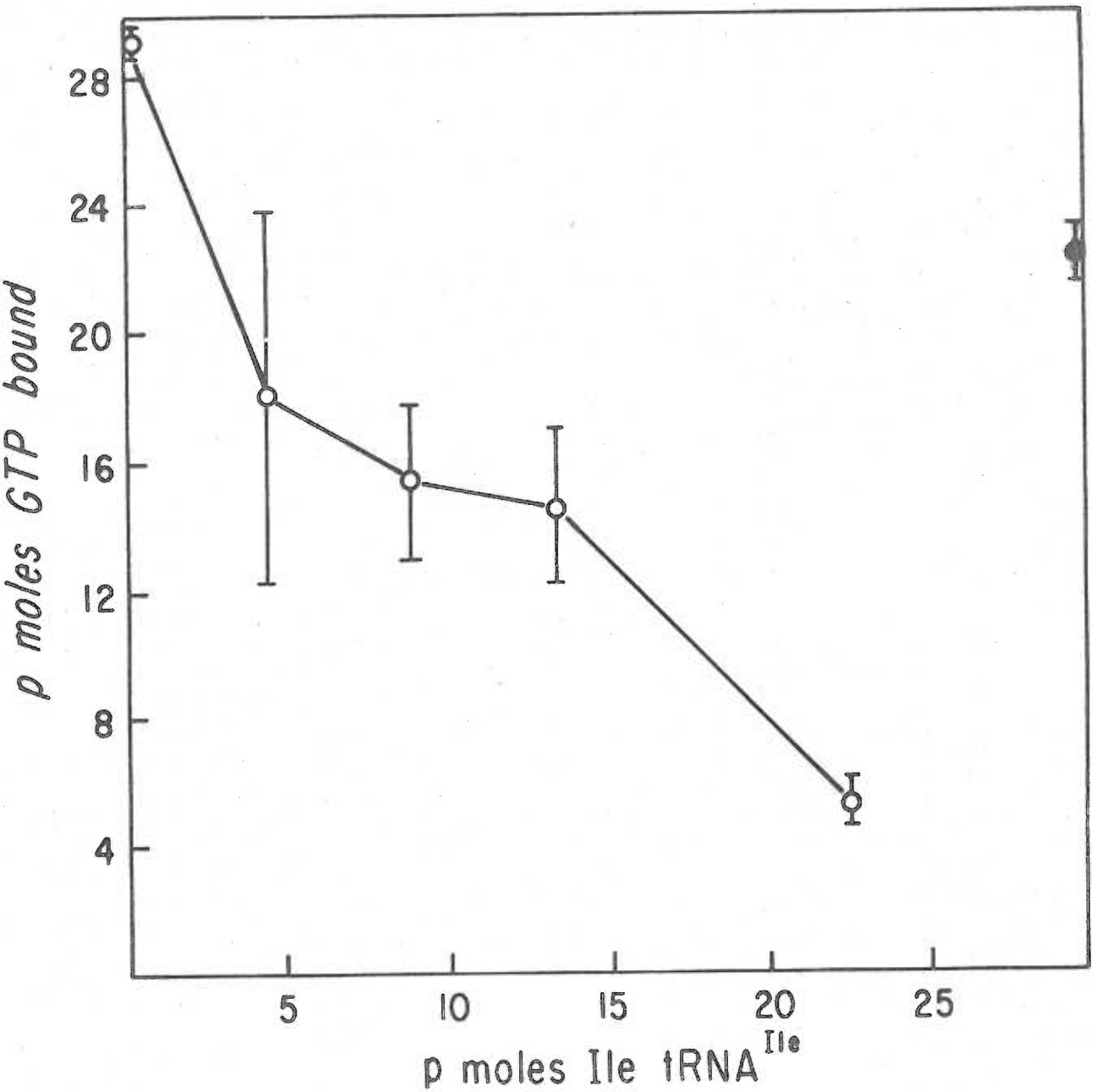

p moles GTP bound
28
24
20
16
12
8
4
5
10
15
20
25
p moles Ile tRNA Ile

Fig. II-2

being utilized. However, it is clear that some ternary complex formation is occurring and is at least qualitatively detectable using this assay.

B. Purification of Mammalian 4S RNA

In the original description of aminoacyl tRNA purification using ternary complex formation, Klyde and Bernfield used gel filtration on columns of Sephadex G-100 as the means of resolving the complex from unreacted tRNA (21). In order to improve resolution and reduce the time and expense involved in a single determination, we decided to attempt to achieve the desired separation using polyacrylamide gel electrophoresis instead. However one initial problem involved the purity of the RNA sample to be analyzed. The original gel filtration separation required purification of the tRNA by two passes through G-100 prior to aminoacylation and complex formation. This is necessary because the ternary complex itself runs very close to the 4S peak and thus any material which might elute in this position must be removed to avoid contamination in the final analytical separation. However on acrylamide gel electrophoresis even RNA which has been twice chromatographed on G-100 still contains a significant 5S rRNA contaminant.

If the final analytical separation of complexed aminoacyl tRNA from free tRNA is to be done on polyacrylamide gels, the purity of the initial RNA sample must be such that it runs as a single component on gels having the same acrylamide composition as those

to be used for the actual analytical separation. Thus 4S RNA must be purified by preparative gel electrophoresis. While there are several approaches to this problem, the most effective one and that which gave the greatest yield utilized electrophoretic elution of the sample. Figure II-3A shows the A_{260} profile of rat liver tRNA which has been electrophoresed in 10% polyacrylamide gels as described in Materials and Methods. The major peak represents the 4S RNA and the smaller peak corresponds to 5S rRNA. By removing that portion of the gel containing only 4S RNA (as indicated by arrows in the figure) and electrophoresing that gel after reinsertion in the original tube now fitted with a dialysis tubing sack fitted over the anodic end, the 4S component can be recovered in the eluate retained within this bag. Recovery of RNA using this method proved to be 85-90% of the applied sample reproducibly. Figure II-3B shows the A_{260} tracing of a tRNA sample which has been purified by this technique and rerun on a 10% gel. It is clear that the 5S rRNA contaminant has now been eliminated and the 4S sample runs as a single component.

If this method of purification is to be used for the purposes intended, it is necessary to establish that tRNA so purified is equally active with respect to amino acid acceptance after electrophoresis. Table II-4 shows the results of in vitro aminoacylation of rabbit liver tRNA both before and after purification. The activity of purified RNA is not significantly different from that of unpurified

Figure II-3

Electrophoretic purification of 4S RNA. Approximately two A_{260} units of rat liver tRNA were applied to each gel. Electrophoresis was as described under Materials and Methods. Each gel was scanned for absorbance at 260 nm in a Gilford spectrophotometer equipped with a linear gel scanning device. (A, unpurified tRNA; B, purified tRNA)

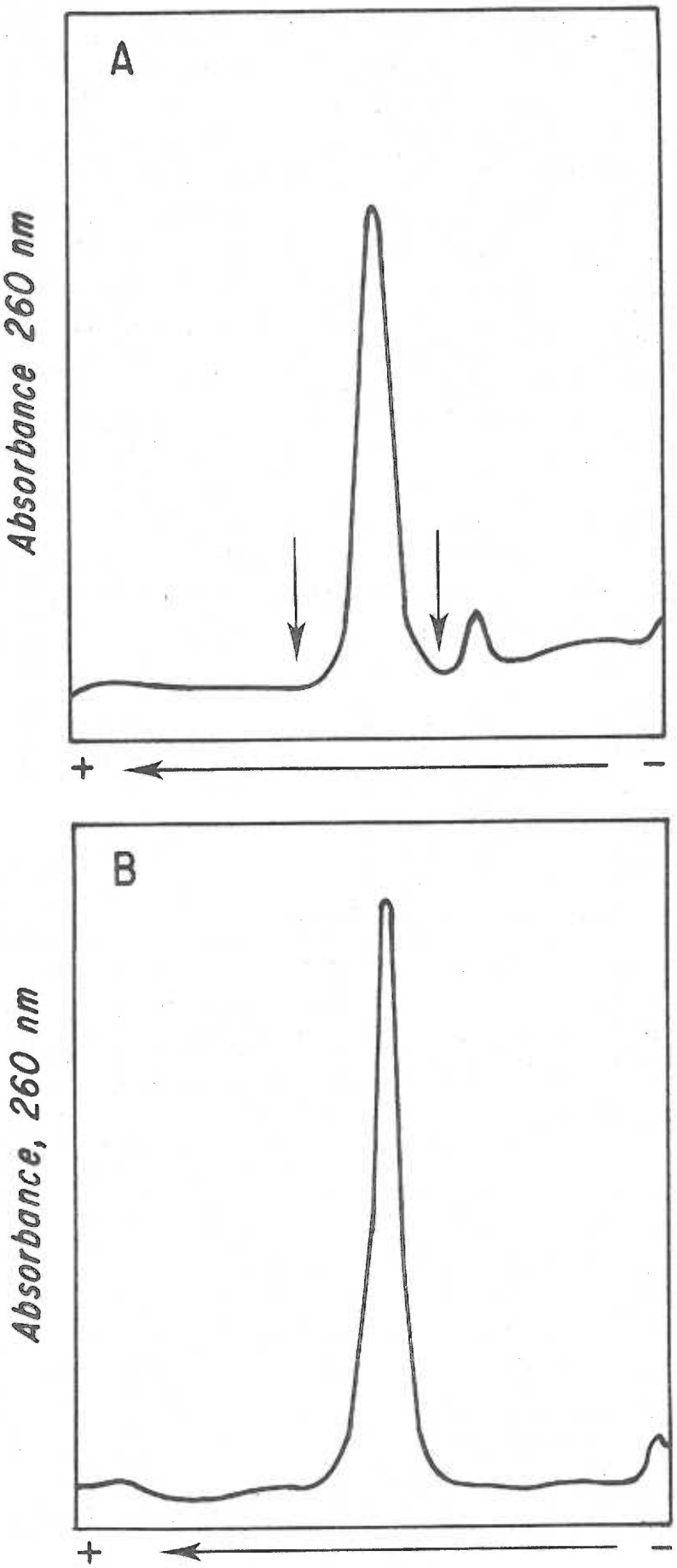


Fig. II - 3

Table II-4. Amino Acid Acceptance of tRNA Before and After Electrophoretic Purification

Amino Acid	Acceptance (pmoles/A_{260}) Before	After
Ile	35	36
Val	100	89
His	25	23

Assays were conducted as described in Materials and Methods. Each value represents the average of duplicate experiments.

RNA. On the basis of this and previously described data, tRNA purified by 10% polyacrylamide gel electrophoresis seems to satisfy all the necessary criteria for use in gel electrophoretic analysis of EF-S_3-GTP-aminoacyl tRNA.

C. Polyacrylamide Gel Electrophoretic Analysis of Ternary Complex Formation

The next step in developing this technique was to show that the ternary complex, once formed, would be stable to conditions of electrophoresis and would be resolved from free uncharged tRNA. EF-S_3-GTP formation and complexation with aminoacyl tRNA were performed in a fashion identical to that used for the filter binding assay except that the final reaction mix was not diluted but rather applied directly to 10% polyacrylamide gels. The detection of ternary complex on gels was assessed by two criteria: 1) the appearance of a separate peak migrating slower than free tRNA which depends upon the aminoacylation of the tRNA and upon incubation with EF-S_3-GTP under appropriate conditions, and 2) the ability to shift aminoacyl tRNA radioactively labeled in the aminoacyl moiety to the position of this second peak dependent upon incubation with activated EF-S_3. Figure II-4 shows the electrophoretic pattern of purified [^{14}C] labeled FL cell 4S RNA which has not been aminoacylated and which has been reacted with EF-S_3-GTP. The direction of migration is from right to left and the anticipated position of the complex will be between 4 and 8 mm into that section of the gel represented in the figure.

Figure II-4

Electrophoretic analysis of ternary complex formation with uncharged FL cell tRNA. 15,000 cpm of [^{14}C] FL cell 4S RNA was complexed with approximately 50 picomoles of EF-S_3-GTP and electrophoresed as described under Materials and Methods.

Figure II-5

Electrophoretic analysis of isoleucyl $tRNA^{Ile}$. 12,000 cpm of [^{14}C] FL cell 4S RNA aminoacylated with approximately 10 picomoles of [^{3}H]-isoleucine (1100 dpm/picomole) and electrophoresed without prior complexation with EF-S_3-GTP as described under Materials and Methods. (●---●, [^{14}C] tRNA; 0---0, [^{3}H]-isoleucine)

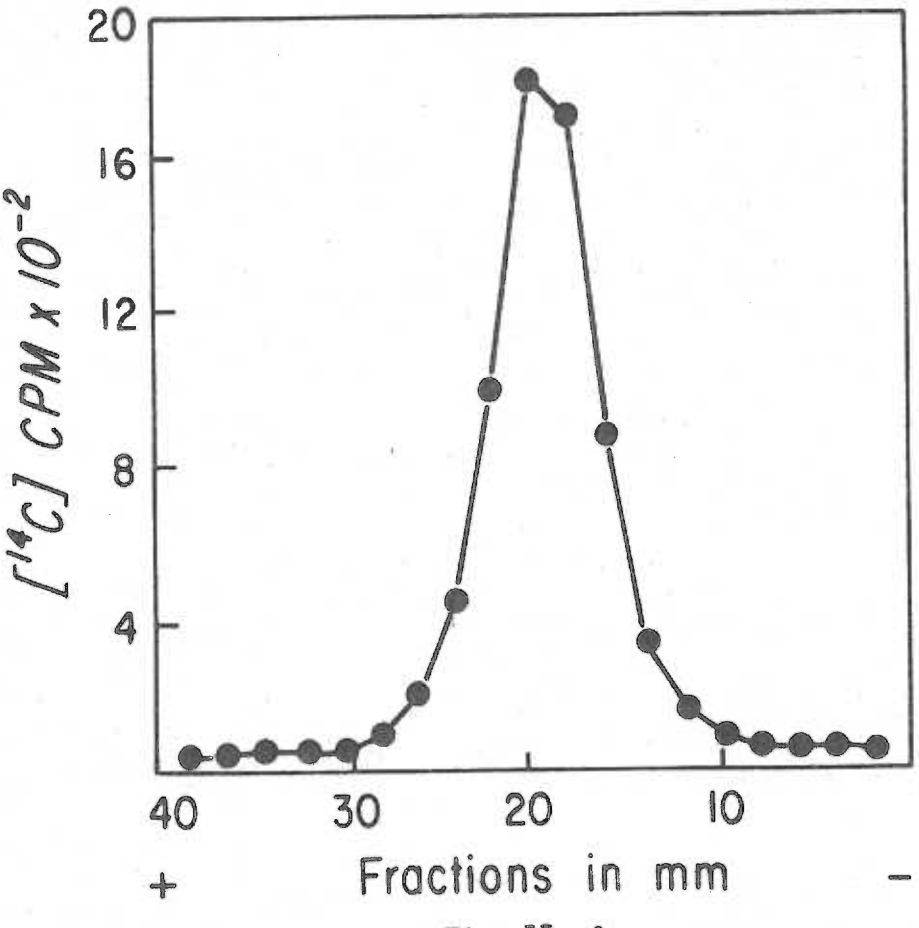


Fig. II-4

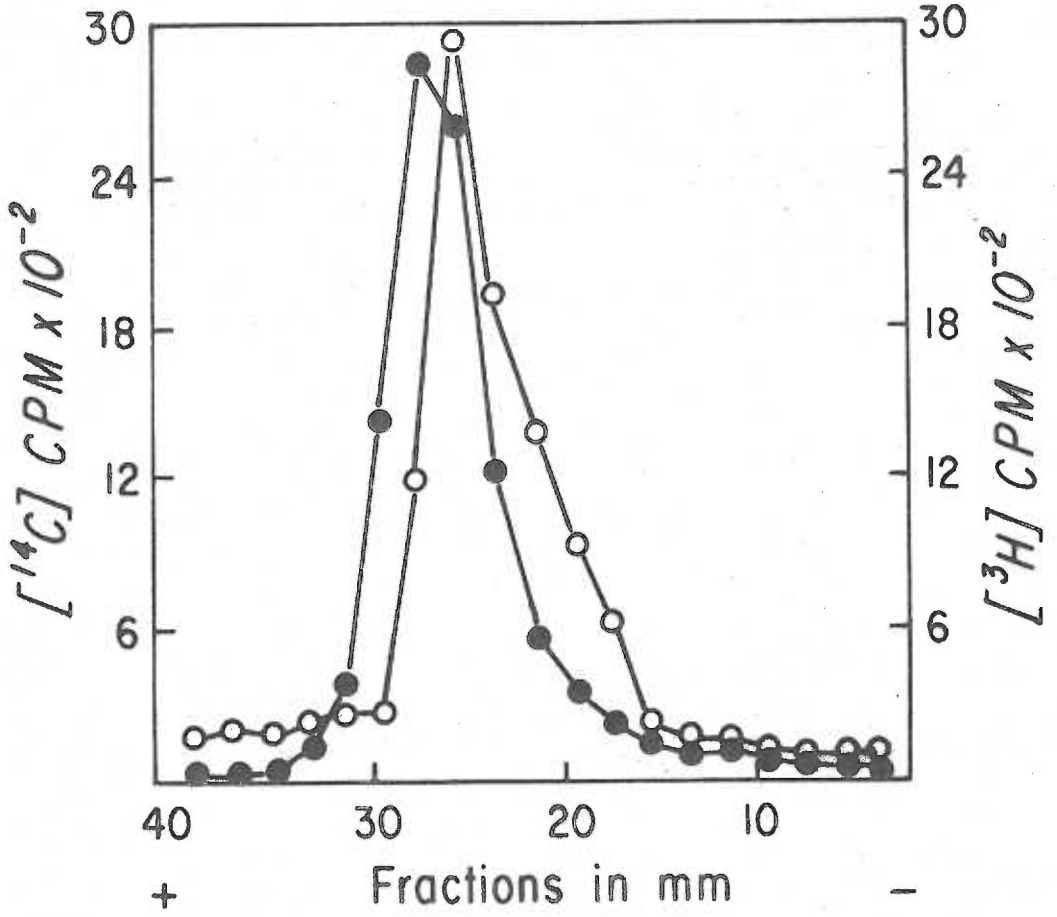


Fig. II-5

It can be seen that there is but a single homogeneous peak and that in the absence of aminoacylation, the tRNA does not form a detectable complex with the activated elongation factor.

Figure II-5 shows a similar electrophoretic pattern of FL cell [^{14}C] 4S RNA which has been aminoacylated with high specific activity [^{3}H]-isoleucine. No EF-S_3-GTP was added before electrophoresis. The [^{3}H]-isoleucyl-[^{14}C]-tRNA^{Ile} appears to migrate at the trailing side of the main 4S RNA band but is still definitely part of this band. This electrophoretic pattern for isoleucyl-tRNA^{Ile} is quite reproducible. In this experiment no radiolabeled material appears to migrate in the position expected of the ternary complex.

Figure II-6 shows the electrophoretic pattern of [^{14}C] FL cell 4S RNA which has been charged with [^{3}H]-isoleucine and reacted with EF-S_3-GTP prior to electrophoresis. As opposed to the migration patterns seen in Figures II-4 and II-5, two fully separate peaks are now observed. There are two separate peaks for the [^{14}C] RNA. The largest, of course, represents the uncharged 4S RNA fraction. The second peak, which represents about 12% of the total [^{14}C] radioactivity on the gel, runs at about the position of 5S rRNA. This is the position expected for the ternary complex. While Klyde and Bernfield were able to show that the Sephadex G-100 elution position of the ternary complex was very close to what could be predicted on the basis of the calculated molecular weight of the complex (21), no such calculation of expected electrophoretic mobility can be made on

Figure II-6

Electrophoretic analysis of EF-S3-GTP-isoleucyl-tRNA^{Ile} ternary complex formation. 11,000 cpm of [^{14}C] FL cell 4S RNA was aminoacylated with [^{3}H]-isoleucine (700 dpm/picomole) and complexed with approximately 50 units of EF-S3-GTP. This mixture was subjected to electrophoresis as described under Materials and Methods. ([^{14}C] tRNA, ●---●; [^{3}H]-isoleucine, 0---0)

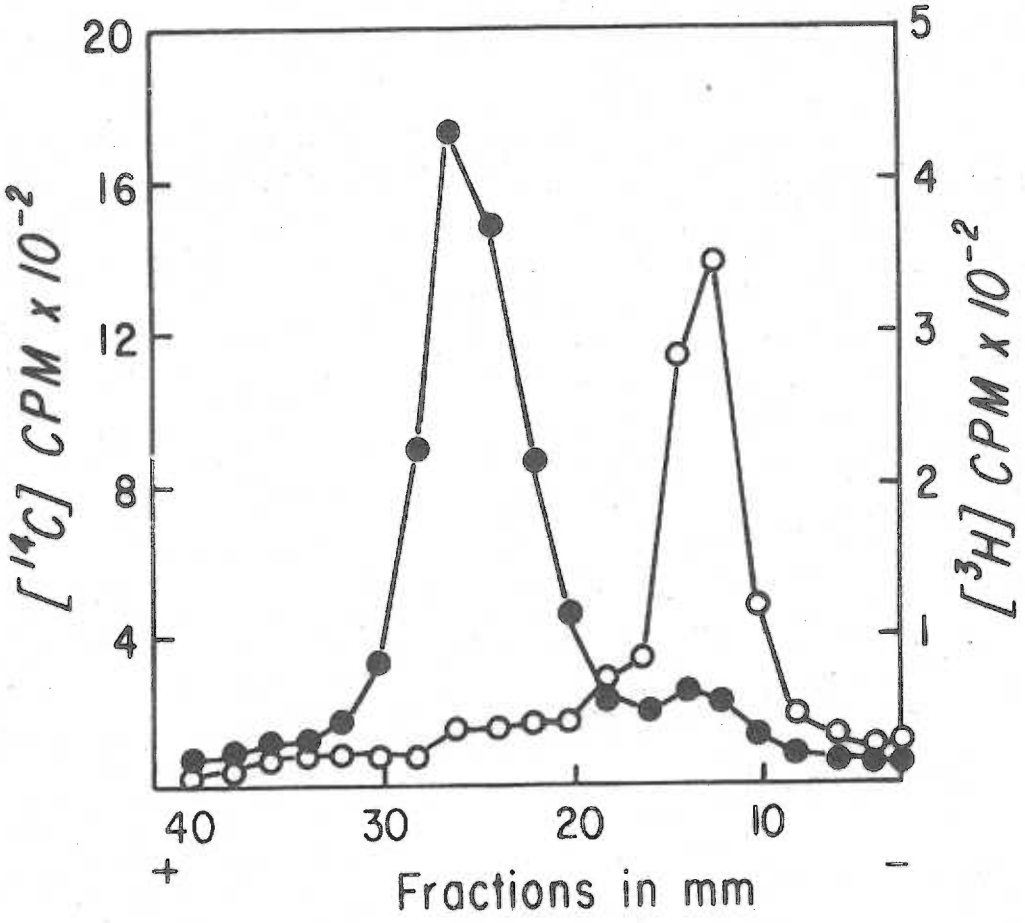

20
16
12
8
4
[14C] CPM x 10-2
5
4
3
2
1
[3H] CPM x 10-2
40
30
20
10
+
−
Fractions in mm

Fig. II-6

the basis of currently available information.] All of the [^{3}H]-isoleucine now migrates with this second smaller peak instead of trailing slightly behind the main tRNA band as seen in Figure II-5. Because of the appearance of a second peak at the expected position and the co-migration of [^{3}H]-isoleucine with this peak dependent upon the reaction with activated EF-S_3-GTP, it seems reasonable to conclude that it represents the EF-S_3-GTP-isoleucyl-tRNA^{Ile} complex. Hence, the electrophoretic technique seems to be adequate for the purposes of separating the ternary complex from unfractionated, uncharged tRNA. In addition, analytical electrophoresis of this nature provides a second independent criterion for the observation of the ternary complex containing activated EF-S_3 and aminoacylated tRNA.

D. Potential of the Technique for Quantitative Analysis

The question of paramount importance in the characterization of this technique is that of its potential value for the quantitative analysis of isoaccepting families of mammalian tRNA. In order for it to be useful for the rapid purification of such tRNA families the amount of the total 4S fraction which migrates in the ternary complex peak should be a direct function of the relative amount of aminoacyl tRNA present in the sample to be analyzed. It was decided to test the performance of the technique with respect to this criterion in several related experiments.

1) If the tRNA is aminoacylated in the presence of most or all

the amino acids, then a majority of the total 4S RNA peak should shift from the first to the second peak. 2) If a single amino acid is added to the charging reaction, then the relative amount of material which shifts to the complex peak on electrophoresis should be directly related to the in vitro amino acid acceptance of that tRNA preparation for the amino acid in question.

While in vitro aminoacylation is certainly not as efficient as probably occurs in vivo, we should reasonably expect to be able to charge a large portion of the tRNA population when a majority of the amino acids are added to the charging reaction. In this regard Waters (43) has shown that a mixture of 17 [^{3}H] labeled amino acids in the appropriate reaction mix resulted in aminoacylation of approximately 70% of the expected theoretical maximum amino acid acceptance, using AKR mouse embryo cells and an enzyme preparation from mouse Krebs II ascites tumor cells. Figure II-7 shows an electrophoretic pattern of [^{14}C] FL cell 4S RNA which has been aminoacylated in the presence of a mixture of 15 amino acids in which isoleucine is labeled with [^{3}H]. The resemblance between this experiment and that presented in Figure II-5 is apparent. While we might conceivably expect to charge at least 50% of the total tRNA, a maximum of 16% of the total [^{14}C] labeled material has shifted to the EF-S_3-GTP-aminoacyl-tRNA peak. The fact that all of the [^{3}H]-isoleucine again migrates with the putative complex peak argues that there was an adequate excess of activated elongation

Figure II-7

Ternary complex formation with FL cell tRNA aminoacylated with 15 amino acids. 15,000 cpm of [^{14}C] FL cell 4S RNA was aminoacylated with 15 amino acids including L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-leucine, L-isoleucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine, and L-histidine, containing [^{3}H]-isoleucine. This charged tRNA was reacted with approximately 50 units of EF-S3-GTP and electrophoresed as described under Materials and Methods. (●---●, [^{14}C] tRNA; 0---0, [^{3}H]-isoleucine)

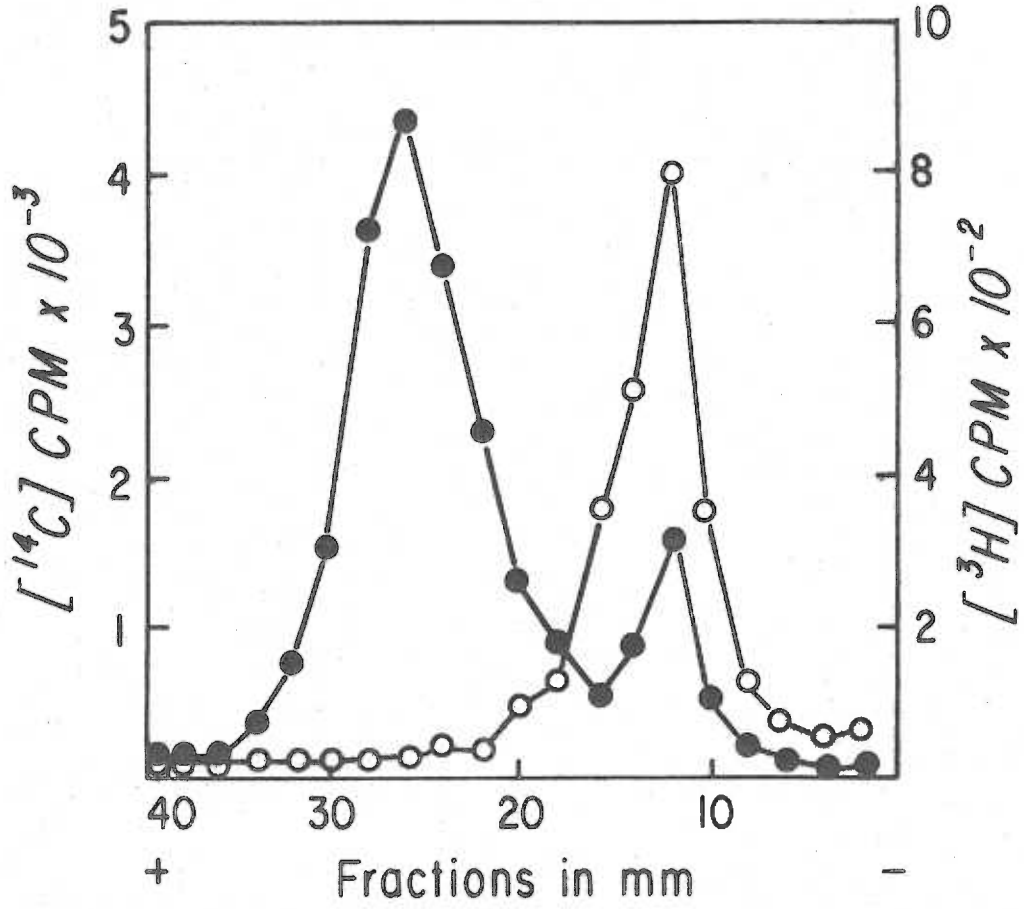

5
4
3
2
1
10
8
6
4
2
[14C] CPM x 10-3
[3H] CPM x 10-2
40
30
20
10
+
Fractions in mm
−

Fig.II-7

Table II-5. Ternary Complex Formation: Dependence Upon the Amount of Amino Acyl tRNA.

Amino Acid Charged	% Shift[a]
Isoleucine	9, 12
Isoleucine, Histidine, Valine	9, 13
15 amino acids[b]	8, 16

a) The % shifted to the ternary complex peak is determined from analytical electrophoresis as described in Fig. II-3.

b) The fifteen amino acids used are listed in the legend to Fig. II-7.

factor present to complex with all available aminoacyl-tRNA. Table II-5 shows the results from a series of experiments in which 1, 3, or 15 amino acids were included in the charging mix. After charging, each sample was reacted with activated EF-S3-GTP and analyzed by 10% polyacrylamide gel electrophoresis as in Figures II-3 through II-5. It is clear that the amount of total 4S RNA which shifts to the ternary complex peak region is apparently independent of the amount or variety of amino acids being added to the charging reaction mix. This observation clearly presents a serious problem with respect to the potential utility of this technique for quantitative analysis as outlined previously.

There are only two reasonable explanations which are consistent with the available data. 1) The assumption of in vitro aminoacylation efficiency is in error; thus the total amount of 4S RNA which is chargeable is relatively small and the amount which actually charges is independent of the quantity or variety of amino acids being added to the reaction mix. 2) The EF-S3-GTP moiety is not able to form a stable ternary complex with most species of aminoacyl tRNA. While neither line of reasoning seems totally consistent with what is known either about in vitro aminoacylation or elongation factor ternary complex formation, they each provide a testable hypothesis.

The first proposal, that concerning the efficiency of in vitro aminoacylation, could potentially result from poor activity

in either of two components of the charging reaction: 1) low amino acid acceptance of the tRNA itself, or 2) low activity of the aminoacyl tRNA synthetase preparation used to charge the tRNA. This problem can be initially approached by measuring the amino acid acceptance of FL cell 4S RNA using conditions identical to those used in the previous experiments. Table II-6 shows the results of a series of assays of FL cell 4S RNA purified by sucrose gradient centrifugation for acceptance of several individual amino acids as well as a mixture of 14. The values for histidine and for leucine are certainly equal to those observed using rabbit liver tRNA or rat liver tRNA and suggest that individual amino acid acceptance is normal for this population of tRNAs. The value for the mixture of 14 amino acids represents approximately 52% of the theoretical maximum acceptance assuming an average molecular weight for tRNA of 27,000 daltons. It is quite clear from these data and those shown in Table II-4 that the preparations of FL cell 4S RNA used in these experiments exhibits normal amino acid acceptance and that the enzyme preparations used for charging contain an adequate supply of most aminoacyl tRNA synthetases to charge a major portion of the available tRNA. These findings argue effectively against inefficient aminoacylation as a reasonable explanation for the observed lack of dependence of ternary complex formation of the quantity of available aminoacyl tRNA.

This now leaves the second hypothesis, that considering

Table II-6. In Vitro Amino Acid Acceptance of FL cell 4S RNA.

Amino Acid	pmoles/A_{260}[a]
Leucine	97,109
Histidine	43,44
14 amino acid mix[b]	1002,987,904

a) Each value represents the average of duplicate experiments made using separate preparations of FL cell tRNA.

b) The 14 amino acids include: L-ala, L-arg, L-asp, L-glu, gly, L-leu, L-ile, L-lys, L-phe, L-pro, L-ser, L-thr, L-tyr, L-val.

the suggestion that EF-S_3-GTP forms primarily unstable ternary complexes with aminoacyl-tRNAs. Klyde and Bernfield provide evidence that some ternary complexes with isoaccepting species of tRNAs are less stable than others and that this instability is apparently temperature dependent (21). However, they have also shown that at least six families of avian aminoacyl-tRNA do form complexes stable enough to be detected by the nitrocellulose filter binding assay (21, 22). If instability of the ternary complexes is the cause of the problem, then on the basis of the previously described results utilizing [^{3}H]-isoleucyl $tRNA^{Ile}$, it would seem reasonable that at least one aminoacyl tRNA does form an acceptably stable complex since all the isoleucyl tRNA migrates with the complex peak. Hence, one would predict that by examination of several aminoacyl-tRNAs we should see some forming stable or partially stable complexes and some forming little or no detectable complex at all. I attempted to do this experiment using the filter binding assay with rabbit liver tRNA aminoacylated with either [^{14}C] leucine, [^{14}C]-isoleucine, or the mixture of fourteen [^{14}C] labeled amino acids as described in Table II-7. In the case of isoleucyl-$tRNA^{Ile}$ the quantity of complex formed agrees well with the quantity of tRNA added to the reaction mixture. Hence complex formation appears to be quantitative and stable. Using leucyl $tRNA^{Leu}$ alone, a significant amount of complex formation is detectable. However the amount of complex detected here is clearly

Table II-7. Filter Binding Assay of Ternary Complex Formation.

tRNA Sample	pmoles aminoacyl tRNA added[a]	pmoles ternary complex formed[b]
Control - none	-	-
[^{14}C]-Isoleucyl-tRNA^{Ile}[c]	4	4,13
"	9	9,13
"	22	21,22
[^{14}C]-Leucyl-tRNA^{Leu}	37	25,27
"	124	60,55
[^{14}C] 14 amino acid mix[d]	43	19
	144	9,23
	288	27

a) The number of pmoles of aminoacyl tRNA added are derived from the total acid precipitable radioactivity present in the sample at the time the assay was conducted.

b) Each value is derived from the decrease in the pmoles of [^{14}C] GTP which bind to filters in the presence of aminoacyl tRNA.

c) Data taken from Fig. II-2.

d) See Table II-6 for a description of the contents of the amino acid mix.

not simply related to the amount of input aminoacyl-tRNA. This difference is partially a function of the nature of the assay. Under normal conditions even a vast excess of aminoacyl tRNA over activated EF-S_3 factor will not reduce the GTP binding to background levels (22). Thus, as the ratio of aminoacyl tRNA to EF-S_3-GTP increases, the ratio of ternary complex detected to input aminoacyl tRNA decreases. In spite of this phenomenon it seems reasonable that the incomplete complex formation may be due to lowered stability of the EF-S_3-GTP-leucyl $tRNA^{Leu}$ complex. Examination of the data obtained using the 14 amino acid mixture reinforce this idea. While the average amount of complex formed seems to be proportional to the amount of tRNA added, the quantity of input tRNA is clearly in vast excess over the detectable ternary complex formed. In each experiment presented there was an excess of EF-S_3-GTP of at least two-fold over the calculated input aminoacyl tRNA. Thus, the very low complex formation observed here is not due to an inadequate supply of elongation factor.

These results clearly indicate that detectable complex formation occurs with some aminoacyl tRNAs but the large majority would appear to either not form the complex at all or to form a complex which readily dissociates under the conditions employed. This conclusion is further supported by the observation that of the three different aminoacyl-tRNA samples (isoleucine, leucine, or the 14 amino acid mix) used in the previous experiment, only isoleucyl-

$tRNA^{Ile}$ is capable of forming a stable complex under the conditions used for polyacrylamide gel electrophoretic analysis of ternary complex formation (M. Litt, unpublished observations). Thus, the stability of the complex appears to depend upon the conditions used for its detection. On the basis of this data and that presented previously, it is concluded that the failure to demonstrate that complex formation, as analyzed by either method described here, is dependent upon the relative proportion of input tRNA which is aminoacylated is due to the inability of EF-S_3 to form stable complexes with most species of murine tRNA under the conditions employed in this investigation.

A second problem which must be considered in any isolation scheme involves evaluation of the degree of purification achieved. In this particular situation the purity of the product is clearly dependent upon the specificity of the aminoacylation reaction. Thus the proportion of total tRNA which electrophoreses in the complex peak position upon reaction with EF-S_3-GTP should depend directly upon the addition of exogenous amino acid to charging reaction mixture. To test this specificity an experiment was done using [^{14}C] FL cell electrophoretically purified 4S RNA charged in a reaction mix lacking only the exogenously added amino acid. This "mock" charged tRNA was reacted with EF-S_3-GTP and analyzed by electrophoresis on 10% polyacrylamide gels as described in Figure II-3. Figure II-8 shows the results of such an experiment. It can

Figure II-8

Ternary complex formation with "mock" charged FL cell tRNA. 10,000 cpm of [^{14}C] FL cell 4S RNA was aminoacylated in the normal reaction mix containing no exogenously supplied amino acid. The "mock charged" tRNA was reacted with approximately 50 picomoles of EF-S_3-GTP and electrophoresed as described under Materials and Methods.

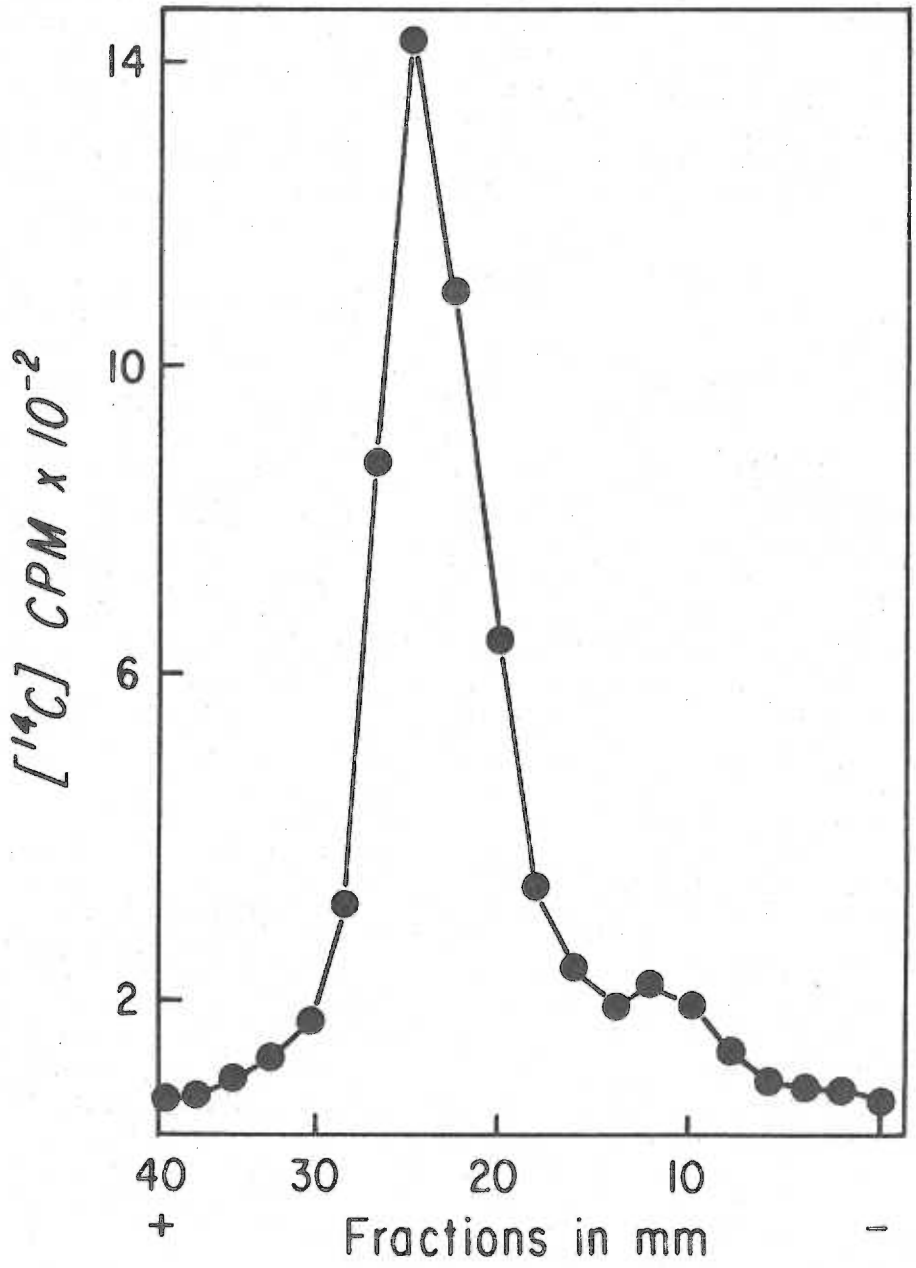


Fig. II-8

be seen that a separate peak does appear in the complex region of the gel and the appearance of this peak is dependent upon the presence of EF-S_3 factor and upon exposure of the tRNA to the charging enzyme. The magnitude of the shift from the 4S peak to the complex peak in mock charged samples is roughly equivalent to that seen when 1, 3, or 15 amino acids are added. This observation was consistently observed in all mock charging experiments. The appearance of the peak in mock charged samples was dependent upon the presence of ATP in the reaction mix (M. Litt, unpublished observations). This apparent lack of specificity then constitutes a second problem of serious import to the continued viability of this technique. Since the goal of this technique is to purify single families of tRNA which represent on the average about 5% of the total tRNA, a non-specific background of 10% is totally unacceptable.

The available data suggest that the problem is localized to the tRNA itself or the aminoacyl-tRNA synthetase preparation. There are several potential explanations for this non-specific shift: 1) While the high background could conceivably be derived from residual aminoacyl-tRNA which escaped the stripping procedures routinely included in the preparation of the tRNA, uncharged tRNA reacted with activated EF-S_3 does not form detectable complex (see Fig. II-4A). Hence, it can be concluded that residual aminoacyl tRNA is not the source of the non-specific shift. 2) A

second suggestion would involve the possibility that there are contaminating RNA species in the 4S RNA preparation which, upon exposure to the charging reaction, are activated in such a way as to form stable complexes with EF-S_3 factor. While this possibility cannot be ruled out on the basis of presently available evidence, there is no support for such an idea in the literature concerning bacterial elongation factor function. In addition one would predict that complex formation dependent upon actual aminoacyl-tRNA would be detectable over and above this non-specific shift. Such is not the case. 3) The remaining proposal considers the possibility that aminoacyl-tRNA is being formed in the absence of exogenously added amino acid. This line of reasoning suggests that the reaction mix contains an endogenous supply of amino acid which, while being quite small, would be sufficient to charge a large portion of the available tRNA. Since the aminoacyl tRNA synthetase preparation is subjected to desalting on columns of sephadex G-50 immediately prior to use in the charging reaction, the source of endogenous contamination must be limited to two possibilities: a) the reagents (i.e., TrisCl, ATP, CTP, or $MgCl_2$) may be contaminated with trace amounts of amino acids, or b) the crude enzyme preparation contains proteolytic enzyme activity which is generating amino acids during the reaction itself.

The first possibility was tested by doing amino acid analyses of each of the concentrated stock solutions used in making

up the charging reaction buffer. To each one was added approximately one nanomole of isoleucine as an internal standard. The results clearly showed that there was no measurable contamination by amino acids (data not shown). Since the stock solutions were diluted by a factor of 10 or greater before use in aminoacylation, it seems reasonable to conclude that reagent contamination was not the source of the non-specific aminoacylation being observed.

The second possibility, that considering protease contamination of the synthetase preparations, was tested in a number of ways. The first approach was to assay for the appearance of primary amines upon incubation of the enzyme at 37°C. Fluorescamine, which reacts with primary amines to give an intensely fluorescent product with an excitation maximum of 390 nm and emission maximum of 480 nm, was used to detect the presence of amino acid (42). Figure II-9 shows the results of a kinetic study of amine generation by a rat liver synthetase preparation incubated at 37°C for several hours. There is a clear increase in detectable primary amine which occurs over the 3 hr period studied. While the enzyme concentration used here was very much higher than used in charging reactions, the amount of amine produced over time is certainly well within the range of amino acid concentration which would seriously affect the specificity of the aminoacylation reaction.

However, it must be kept in mind that this experiment detects only the appearance of primary amines and exhibits no

Figure II-9

Primary amine generation by a rat liver crude aminoacyl-tRNA synthetase preparation. 250 µl of an aminoacyl tRNA synthetase preparation containing 4.9 mg protein/ml was incubated at 37°C for 4 hr. At 0, 1.5, and 3.5 hr after the start of the incubation, a 50 µl aliquot was removed and treated as described under Materials and Methods. The values used in this figure were derived from a plot of isoleucine concentration versus relative fluorescent intensity obtained as a standard curve.

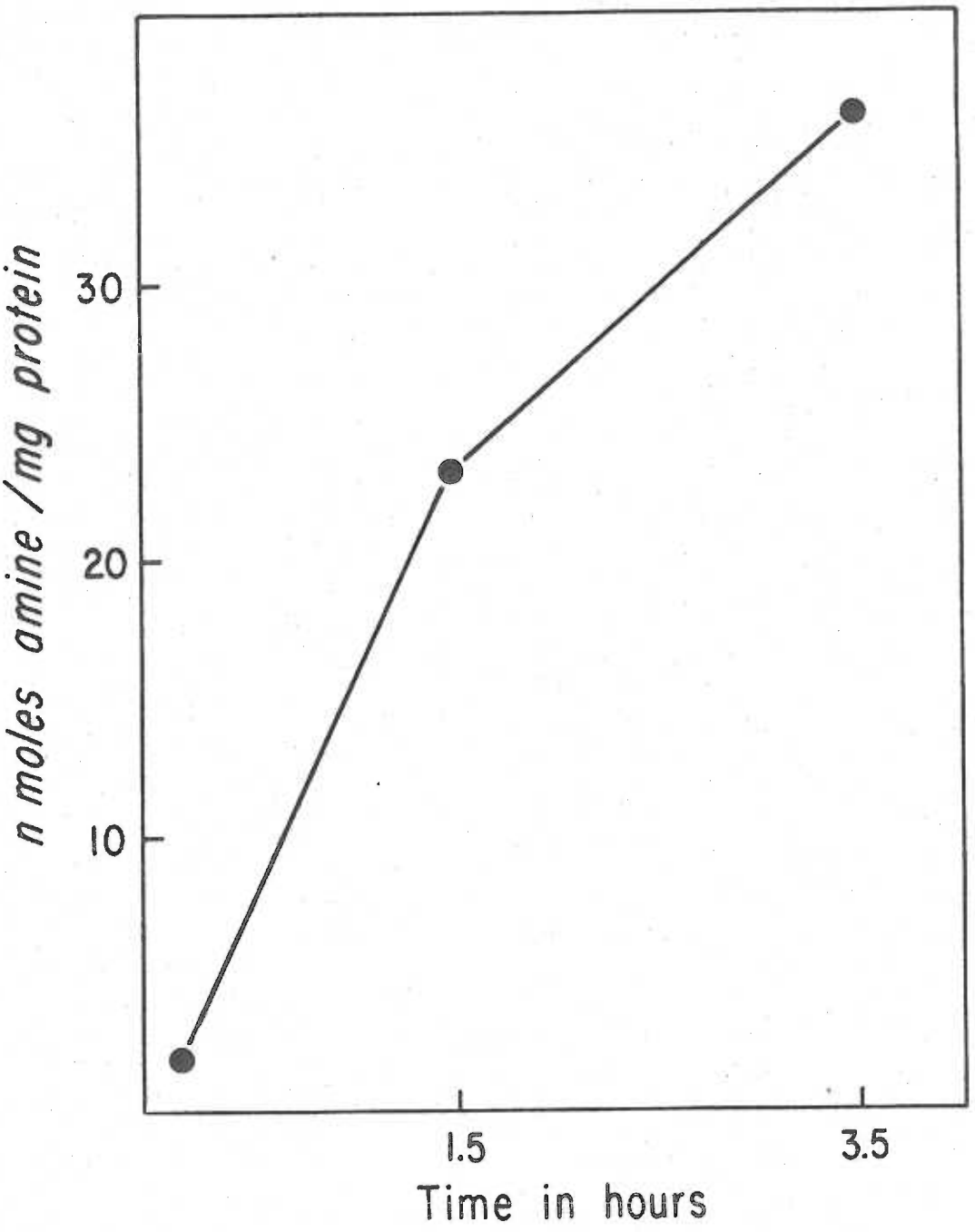


Fig.II-9

selectivity for biologically important amino acids. In order to examine the specific nature of the amine being generated by the enzyme it is necessary to do amino acid analysis of the acid soluble material. For this experiment a 1 ml mock charging reaction was prepared and incubated at 37°C for 1 hr. The reaction was terminated by precipitation with TCA and the supernatant was subjected to amino acid analysis. As a control a sample containing the reaction buffer but no enzyme was treated identically. The only significant difference between the control and experimental samples was the appearance of a very large peak of ammonia in the sample containing enzyme (data not shown). This suggests that the production of primary amine observed in the fluorescamine experiments could have been due simply to the production of ammonia during the reaction. This conclusion is somewhat tentative because both samples showed evidence of contamination as there were small but roughly equivalent amounts of several amino acids (between 1 and 10 nanomoles) on each chromatogram.

While this originally suggested reagent contamination, subsequent amino acid analysis of each reagent was entirely negative (see above).

These experiments do provide an upper limit for the amount of true amino acid generation occurring in the charging reaction of not greater than 1 - 2 μM. Since the Km for in vitro aminoacylation varies between 1 and 100 μM depending on the amino acid, these

values fall within a range which could potentially cause some low level of non-specific acylation (44). However, when the magnitude of the non-specific shift is considered (see above and Fig. II-6) it suggests that amino acid generation by the enzyme could not be entirely responsible for the behavior of the mock charged tRNA samples upon electrophoretic analysis of ternary complex formation.

This indication is further supported by the ability to detect, using a mixture of fourteen [^{14}C] labeled amino acids, more than 50% of the available tRNA in a charged condition (see Table II-6). If large amounts of amino acid were being produced, the specific activity of each amino acid should be significantly lowered, thus making detection of this amount of aminoacyl-tRNA very difficult. In addition recent experiments using DBAE-cellulose to separate charged from uncharged tRNA suggest that mock charged samples contain little or no aminoacyl-tRNA (M. Litt, unpublished observations). Thus while amino acid generation by the enzyme seems to be an unlikely candidate for the source of the problem under investigation, it cannot be ruled out entirely upon the basis of the data presented above.

IV. Discussion

The work reported in this presentation was initiated in an attempt to develop a technique for routine use which would allow for the rapid purification of isoaccepting families of tRNA. In vitro aminoacylation, catalyzed by preparations of aminoacyl-tRNA synthetases, provides the ability to specifically mark that proportion of a total population of tRNA which codes for a particular amino acid. Hence the problem is reduced to finding a technique which can distinguish between charged and free tRNA and separate the two species. While there are a variety of reported techniques which provide an apparent solution to this problem, all but one are dependent upon purely chemical recognition and thus are burdened by the non-specific interactions which often characterize such methods (10, 13-20). Instead I chose a recently reported technique which relies upon the biological specificity inherent in the recognition of aminoacyl-tRNA by bacterial elongation factor Tu (21, 22). As described above, the utility of this technique for quantitative analysis of tRNA families has been shown to be highly questionable.

The elongation factor used in most of these experiments was isolated from *B. stearothermophilus*. While this protein may differ from EF-Tu (isolated from *E. coli*) in many respects, the function and performance in these experiments were essentially indistinguishable from $EF\text{-}S_3$.

The initial demonstration of complex formation by use of either the nitrocellulose filter binding assay or analysis by polyacrylamide gel electrophoresis provided no remarkable findings. The results, using exclusively isoleucyl-tRNA^{Ile} isolated either from rat liver or from FL cells, showed that this aminoacyl-tRNA was participating in complex formation in a quantitative fashion. Thus, under conditions where the amount of aminoacyl-tRNA was limiting relative to EF-S$_3$-GTP, the ratio of complex formed to input aminoacyl-tRNA was close to one. However upon initiating experiments to assess the potential of the technique for quantitative analysis, it became quite clear that these initial observations were extremely misleading.

The value of this technique to provide adequate purification of isoaccepting families of tRNA depends upon its performance in two related areas. 1) The proportion of the total tRNA population which participates in complex formation should depend in a linear fashion upon the relative amount of the total tRNA which is aminoacylated. 2) The formation of acylated-tRNA and/or complex should be specific such that the degree of purity achieved is very high. The available data suggest that this technique must be rated very low with respect to both criteria.

By examining the amount of total tRNA which participates in complex formation following aminoacylation of the tRNA with 1, 3, or 15 amino acids, it was observed that the percentage which formed the ternary complex was independent of the amount of aminoacyl-tRNA

present. Since the tRNA was shown to be quite active with respect to amino acid acceptance, the problem was localized to the elongation factor itself. The amount of complex formation detectable depended upon the amino acid which was esterified to the tRNA and the method used to measure complex formation. This suggests that many of the ternary complexes formed are relatively unstable and are dissociating before they can be detected. There is evidence in support of this concept in the literature. Arai et al. examined the dissociation constant of *E. coli* EF-Tu-GTP-phenylalanyl-tRNA^{phe} via the nitrocellulose filter binding assay (45). They reported a dissociation constant of 7.2×10^{-8} M. On the basis of this value it can be calculated that about 75% of the aminoacyl tRNA added to a solution of EF-Tu-GTP should be in the form of ternary complex under the conditions employed in the present study. This suggests that even a 10-fold dilution could drastically shift the equilibrium in favor of dissociation. The distinction should be made here between the dissociation constant and the rate of dissociation of the ternary complex. While the dissociation constant will determine the extent of ternary complex formation at equilibrium, the rate at which the complex dissociates when subjected to conditions which may favor dissociation becomes the important parameter to consider in either assay of complex formation employed here. Since both assays result in the separation of ternary complex from other key components of the reaction mixture, the complex is subjected to conditions

which vary significantly from those under which it was formed. Hence, the rate of dissociation of the complex relative to the time required for separation will determine the actual amount of ternary complex detected in either assay. Since some complexes (e.g., leucyl-tRNA^{Leu}) show some complex formation in the filter binding assay but none at all when assessed on polyacrylamide gels run for 2 hr, it would seem that the rate of dissociation is such that the relatively fast filter binding assay is able to catch some complex before full dissociation has occurred. In other cases (e.g., isoleucyl-tRNA^{Ile}) the rate of dissociation must be slow enough to allow detection on gels electrophoresed for as long as 8 hr.

While dissociation constants and rates of dissociation for the ternary complex probably vary significantly from one tRNA to another, it is reasonable to suspect the concentration range in which we are necessarily working is such that complex stability is at least partially limited. In addition, Klyde and Bernfield, the originators of the technique, reported that with seryl-tRNA^{Ser}, the ternary complex showed evidence of dissociation in several experiments (21). The dissociation which they observed allowed isolation of two separate populations of seryl-tRNA^{Ser}, each of which was enriched for different isoaccepting species. This indicated that even within a family of isoaccepting tRNAs the ternary complex exhibits variable stability. On the basis of these considerations it is concluded that the use of this technique for the analysis of

aminoacyl tRNA is limited by the apparent instability of ternary complex. While this instability may result from an unfavorably fast rate of dissociation, other contributing factors cannot be ruled out on the basis of available data.

The second problem with the technique is related, as mentioned previously, to the purity of the product tRNA in the isolated complex. If the tRNA is highly purified by this procedure, then the relative amount of the total tRNA population which forms complex should reflect the amino acid acceptance of that population of tRNA for the particular amino acid included in the reaction mixture. However, mock charged tRNA samples (in which no exogenous amino acid was added to the mix) exhibit the same or nearly the same extent of ternary complex formation as samples to which 1, 3, or 15 amino acids have been added. Hence the specificity of the product would appear to be relatively low. Since the tRNA samples which have not been exposed to the charging enzyme do not form detectable complex with EF-S_3-GTP, the source of the problem is localized to the charging reaction itself.

The most likely explanation of this problem would invoke amino acid contamination of reagents or amino acid generation by proteolytic contaminants in the enzyme. There is some evidence to support this concept. Powers and Peterkofsky, in applying BD-cellulose chromatography to the separation of free and charged *E. coli*

tRNA, found it necessary to partially purify the charging enzymes before the non-specific aminoacylation background could be lowered to acceptable levels (23). However experimental examination of this problem as described above suggests that this is not the source of the problem. Amino acid analysis of the charging reaction reagents showed them to be functionally free of amino acid contamination. While the enzyme appears to generate a significant amount of primary amine, amino acid analysis of the acid soluble products of the reaction indicated that the primary amine generation could probably be accounted for by ammonia generation. In addition the tRNA from mock charged samples appears to contain essentially no aminoacyl-tRNA by the independent criteria of DBAE-cellulose analysis. Klyde and Bernfield used a crude enzyme preparation very similar to that used in the present study and found the average purity of the recovered aminoacyl-tRNA to be greater than 75% (21). Thus the available evidence argues against protease contamination as the source of the non-specific complex formation observed in mock charged samples. This leaves only a somewhat unattractive hypothesis to consider; that some factor in the elongation factor preparation, perhaps EF-S_3 or EF-Tu itself, is able to form a stable complex with some polynucleotide in the tRNA sample in a fashion which is dependent upon exposure to the charging enzyme in the presence of ATP. If this is the case, which seems unlikely, it is necessarily a problem which depends upon the use of this particular technique. Since the utility

of the ternary complex analysis of aminoacyl-tRNA is of clearly limited value, further experimental pursuit of this particular problem should be considered in terms of its potential return.

In light of the evidence presented above it can be concluded that the potential use of this procedure for the quantitative analysis of picomole quantities of aminoacyl tRNA is severely limited. While it may have application where fairly large quantities of tRNA are available, as with animal or plant tissues or organs, it is not of any clear value for the analysis of tRNAs for cultured eukaryotic cells.

V. Summary

The work discussed in the second section of this dissertation represents an attempt to characterize and evaluate a technique designed for the purpose of purifying single families of isoaccepting tRNAs. This technique depends upon the biological specificity inherent in both aminoacyl tRNA synthetase enzymes and in the bacterial elongation factor EF-Tu. Hence, specifically aminoacylated tRNAs can be isolated in a relatively pure state by complex formation with EF-Tu-GTP followed by separation of this ternary complex from uncharged tRNA. I have demonstrated the formation of ternary complex by two criteria, one of which involves the separation of complexed tRNA by polyacrylamide gel electrophoresis. In attempts to verify the potential of this technique for quantitative analysis of tRNA populations, I have observed that complex stability with a majority of the tRNA species is quite limited. In addition, the product purity is also quite poor in contrast to that predicted by the use of such specific biological components. It is concluded that this technique is of very limited value for the purposes of purifying picomole quantities of single families of eukaryotic tRNA.

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