STUDIES ON THE VARIATION OF tRNA WITH SPECIAL REFERENCE TO INHIBITION OF AMINOACYLATION IN CERTAIN INVERTEBRATES

A Thesis Submitted to the University of Hyderabad

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DOCTOR OF PHILOSOPHY

By

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TO MY PARENTS

DECLARATION

The candidate declares that this work has been carried out by him under the supervision of Dr. R. MAYASUNDARI, School of Life Sciences, University of Hyderabad, Hyderabad - 500 134, India, and that this work has not been submitted for any degree or diploma of any other University.

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CERTIFICATE

This is to certify that the thesis entitled "STUDIES ON THE VARIATIOIN OF tRNA WITH SPECIAL REFERENCE TO INHIBITIOIN OF AMINOACYLATION IN CERTAIN INVER-TEBRATES" is based on the results of the work carried out by Mr. P. SIVARAM, M.Sc., for the degree of DOCTOR OF PHILOSOPHY under my supervision. This work has not been submitted to any degree or diploma of any other University.

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LIST OF ABBREVIATIONS

A	:	Adenine
A_{260}	:	Absorbancy at 260 nm
A_{280}	:	Absorbancy at 280 nm
ATP	:	Adenosine 5'-triphosphate
C	:	Cytosine
CTP	:	Cytidine 5'-triphosphate
CPM	:	Counts per minute
DNA	:	Deoxyribonucleic acid
DEAE	:	Diethyl aminoethyl
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra acetic acid
G	:	Guanine
GTP	:	Guanosine 5'-triphosphate
min.	:	Minute
mRNA	:	Messenger ribonucleic acid
β-ME	:	β -mercaptoethanol
PPO	:	2,5-Diphenyloxazole
POPOP	:	1,4 bis[2(5-phenyloxazolyl)]-benzene
RPC	:	Reverse phase chromatography
RNA	:	Ribonucleic acid
SD	:	Standard deviation
T	:	Thymine
tRNA	:	Transfer Ribonucleic acid
TRIS	:	Tris[hydroxymethyl]-aminomethane
TCA	:	Trichloro acetic acid

U	:	Uracil
λ	:	Lambda

Modified Nucleosides:

D	:	Dihydrouridine
I	:	Inosine
i^6A	:	N^6-Isopentenyladenosine
m^1A	:	1-Methyladenosine
ms^2i^6A	:	2-Methylthio-N^6-isopentenyl-adenosine
mt^6A	:	N-[(9-β-D-Ribofuranosylpurin-6-yl) N-methylcarbamoyl] threonine
t^6A	:	N-[(9-β-D-Ribofuranosylpurin-6-yl) carbamoyl] threonine
m^7G	:	7-Methylguanosine
mcm^5U	:	5-(Methoxycarbonylmethyl) uridine
s^4U	:	4-Thiouridine
mcm^5s^2U	:	5-(Methoxycarbonylmethyl)-2-thiouridine
mnm^5s^2U	:	5-Methylaminomethyl-2-thiouridine
Q	:	Queuosine
T	:	Ribothymidine
yW	:	Base 'Y'
Ψ	:	Pseudouridine

tRNAs :

$tRNA_f^{met}$	:	unacylated prokaryotic initiator tRNA
$tRNA_i^{met}$	:	unacylated eukaryotic initiator tRNA
$tRNA^{aa}$	:	Unacylated tRNA
$aa\text{-}tRNA^{aa}$	:	aminoacylated-tRNA
$*aa\text{-}tRNA^{aa}$	:	Misacylated-tRNA

CONTENTS

CHAPTER - I

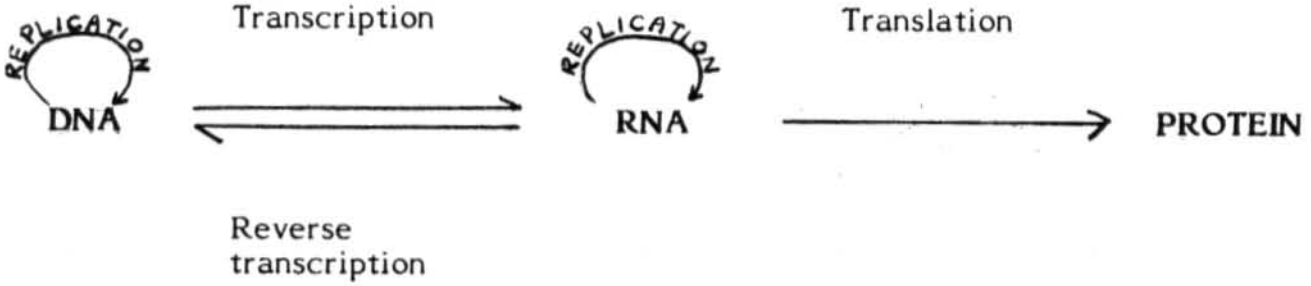
INTRODUCTION

1.1. Historical background

The discovery of the structure of DNA by Watson and Crick (1) is one of the most influential events in biology in the past century and is considered to mark the birth of modern Molecular Biology. By this time it was already established that DNA is the genetic material. The history of events dates back to 1867 when the fundamental investigations which led to the discovery of nucleic acids were made by Miescher (2). These investigations were further continued by Altman (3) who described a method for the preparation of protein free nucleic acid. Later Kossel, Levene, Jacobs and others (4,5,6) further contributed to our understanding of nucleic acid chemistry and showed the existence of two types of nucleic acids, DNA and RNA. The idea that DNA is the genetic material first took its birth from the observations of Griffith (7) in 1928 who showed that heat killed 'S' strain of pneumococcus transformed non lethal 'R' strain to live 'S' strain. It was concluded that the cell extract contained a transforming principle which was not characterized further. The next development occurred 15 years later when Avery et al (8) partially purified the transforming principle and demonstrated that it was DNA. Most biologists remained unconvinced that DNA is the genetic material until five years later, when Erwin Chargaff (9) showed that, a wide variety of chemical structures in DNA were possible, by determining the molar concentration of each base, thus allowing biological specificity. The final and concrete proof for the genetic nature of DNA was given by Hershy and Chase (10) in 1952 who showed that in the T_2 phage infection of E.coli it was DNA but not the protein that enters the cell.

In the Watson and Crick model, DNA is depicted as a double helical molecule in which two antiparallel right handed helices are coiled about

a single axis. An important result of this structure was that the complementarity of base pairing between DNA strands through hydrogen bonds, suggested a copying mechanism for the genetic material i.e. the semi-conservative model in which one parental strand is always conserved during replication. The first experimental evidence for the semi-conservative replication was obtained from the landmark experiment of Messelson and Stahl (11). The mechanism of DNA replication was revealed later by the discovery of a variety of enzymes starting from the famous Kornberg enzyme (12). DNA molecules are not the direct templates for protein synthesis. The relationship between DNA and protein was first summarized by the central dogma proposed by Crick (13), where the flow of information is unidirectional i.e. from DNA to RNA to protein. For a long time it was thought that this process is irreversible until the discovery of reverse transcriptase, by Temin et al. (14), the enzyme which makes DNA from RNA template. So the modified central dogma can be represented as:



Among the earliest reports on the nature of the transcription apparatus was the detection of DNA dependent RNA polymerase in bacteria by Hurwitz et al (15), where the synthesis of RNA involves complementary base pairing with DNA. The speculation on the necessity for a small molecular weight RNA and on aminoacylating enzyme came from Crick in his adaptor hypothesis (13). It was soon demonstrated by Hoagland and others (16) that such an

adaptor function is provided by tRNA and Berg et al (17) showed that the enzymes are the aminoacyl-tRNA synthetases. The studies of Yanofsky et al (18) established that the base sequence of DNA maps colinearly into corresponding polypeptides. A non-overlapping triplet code read from a fixed starting point was first suggested on the basis of genetic studies by Crick (19). Subsequent studies by several groups of workers including Nirenberg, Leder, Matthaei, Khorana, Nomura and others established the different codon assignments for amino acids, ribosome function and different factors involved in protein synthesis (20-24). The genetic code thus obtained is degenerate. 61 codons represent the twenty amino acids. Often the base in the third position of the codon is not significant. Crick (25) proposed the 'Wobble hypothesis' to explain the degeneracy in the code. It says that the third base of the codon is involved in a loose or 'wobble' pairing with the corresponding base of anticodon. The genetic code though originally elucidated in E.coli, was later shown to be universal, by employing several heterologous translation systems. However, recent studies have shown exceptions to this generalisation. Studies with mitochondrial genomes have shown that UGA, which is a termination codon in all other systems codes for tryptophan in mitochondria. In yeast CUA codes for threonine instead of leucine. In mammals in addition to AUG, AUA acts as an initiation codon, also the codons AGA and AGG cause termination instead of representing arginine (26).

1.2. Protein synthesis

The events of protein biosynthesis can be broadly divided into 3 stages: initiation, elongation and termination. Initiation of protein synthesis involves attachment of mRNA to the 30S subunit, a process requiring Mg^{++} as well as initiation factor IF_3. Subsequent attachment of fmet-tRNA in response to initiation codon AUG to form the complex requires GTP and

initiation factors IF_1 and IF_2. The 50S ribosomal subunit adds to this complex to form the functional 70S ribosome. In elongation, recognition of internal codons involves factors EF-Tu, EF-Ts and GTP. The EF-Tu-GTP-aa-tRNA complex binds to the ribosome in response to the corresponding codon. Peptide bond formation occurs by transfer of the fmet or peptidyl group from the tRNA on the P site to the aminocyl-tRNA on the A site, catalyzed by peptidyl transferase. Translocation involves several coordinated processes; release of deacylated tRNA from the P site, one codon movement of mRNA and ribosome with respect to each other, precise positioning of the next codon for translation; and coordinated movement of peptidyl-tRNA from the A to P site. By repetition of the codon recognition step, next aminoacyl-tRNA enters the A site in response to the codon. Transpeptidation and translocation produce the complete protein which is still attached to the tRNA. In termination when the termination codon is reached, in response to certain releasing factors the completed protein is released. After termination the ribosome is dissociated into 30S and 50S subunits with release of mRNA (27).

1.3. Regulation of protein synthesis

All the living cells possess accurately programmed mechanisms for regulating the relative amounts of different proteins synthesized. Based on the genetic and biochemical studies, Jacob and Monod (28) first put forward the 'operon' model to explain the regulation of gene expression in prokaryotes. In eukaryotes the regulation of protein synthesis has another fundamental role i.e. as a means for the differentiation of cells, having different ultrastructure, molecular composition and biological function. Though all the cells of higher organisms contain the complete genome, in any given cell type only a small fraction of the total genome is expressed and the rest of the structural genes are predominantly repressed. Since transcription and translation

are spatially and temporarily separated in eukaryotes unlike in prokaryotes, there are atleast two levels at which protein biosynthesis is regulated, i.e. transcriptional level and translational level.

1.3.1. Regulation at transcriptional level

Regulation at this stage can occur by any of the following mechanisms.

1.3.1.1. Alkylation

A definite portion of the genome can be made inaccessible for transcription by chemical modification of DNA itself. The most well known modification of DNA is alkylation, particularly methylation (29,30). The existence of DNA methylases is well established (31,32) and about 2-7% of the cytosine residues of animal DNA are methylated. Gene expression is often speculated to be associated with demethylation (33,34).

1.3.1.2. Histones

The chromosome structure and hence gene expression can be regulated by histones which are closely associated with DNA in the native nucleoprotein complex. Histones cover and repress most of the DNA, leaving only a certain part accessible to the transcribing machinery. Any change in the binding affinity of histones and DNA may affect the functional status of specific segment of DNA. Stedman et al (35) proposed that the amount of histones, in actively growing and non growing tissues, vary characteristically. Bonner et al (36) showed that preparations of histones could inhibit the ability of DNA to serve as a template for the synthesis of RNA by RNA polymerase.

1.3.1.3. Heterogeneous nuclear RNA (hnRNA)

The eukaryotic nucleus contains pools of large RNA molecules often called the hnRNA. They consist of a complex mixture of many RNAs that

are very much larger than the mRNAs. hnRNAs also turn over very rapidly and they are not usually transported to the cytoplasm. Though the major function of it is not known it is speculated to be involved in regulation of gene expression (37).

1.3.1.4. Role of stable complexes

The role of stable complexes that repress and activate eukaryotic genome has been put forward by Brown (38), based on the results obtained from 5S RNA genes. A region of about 50 nucleotides within 5S RNA gene which is referred to as the 'internal control' region is formally analogous to promoter of prokaryotes, because it is required by RNA polymerase III to initiate transcription. However, RNA polymerase does not recognize this DNA sequence directly, but recognizes a complex that includes DNA in the control region and three or more other molecules. One of these has been partially characterized and called TF III A or 40 kd factor. In the absence of this protein a 5S RNA gene can be stably repressed by addition of histones. According to this hypothesis genes are in a state of 'determination' or 'commitment' with the formation of a stable complex, but the expression of gene that is already determined can be modulated and expressed by different factors like hormones, a process called 'differentiation'.

1.3.2. Regulation at translational level

Translation is the process by which the genetic message present in the form of nucleotide sequence of a specific messenger RNA is converted into the amino acid sequence of a protein. Various components are known to be required for translation, such as ribosomes, mRNA, chain initiation, elongation and termination factors, tRNAs, aminoacyl-tRNA synthetases and amino acids in addition to various energy donating molecules like ATP,

GTP etc. The modification or limitation of some of these factors may lead to preferential translation of specific messengers leading to differential gene expression. Endogenous control of protein biosynthesis has long been known both in unicellular and multicellular organisms. The surprisingly large number of naturally occurring inhibitors of protein synthesis suggests this site as the 'Achilles heel' of cell metabolism (39). In reticulocytes, initiation of globin synthesis depends on the presence of hemin and is inhibited by oxidized glutathione. A complex translation inhibitory system, the hemin controlled repressor, involving the activation of a cAMP dependent protein-kinase which phosphorylates eIF-2 was discovered (40). Interferon seems to exert its antiviral effect at least in part by controlling translation of viral mRNAs. Treatment of cells with interferon induces translational inhibitory system that can be activated by double stranded RNA (ds RNA). ds RNA activates an enzyme that converts ATP into a potent nucleotide inhibitor of translation (41).

1.4. Transfer RNA

Transfer RNAs play a central role in protein biosynthesis being involved at every stage, starting from aminoacylation to termination of polypeptide synthesis. The tRNA molecule is an ancient component of biological systems. It probably arose some four billion years ago in the early prebiotic period (42). The following few pages throw light on some of the structural and functional aspects of transfer RNA and aminoacyl-tRNA synthetases.

Transfer RNA is the term first coined by Allen and Schweet (43) which describes a group of low molecular weight RNA molecules that play a vital role in protein biosynthesis. The existence of this type of molecules had been predicted by Crick in 1955 (13). He suggested that, since DNA and

RNA do not act as direct templates for amino acids, 'adaptor molecules' were necessary to translate the information carried by nucleic acids into a protein. The hypothesis required that there was one adaptor molecule for each amino acid, with a hydrogen bonding surface to bind the nucleic acid templae at one end and a site for specific attachment of amino acid, which is an enzyme catalyzed reaction. He further suggested that the nature of the link between amino acids and adaptor would likely provide the energy for amino acid polymerization. Transfer RNAs (initially called soluble RNAs or sRNAs because they occur in soluble cytoplasmic fraction of cells) were assigned the role of adaptor molecules by Hoagland et al in 1958 (16).

1.4.1. Structure of tRNA

There are about 40-60 tRNA species found in prokaryotes or in the cytoplasm of eukaryotic cell with distinctly different sequences for the 20 amino acids. Hence there are several tRNAs for each amino acid. These multiple tRNAs for a single amino acid are called isoacceptors (17). Since their function is to translate the several different codons for an amino acid, they essentially differ in their anticodon sequence. They are usually distinctly different in their sequences although sequence homology can be found in related species. In addition to the cytoplasmic tRNAs, there are 20-30 tRNAs representing almost all amino acids, in organelles such as mitochondria and chloroplasts (46). The tRNAs found in organelles are used for the synthesis of organelle specific proteins. The sequences of organelle tRNAs show more homology to that of prokaryotes than the eukaryotes showing their prokaryotic origin (45,103).

1.4.1.1. Primary sequence

Holley et al (44) were the first to determine the complete sequence

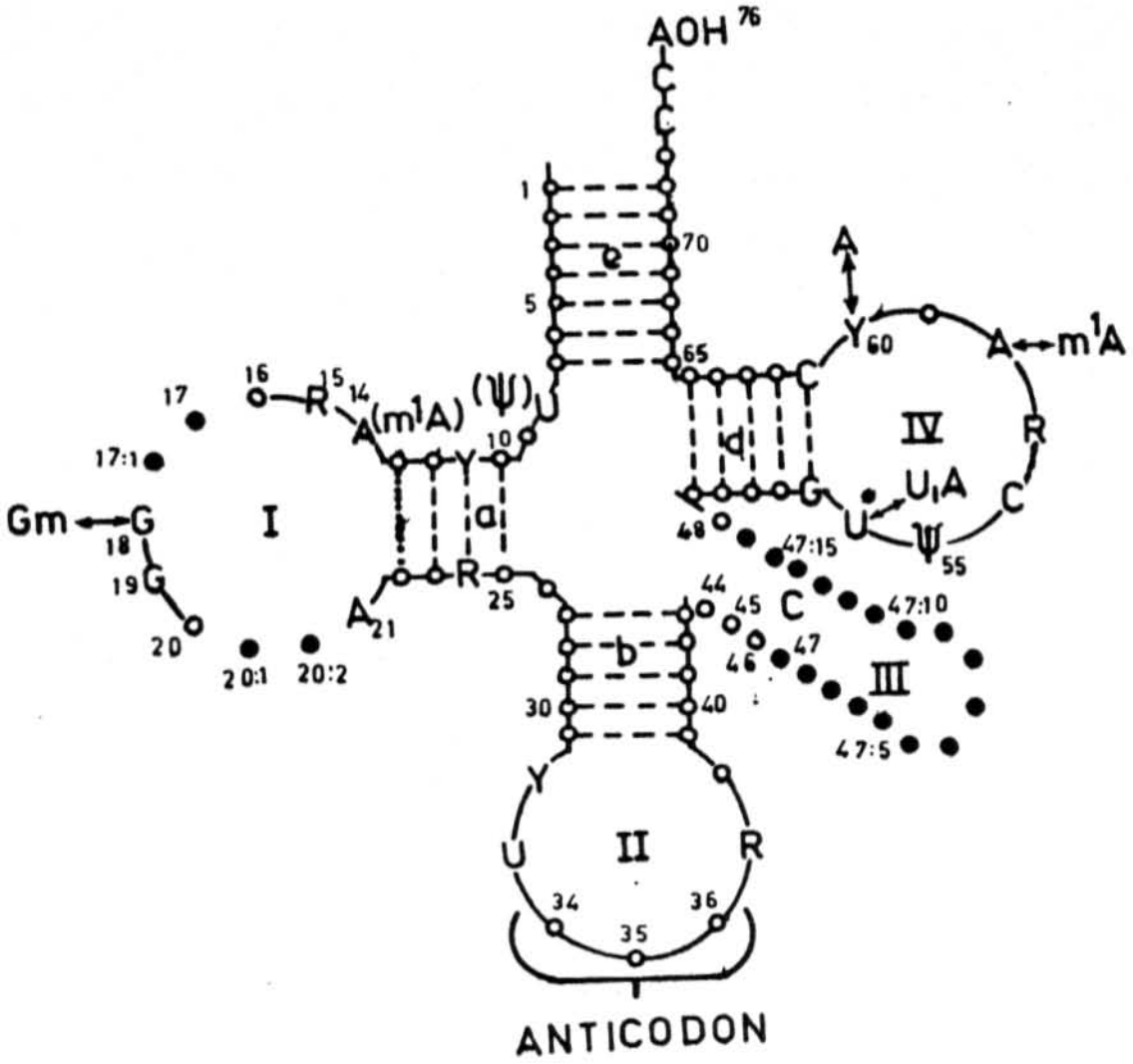


Fig. 1. Cloverleaf model of tRNA showing common features of cytoplasmic elongator tRNAs of eukaryotes. Nucleoside positions are indicated by circles or by letters. Invariant or semi-invariant bases are indicated by letters. Nucleoside positions always present are indicated by open circles and those that may or may not be present by filled circles. A solid line connecting the nucleosides represents the phospho-diester backbone and the base pairs are indicated by dashed lines (the dotted line in stem a represents a base pair that is not always present). An arrow between two nucleosides means the residue outside the loop is found two times or more. A nucleoside in brackets is the single exception to the commonly found nucleoside. The 5'-end of the molecule is position 1. Y, uridine, cytidine, or their modified forms; R, guanosine, adenosine, or their modified forms; U*, modified uridine.

of a tRNA molecule. Since then extensive work has been done on this aspect and currently over 300 distinct tRNA sequences are known from prokaryotes, lower and higher eukaryotes and from eukaryotic organelles (45). The list includes numerous examples of isoacceptors from a single species, tRNAs specific for the same amino acid from different species and tRNAs from the same species for different amino acids. Many workers attempted to compare and draw sequence homologies in related species (98-104). Though some relationships can be deduced, no clear principles have emerged. tRNA molecules contain about 73 to 90 nucleotides in a single polynucleotide chain.

1.4.1.2. Secondary structure

All the known sequences of tRNAs can be arranged into a two dimensional 'clover leaf' form first proposed by Holley et al (44) as shown in Fig 1 (46). The clover leaf structure consists of 4-5 double stranded helical regions (a-e) called stems, connected by four single stranded regions (I-IV) called loops. A tRNA arm consists of its loop and its stem and is denoted by its loop number. The presence of stem c is variable and occurs only if sufficient residues are present in arm III and this is often called the extra arm. The 3'-C_pC_pA terminus is always present and it is attached post-transcriptionally by the enzyme nucleotidyl transferase (47). The amino acid is attached to the terminal A of the -CCA sequence. The anticodon triplet is located in the center of the 7-membered loop II (also called anticodon loop) at the end of stem b. The size of each element of the clover leaf is constant except at loops I, III and stem c where all the variation in length (73-94 bases) of tRNA is accommodated. Majority of tRNAs have a small arm III consisting of 4-5 residues. Only $tRNA^{leu}$, $tRNA^{ser}$ and prokaryotic $tRNA^{tyr}$ have a large arm III which ranges from 13-21 residues.

here are a number of constant features. The acceptor stem generally has seven base pairs. The stem d consists of five base pairs and there are seven nucleotides in the loop IV. This is the most highly specified stem and loop. Those nucleotides which are constant in particular positions for chain elongating tRNAs are indicated by letters in Figure 1. There are about 14 such invariant bases present in tRNA. The preservation of certain nucleotide sequences (e.g. G-T-Ψ-C etc) in a large number of different molecules suggests that they may be there for reasons related to the three dimensional conformation of the molecule or because they provide a constant mode of interaction with other molecules (42). In addition to the invariant bases a number of semi-invariant positions of several types are found (46).

(i) With a few exceptions, at positions 11,32 and 60 only pyrimidines are found.

(ii) Position 14 is always A or sometimes m^1A

(iii) Positions 15,24,37 and 57 are always purines in most of the cytoplasmic tRNAs.

(iv) The variations at positions 18 and 58 occur frequently

(v) U_{54} is usually modified to T,Ψ or Tm.

There are many exceptions to rules of invariant and semi-invariant positions when elongator tRNAs of organelles and prokaryotes are examined. Stem a can be as small as 2 base pairs, loop I as small as 3 residues and invariant G_{18}, G_{19} is absent in all but $tRNA^{leu}$. In $tRNA^{ser}$ arm I has disappeared altogether (48).

1.4.1.3. Minor bases

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1.4.1.3. Minor bases

One surprising feature of tRNAs is, the presence of a number of unusual bases, made sometimes by quite elaborate modifications of one of

.e four normal nucleotides. More than 50 such bases have been identified in sequenced tRNAs todate (49,50,51). They usually occur at restricted sites in tRNA, and in many cases a particular base is found only at a single position. Positions 34 and 37 are particularly favoured sites since they play a role in codon-anticodon recognition. The most frequently occurring modified nucleotides are I, ψ, S^4U and a number of methylated nucleosides like methylated uridine (ribothymidine) and methylcytosine. In all cases, except one, the modification reaction involves the alteration of, or addition to, existing bases in the tRNA. The exception is the synthesis of Q bases, where an enzyme has been found that is able to exchange free queuosine with a guanine residue in the tRNA. The reaction involves breaking and remaking of bonds on either side of the nucleoside (52). Though modified nucleosides are present in all tRNAs no specific roles have been assigned to them so far (49). The main effect of base modification appears to be an increase in the solvent accessible surface of the molecule. The increase in the overall exposure area is upto 20%, suggesting that the modifications exist for recognition by various proteins (55).

1.4.1.4. Tertiary structure

The reasons for the large number of conserved residues in tRNAs remained unknown until the elucidation of the three dimensional structure of yeast phenylalanine tRNA. The first crystal structure of tRNA at 3A° resolution was determined in 1974 (53,54). The crystal structure of four tRNAs are now known (46). The molecule consists of two helical stems at approximately right angles to each other. These are held together by a variety of tertiary structural interactions, giving it an overall 'L' shape. The length of each arm is approximately 70 A° and the thickness is about 22 A°. It is a flat molecule. The amino acid binding end is at the right extreme of the

molecule and the anticodon at the extreme bottom. The loop IV sequence is suggested to be involved in ribosome binding. The horizontal helix is made up of stems e and d in which the 3' end chain is continuous. The vertical more irregular helix consists of stems a and b plus additional bases. The central part of the molecule, including the region where the two helices come together, is stabilized by a variety of tertiary interactions. These tertiary interactions involve most of the invariant and semi-invariant bases and thus explain their existence. The structure may also explain why all of the variations of tRNA size is confined to the two regions of loop I and III. Since the rest of the molecule is so involved in either H-bonding or stacking interaction, these are the only places where variation can occur.

1.4.2. Solution structure and conformational changes during protein synthesis

Studies on $tRNA^{phe}$, as well as other tRNAs by a variety of physical and chemical methods hae shown that the same tertiary structure exists in solution also (55). Two kinds of conformational changes in tRNA have been proposed to take place when tRNA binds to ribosome. The first is related to the interaction of loop IV with ribosomal RNA at the A site (56). Strong evidence is available for the interaction of loop IV, in particular the common GTΨC sequence, with ribosomal RNA at the A site (56). Hence the loop I and IV which are usually strongly complexed separate out. The second conformational change is in the anticodon loop. Some recent results disproved this idea (57).

1.4.3. Adaptor role of tRNA (tRNA structure-function relationships in ribosome mediated protein synthesis)

1.4.3.1 Codon-Anticodon recognition

One of the early observations during deciphering of the genetic code

was that, a single tRNA species could recognize more than one codon. In order to account for this, Crick proposed the wobble hypothesis (25), which stated that the 5' end of anticodon, G.U, I.U and I.A base pairs are found in addition to standard G.C, U.A and U.I pairs. This allows the 64 assigned codons to be read by a minimum of 32 tRNAs. This follows directly from the fact that two tRNAs are needed for each of the 16 sets of codons of the form XYN, plus one initiator tRNA minus one tRNA for the termination codon pairs UA $\begin{matrix}A\\G\end{matrix}$. However more than 32 tRNAs are used. This is because the tRNAs for the 5 amino acids (arginine, glutamine, glutamic acid, leucine and lysine) whose codons are XY $\begin{matrix}A\\G\end{matrix}$ do not use U_{34} corresponding to the wobble rules. Instead, a modified U as mcm^5s^2U, mcm^5U or mnm^5s^2U is used. This residue does not pair with G, so that a second C_{34} containing tRNA is used. Nishimura (49) has suggested that the reason for an unmodified U_{34} not allowed in these cases is that it might too readily mispair with XYU which would be lethal since those codons stand for a different amino acid. Three classes of modified bases are found next to the 3' side of the anticodon (N_{37}).

(i) When the 3' base of anticodon is A_{36}, N_{37} is i^6A, ms^2i^6A or yW. These are bulky hydrophobic bases.

(ii) If U_{36} is present N_{37} is t^6A, mt^6A or ms^2t^6A. These are also bulky but hydrophilic bases.

(iii) When C_{36} or G_{36} are present, methylated G_{37}, I_{37}, A_{37} or unmodified A are present.

Evidence exists that 1st and 2nd class of modifications are necessary for optimal ribosomal decoding to occur (58). It has been suggested that

N_{37} modifications are designed to modulate the strength of codon-anticodon interaction, so that all tRNAs, irrespective of the number of GC base pairs, will form a codon-anticodon complex of approximately equal strength (59). Yarus (60) has put forward a hypothesis that coding performance of triplet anticodon is enhanced by selection of a matching anticodon loop and stem sequence. The anticodon plus the nearby sequences which is called the 'extended anticodon' contains more coding information than the anticodon alone and can perform more efficiently and accurately at the ribosome. This idea successfully accounts for the relative efficiencies of many tRNAs.

1.4.3.2. Interaction with initiation and elongation factors

The interaction of bacterial polypeptide chain initiation factor 2 (IF_2) with initiator tRNA was studied in E.coli (61). The structural requirements of the tRNA for binary complex formation revealed by studies with modified fmet-$tRNA_f^{met}$, a number of N-blocked and normal aminoacyl-tRNAs suggests that IF_2 selects the initiator by recognizing the fmet moiety and IF_2 alone is not capable of distinguishing the nucleotide sequence of tRNAs. In rabbit reticulocyte system, misacylated tRNAs like ile-$tRNA_i^{met}$ of yeast failed to initiate protein synthesis showing that methionyl residue may play a role in the eIF_2 recognition of the tRNA (62). eIF-2 distinguishes between unformylated prokaryotic $tRNA_f^{met}$ and eukaryotic $tRNA_i^{met}$ and also between eukaryotic initiator and non-initiator methionyl-tRNA (63).

In E.coli, removal of the 5'-phosphate from several normal aminoacyl-tRNAs was found to drastically reduce the ability of these tRNAs to form stable ternary complexes with EF-Tu (64). The requirement for base pairing at the end of the acceptor stem therefore apears to result from a requirement for a specific spatial orientation of the 5' terminal phosphate for T factor

binding. Very little is known about specific features in tRNA recognition by eukaryotic elongation factor 1. EF-1 does not recognize deacylated or N-acylated aminoacyl-tRNAs as in prokaryotes (65).

1.4.3.3. Ribosome

The main recognition feature in tRNA for ribosome binding in both prokaryotes and eukaryotes is considered to be the GTΨCR sequence in loop IV. It is usually thought to interact with 5S RNA by forming complementary base pairs (56). The rat liver ribosomal proteins L6, L35a and S15 were shown to form specific complexes with yeast elongator tRNAs and hence they may be involved in the recognition process (66).

1.4.4 Involvement of tRNAs in other cellular processes

In addition to the adaptor role it performs in ribosome mediated protein synthesis, tRNA participates in several other cellular processes, detailed below:

1.4.4.1 Transfer of amino acids to specific acceptor molecules

These are reactions which are catalyzed by a group of enzymes known as aminoacyl-tRNA protein transferases (67). Addition of an amino acid to the amino terminus of specific proteins or peptides has been demonstrated *in vitro* in both prokaryotes and eukaryotes. The transfer of arginine from arg-$tRNA^{arg}$ in eukaryotes may play a role in membrane modifications or in regulation of chromatin activity since both erythrocyte ghost membrane proteins and chromosomal proteins may be terminally arginylated *in vitro* (68,69). The structural requirements of the tRNA for these reactions is not clear. The amino acid moiety is apparently more important than tRNA in defining specificity. Since mischarged phe-$tRNA^{val}$ may act as a donor but

not val-tRNA^{val}. A free amino group on the amino acid is also required. tRNA also plays some role since phe-tRNA^{val} is a less efficient donar to the acceptor protein than is phe-tRNA^{phe}.

Phosphatidyl glycerol will accept lysyl and alanyl residues from tRNA forming O-lysyl-(or alanyl) phosphatidyl glycerol by transferase catalyzed esterification of the glycerol moiety. This is probably associated with membrane modifications (70).

The formation of peptide bridges between L-lysine (ϵ-amino) and D-alanine (carboxyl) residues on tetrapeptides on adjacent strands of peptidoglycan in bacterial cell wall biosynthesis is catalyzed by the aminoacyl-tRNA-N-acetyl muramyl-penta-peptide transferases. Investigations of the tRNAs from various organisms which accept amino acids involved in these reactions (e.g. glycine, threonine, serine) had given some idea on the structural requirements of the tRNA in this reaction. Of the 7 serine isoacceptors in S. epidemis one does not function in ribosome mediated protein synthesis and one is inactive in peptidoglycan synthesis (71). Of the five isoaccepting glycine tRNAs, all could transfer glycine to peptidoglycan but two, tRNA$^{gly}_{1A,B}$ failed to act as adaptors in protein synthesis. Subsequent sequence analysis of these tRNAs showed that these have distinct differences from other tRNAs that participate in ribosomal protein synthesis being minimally modified and lacking many of the invariant bases, including the TΨCG sequence implicated in ribosome binding (72).

1.4.4.2. Regulation of amino acid biosynthesis

Aminoacyl-tRNAs are involved in regulation of the biosynthesis of a number of amino acids (73,74,75). The well defined example is the

his-tRNA^{his} which acts as a co-repressor in the regulation of histidine biosynthesis in Salmonella typhimurium. Six classes of regulatory mutants his O, his R, his S, his T, his U and his W have been identified (76,77). The extent of repression of the histidine operon in vivo correlates with the concentration of his-tRNA^{his} except in his T mutants where the operon is derepressed although the levels of tRNA^{his} and his-tRNA^{his} are the same as in the wild type (78). The tRNA^{his} from this mutant has been sequenced and found to lack the pseudouridine present at positions 37 and 38, thus function normally in protein synthesis but defective in the activation of repressor. This also shows that modified bases play a role in regulation.

1.4.4.3. tRNA like structures in viral RNAs

Several viral RNAs may be enzymatically aminoacylated. Most of these are plant viruses (79-82). Aminoacyl-tRNA synthetases from E.coli, yeast, plants and mammals can catalyze this reaction. Esterification is via a normal aminoacyl-tRNA bond to the 3' terminal A of the -CCA end of RNA. There is a specificity for the amino acid added, which depends on the particular RNA. So far, histidine, tyrosine and valine have been found to acylate plant viral RNA. Complete primary structure of the 3' regions of some RNAs have been determined. They only resemble crudely the clover leaf structure. No modified nucleosides are present (83). The reasons for the presence of these structures are not known. Haenni and Chapville (83) speculated that their role may be to inhibit host protein synthesis possibly by competing for one of host isoacceptor tRNA not needed for virus synthesis.

1.4.4.4. Primer for reverse transcriptase

Replication of the retroviruses requires a tRNA primer for initiation of synthesis of the DNA strand complementary to the viral RNA (84). The

host cell tRNAs act as primers. Priming occurs by reverse transcriptase induced hybridization of the 3' region of the tRNA (60-75 residues) to a region near to the 5' end of the viral RNA. Reverse transcriptase binding of tRNA requires most of the sequences on tRNA (except 67-76) and the binding is highly specific. Only certain tRNAs mostly participate in priming.

1.4.4.5. Transfer RNAs and the regulation of cell growth and development

tRNAs show many qualitative and quantitative changes in distribution, modification and metabolism under various physiological conditions (85,86). Changes during viral infection, growth condition, neoplasia, with tissue type, with hormones, during special protein synthesis etc. have been observed. The activity and/or levels of enzymes acting on tRNAs are also often changed to function more efficiently in the synthesis of a particular protein (87,88). The differentiation of silk glands of Bombyx mori leads to massive synthesis of 2 silk proteins fibroin and sericin at the end of 5th instar. Quantitative changes in tRNA population occur at this time which match with the predominant amino acids of fibroin i.e. glycine, alanine and serine (89). Christner and Rosenbloom (90) compared the amino acid acceptor activity for each of the 20 amino acids between collagenous tissues like tendon and non-collagenous tissues like brain and liver in the embryonic chick. They found an increase in acceptor activity of tendon tRNA for glycine (33%), arginine (40%) and proline (83%). RPC elution profiles of $tRNA^{gly}$ demonstrated that the increase in $tRNA^{gly}$ could be accounted for by an increase in one of the four major isoacceptors. These results suggest that a characteristic complement of tRNA species may be associated with collagen synthesis, which match with the predominant amino acids of collagen, that is glycine (33%) proline, hydroxyproline (23%) and alanine (11%). Although nucleotide composition analyses have been made (91,92), investigations of these observed differ-

ences in tRNA populations have not generally yet been pursued to the level of detailed analysis of structural differences. Such analyses may well reveal only small and subtle differences in structure, which often produce significantly large differences in chromatographic behaviour to allow its detection as well as having a profound effect on the ability of tRNA to function in regulation by, for example, its interaction with specific regulatory proteins.

1.5. tRNA Biosynthesis

Much of the knowledge about the process of tRNA biosynthesis has come from studies with microorganisms, where RNA sequence determination and mutant isolation are more readily carried out. E.coli has about 60 tRNA genes and possesses one or a few copies of a particular gene for each tRNA sequence (93). The genetic mapping experiments using suppressor tRNAs have shown that tRNA genes are clustered at several locations on the genome. Eukaryotes have much higher number of tRNA genes e.g. about 400 in Yeast (93) and 8000 in Xenopus laevis (94). Except for mitochondrial tRNAs, other tRNA molecules arise as a result of transcription from nuclear DNA. The initial product of transcription of a tRNA gene is a longer tRNA molecule (precursor) that must be processed to yield mature tRNAs. Sometimes these precursors contain more than one tRNA. e.g. proline-serine precursor from T_4 bacteriophage. Highly specific enzymatic reactions are involved in the processing, where exonucleases, RNase P and other enzymes participate. Some tRNAs of eukaryotes contain introns of 14-34 base pairs, at the 3' side of anticodon loop junction, which are spliced during processing. The -CCA end is added post-transcriptionally by the enzyme nucleotidyl transferase (47). Minor bases are all made by modifications of their parent in the precursor polynucleotide chain except for the Q base which is inserted by base exchange with G. Processing does not require modifications to be present

in precursor molecule but experiments with Xenopus have shown that at least four modifications occur in the nucleus at defined stages of the processing (95).

1.6. tRNA and evolution

The tRNA molecule is an ancient component of biological systems. It probably arose some four billion years ago in the early prebiotic period (42). tRNA makes a very interesting subject for the study of evolution because of several aspects related not only to its function but also to its structure. Its role as an adaptor leads to the idea that a similar molecule must have been present in the earliest stages of molecular evolution. Other considerations such as those regarding the length of a primordial molecule which allowed stability against degradation and conservation of the information content to a large extent through successive self replicative cycles in prebiotic conditions, lead toward a tRNA-like molecule that has kept its position in the process of flow of information from nucleic acids to proteins upto the present (96) and has acquired further functions within the cellular environment during evolutionary history. Environmental conditions have imposed several constraints to the evolution of tRNA and as a result, one can observe certain regularities in the sequence and conformation of these molecules. The biochemical evolution using tRNA as model can be studied in two ways.

1. Sequence comparison
2. Heterologous charging

1.6.1. Sequence comparison

As mentioned earlier there are over 300 tRNAs that have been sequenced so far (45). Using this sequence information many groups of scientists

attempted to construct phylogenetic trees and to draw species relationships. One evidence for a single common evolutionary origin of tRNA is its uniform secondary structure and the presene of identical bases at homologous sites (97). Holmquist et al (98) compared forty three tRNAs, divided about equally between 23 prokaryotes (principally E.coli) and 20 eukaryotes (yeast). Pairwise comparisons of these tRNAs reveal additional similarity, greatest for certain pairs of tRNAs for the same amino acid in the same organism. Larue et al (99) constructed phylogenetic trees of $tRNA^{met}$ and $tRNA^{phe}$. It has been shown from several reports (100,101) that $tRNA^{phe}$ and the initiator tRNA structures are most conserved. Ghosh (102) compared the wheat germ initiator tRNA sequence with several other initiator tRNAs. Wheat germ is more homologous to eukaryotic tRNA (similarity 75-90%) than to tRNAs of prokaryotes or organelles (51-68%). Among eukaryotes wheat germ is closely related to vertebrates and Drosophila (87-90%) than to fungi (73-76%). Hecker et al (103) compared the nucleotide sequences of blue green algae $tRNA^{phe}$ with other prokaryotic and eukaryotic sequences. Sequences of Euglena gracilis, bean leaves and spinach chloroplast $tRNA^{phe}$ were compared to $tRNA^{phe}$ from eukaryotes and prokaryotes and shown that chloroplast $tRNA^{phe}$ is more homologous with those of prokaryotes than those of eukaryotic cytoplasm. This suggests that present day $tRNA^{phe}$ within the chloroplast genomes evolved from a prokaryotic gene and unlikely from a eukaryotic genome. Rodriguez et al (104) constructed phylogenetic trees of tRNAs specific for phenylalanine, methionine, glycine and valine. Although the exact relationships between taxa cannot be obtained from the mere analysis, several features of the tRNA evolution could be inferred. Thus, though only gross phylogenetic relationships can be obtained by analysis of the sequences of tRNA, such analysis helps to increase our knowledge of the evolutionary history of the molecule and its influence in the process of evolution.

1.6.2. Heterologous charging

The combination of synthetase from one organism and tRNA from another (heterologous combination) first demonstrated the existence of isoacceptors (17,106). This phenomenon has provided insight into the adaptor role of tRNA, location of recognition site on tRNA and changes that appear in tRNA in the course of evolution. Species specificity is often the main interest of such studies (105). In studies of this kind many artifacts come into picture thus making interpretation a lively and imaginative pasttime (105). Patterns of codon recognition by isoacceptors from wheat germ were determined by Hatfield et al (107). Such studies in conjunction with coding studies from other species (106,108) provide an evolutionary record of the changes which have occurred in the patterns of codon recognition between different organisms. Patterns of codon recognition of major isoleucine and valine isoacceptors in wheat germ are similar to those in mammals (109) but differ from those in E.coli (110). These studies have also been proved from sequence data. One drawback of such studies is that the conditions that are optimal for homologous charging may not be so for heterologous charging (105). Alanine tRNA in general shows high aminoacylation activity in all heterologous combinations indicating that synthetase sites for $tRNA^{ala}$ species began to the conserved in evolution at a very early stage (158).

Despite these limitations heterologous charging of tRNA from a variety of organisms provide a better understanding of evolutionary changes which have occurred in tRNA-synthetase recognition.

1.7. Aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases which probably arose over one billion years ago were discovered only some 25 years ago (16). They catalyze the

esterification of an amino acid to its cognate tRNA species to form aminoacyl-tRNA. There is only one synthetase in the prokaryotes per amino acid (111) and the same is probably true for eukaryotes. Though the existence of synthetases has been shown in mitochondria and chloroplast, in all cases they are made in the cytoplasm using nuclear genes (112,113).

1.7.1. Subunit structure

Though all synthetases catalyze the same reaction they differ markedly in their physical properties. The subunit size ranges from 37 to 120 kd and four types of quaternary structures are found i.e. α, α_2, $\alpha_2\beta_2$ and α_4. Some data show that, while synthetases are heterogeneous amongst themselves with respect to quaternary structure, a particular enzyme's quaternary structure is the same in various organisms (46). The smallest enzyme is E.coli trp-tRNA synthetase, an α_2 protein with subunit molecular weight of 37,000. Each subunit of this enzyme appears to have its own sites for amino acid, adenylate and tRNA (114). Therefore, the minimum size of the polypeptide chain required to carry out aminoacylation is about 330 amino acids. The longest polypeptide chains are associated with E.coli ile and val-tRNA synthetases, each is an α enzyme with molecular weight of about 110,000 (115) and the largest synthetase quaternary structure characterized so far is E.coli ala-tRNA synthetase, a α_4 protein with molecular weight 380,000 (116).

There are three explanations for the heterogeneous subunit sizes and quaternary structure (117). These enzymes may have evolved by convergent evolution from unrelated ancestors and have different polypeptide sizes and quaternary structure. This hypothesis is difficult to prove or disprove. Secondly, synthetases may be more similar than is apparent by simply comparing sizes. Several reports on large synthetases (subunit size MW $>$ 60,000) state that

polypeptides contain repeated sequences (115). However doubt has been cast recently on the existence of repeated sequences (117). The third possibility is that synthetases have different sizes, partly because they perform other functions in addition to aminoacylation, which vary from protein to protein. For example one synthetase has been found to act as a regulator of transcription and this role is believed to depend upon the particular quaternary structure (118).

1.7.2. Multi-enzyme complexes

While in prokaryotes, synthetases exist free or in complex with tRNA, in eukaryotes many synthetases are found as components of high molecular weight complexes with MW 1-5 x 10^6 daltons. These complexes have been reported in a wide variety of tissues (119-123). The occurrence of these high MW complexes appears to be ubiquitous in higher eukaryotes (124). The synthetase composition of the complex varies although enzymes for arginine, glutamine, glutamic acid, leucine and lysine are generally present. Several synthetases are shown to contain carbohydrate moieties suggesting the possibility that carbohydrates may be involved in complex formation (125). The functional significance of these high molecular weight complexes are unclear but speculated to be involved in control of DNA replication, transcription and translation (126).

1.7.3. Homologies between synthetases

Bacillus stearothermophilus trp-tRNA synthetase, a 327 amino acid polypeptide was the first to be sequenced completely (127). Sequences of other synthetases have recently been reported either by DNA sequencing or protein sequencing techniques (128-131). There is a considerable homology between B. stearothermophilus trp-tRNA synthetase and its E. coli counterpart

(127,129). These homologies are more at the N-terminal end. E. coli ala-tRNA synthetase (875 amino acids/subunit) has been compared with the partial sequence of E. coli tyr-tRNA synthetase (about 420 amino acids/subunit). A significant homology occurs between 370-382 of alanine enzyme and residues 9-25 of tyrosine enzyme. This region in alanine enzyme is speculated to be involved in aminoacylation function. In the E.coli tryptophan enzyme, 15 of 18 consecutive nucleotides in the carboxy terminal half match with an 18 nucleotide stretch in C-terminal half of alanine enzyme. The significance of this homology is still unknown (117). Schimmel et al have shown that separate domains exist in synthetase to carry out the different reactions of aminoacylation (132).

1.8. Aminoacylation

1.8.1. Specificity of reaction

Proof of the adaptor hypothesis that once attached to a tRNA, amino acid no longer has any effect on codon selection has long been available (133). The consequence of this fact is that synthetases must be highly accurate both in their selection of an amino acid and of a tRNA. The upper limit to the degree of error tolerated in finished proteins was obtained as 3×10^{-4} (134). The demands on synthetases are much greater than for many other enzymes. Error correction, editing or proof reading with respect to amino acid specificity appears to occur before aminoacyl-tRNA and synthetase separate out. Synthetase specificity can be conveniently divided into two parts namely specificity for the small ligand, the amino acid, and for the large ligand, the tRNA.

1.8.2. Amino acid recognition

An α-amino group is essential both for substrate or inhibitor activity.

The carboxyl group, on the other hand, is not necessary. All enzymes are L-isomer specific. Synthetases are speculated to follow a double sieve editing mechanism (135) for the selection of a given amino acid from the other naturally occurring ones of similar structure. First amino acids larger than the substrate are excluded by the usual steric mechanisms. Amino acids smaller are only partially excluded (poorer fit). But in cases where they are similar in size e.g. valine and isoleucine a special hydrolytic mechanism comes into picture. The incorrect amino acid is transferred to tRNA and then rapidly hydrolyzed while the aminoacyl-tRNA is still enzyme bound. This appears to occur at an amino acid binding site distinct from the original amino acid recognition site, since enzyme bound aminoacyl-AMP does not interfere with this process. Use of tRNAs with a deoxyadenosine at the 3' end has revealed another interesting feature of this editing mechanism. In a number of cases the absence of one of the 3' terminal hydroxyl groups block the hydrolytic editing function and allows the incorrect aminoacyl-tRNAs to accumulate (136). This has prompted Crammer and his colleagues (136) to propose that the hydroxyl group is directly involved in the hydrolytic reaction which they called 'chemical proof reading'.

1.8.3. Recognition sites on tRNA

Many studies have shown that synthetases in general bind in and around the inside of L shaped tRNA. While tRNAs vary considerably in length and nucleotide sequence, all cytoplasmic sequence have uridine or 4-thiouridine at position 8 (45). This uridine is located at the inside of the vertex of the L where it is well positioned to interact with bound synthetase. This interaction results in a synthetase-catalyzed exchange of pyrimidine ring H-5 atoms on U-8 (137). A trinucleotide UAG which is the sequence around U-8 is a specific inhibitor of aminoacylation (138). Schimmel et al suggsted that U-8

might be a kind of bench mark for synthetase, once recognition has occurred. That is, by anchoring to the tRNA at this point the proper placement of the 3' end of the tRNA at the catalytic center would be assumed. In addition to uridine-8 there must be other nucleotide sequences which determine the recognition of tRNA. These sequences would permit or prevent the enzyme's access to U-8 depending upon whether the enzyme contacts cognate or non-cognate tRNA. The lack of H-5 exchange at U-8 in a non-cognate tRNA-synthetase mixture is consistent with this view. Much work was done on the recognition process of $tRNA_f^{met}$ from E. coli by its cognate methionyl-tRNA synthetase. Various procedures for attachment of protein affinity labelling reagents of variable length and amino acid specificity to internal sites in $tRNA^{met}$ have been developed (139,140). The results indicate that recognition of $tRNA_f^{met}$ requires highly specific interaction of methyionyl-tRNA synthetase with functional groups on the nucleotide bases of the anticodon sequence, suggesting that anticodon sequences may be important for accurate discrimination between cognate and non-cognate tRNAs by aminoacyl-tRNA synthetases (141).

1.8.4. The aminoacylation reaction

The process of aminoacylation can be represented as shown below.

$$AA + ATP + E \rightleftharpoons E - AA - AMP + PPi \qquad (1)$$

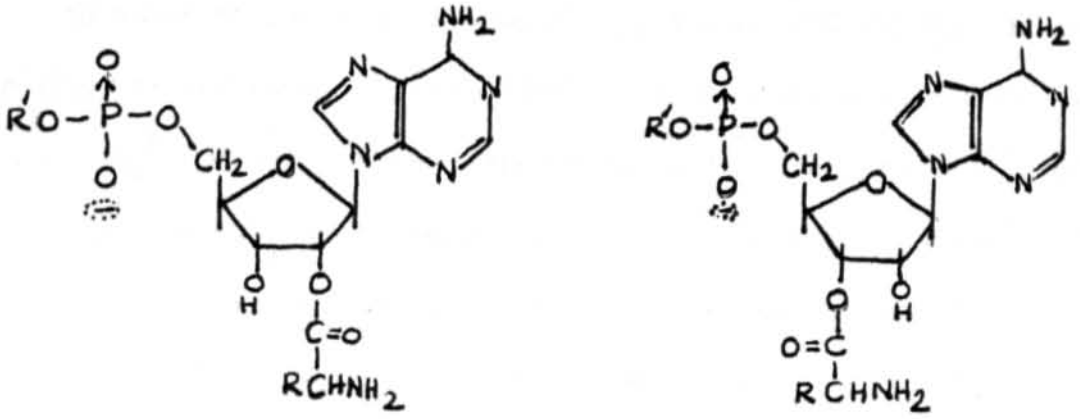
$$E\text{-}AA - AMP + tRNA \rightleftharpoons AA\text{-}tRNA + AMP + E \qquad (2)$$

$$AA + ATP + tRNA \rightleftharpoons AA - tRNA + AMP + PPi \qquad (3)$$

In the first step amino acids are activated by formation of an enzyme bound aminoacyladenylate with the liberation of pyrophosphate. In the second

step the amino acid transferred to tRNA with the liberation of AMP and the regeneration of the enzyme. The overall stoichiometry is correct and the reaction is reversible (17). The aminoacyl-tRNA formed preserves the high group transfer potential of ATP (142). This high group transfer potential finds expression in the facile reaction with nucleophiles such as the amino group of amino acids in peptide bond formation as suggested by Crick (13,14).

The structure of aminoacyl-tRNA is shown in Figure 2.



2' ⇌ 3' isomerism

R-amino acid side chain

R'-reminder of tRNA

Figure 2: STRUCTURE OF AMINOACYL-tRNA

The amino acid is attached via its carboxyl group in an ester linkage to either the 2' or 3' hydroxyl group of the 3'-adenosine of the tRNA. Although in solution the amino acid equilibrated rapidly (in milliseconds) between the two positions it is now known that synthetases specifically select one hydroxyl or other for the acylation in a manner keyed to their amino acid specificity. Use of 2' or 3' deoxy tRNA analogues, deoxyadenosine at the 3' end of tRNA, showed that there is about an equal distribution of 2' Vs 3' specificity although chemically there is 2:1 bias in favour of the 3' isomer (143,144). The specificity for a given amino acid is preserved on going from prokaryotes to higher

eukaryotes. The source of specificity has been shown to reside in synthetase except in case of tryptophan where specificity appears to reside in the tRNA (145). The specificity between 2' and 3' is believed to be randomly chosen and then became fixed at some point in evolution (46)

1.8.5. Mechanism of the reaction

According to equations 1 and 2 enzyme bound aminoacyl-adenylates are obligatory intermediates in the synthesis of aminoacyl-tRNA. The original work and some recent studies have clearly established the role of aminoacyl-adenylates as obligatory intermediates in many systems (146,147). Reaction 1 requires Mg^{2+} which can be replaced to some extent by Mn^{2+} but not by polyamines (148,149). Reaction 2 does not require divalent cation in the presence of sufficient monovalent cations or polyamines which stabilizes the structure of tRNA (148). The order of addition of reactants has been studied for a number of synthetases. There do not seem to be a unified order of substrate binding and product release among various synthetases studied. In some cases addition is random and in others ATP or amino acid adds first (46).

The stereochemistry of transfer of AMP from ATP to amino acid is known for methionyl-tRNA synthetase and tyrosine-tRNA synthetase of E. coli. In both the cases there is a net inversion of configuration at α-phosphorous of ATP, and there is direct in line displacement (150). There is also preliminary evidence for covalent bond formation between tryptophan and bovine tryptophan-tRNA synthetase subsequent to aminoacyl-adenylate formation and before transfer to tRNA. The bond is thought to be a mixed anhydride between carboxyl group on the enzyme and the carboxyl group of amino acid (151). Though the equations (1) and (2) are true under in vitro

conditions it is likely that the unacylated tRNA and aminoacyl-tRNA are present as a complex with the synthetases in the cell. At least in prokaryotes where approximately equivalent micromolar quantities of each tRNA and synthetases are found, the association constants ensure that there is essentially no free tRNA in the bacterial cell (152). The situation is less clear in eukaryotes.

1.8.6. Inhibition of aminoacylation

As described earlier, cell growth is often regulated via qualitative and/or quantitative modification of protein biosynthesis. In addition to the control mechanisms that operate at the ribosomal level, protein synthesis is also regulated at the very first step i.e. the formation of aminoacyl-tRNA. This is brought about either by affecting one of the three substrates namely tRNA, amino acid or ATP or by altering the enzyme levels. Whelly et al (153) reported that in the absence of estradiol the translation of mRNA was inhibited in chick oviduct. They have isolated a heat labile protein of MW 43-65 kd which inhibits aminoacylation of tRNAs by causing reversible alterations to tRNAs. Shapiro et al (154) found that addition of supernatant factors from the uteri of estradiol treated guinea pigs resulted in a 50% stimulation of protein synthesis of uterine ribosomes. There are very few inhibitors known for aminoacylation as opposed to the large number of inhibitors known for ribosome mediated translation (26,155). Most of the aminoacylation inhibitors described belong to the category of substrate analogues in addition to a few antibiotics (155,156). The following table gives an account of the various inhibitors that act at aminoacylation (155).

Table 1. Inhibitors of protein synthesis acting on steps taking place prior to translation

Inhibitors of aminoacyl-tRNA formation		
Inhibitors of amino acid activation[a]	Inhibitors of amino acid transfer to RNA[a]	Inhibitors which are transferred to tRNA leading to synthesis of abnormal proteins[a]
7-Azatryptophan	Aminoalkyl-adenylates	Ethionine
Tryptazan	Borrelidin[b]	Norleucine
6-Fluorotryptophan	Furanomycin[b]	Alloisoleucine
5-Fluorotryptophan	Minosine[b]	Azetidine-carboxylic acid
Norvaline	4-Oxalysine	Canavanine
α-Amino-β-clorobutyrate	2,6-Diamino-4-hexynoic acid	N-Ethylglycine
α-Aminobutyrate	Trans-4-dehydrolysine	O-Methyl threonine
Selenomethionine		2-Fluoro-L-histidine[c]
Ethionine		
Norleucine		
Methyl-ester of serine		
Ethyl-ester of serine		
Aminoalkyl-adenylates		
Tiramine		
L-Tyrosinol		
L-Tyrosine amide		
L-Tyrosine methyl ester		

[a]Amino acid analogs specifically replace or compete with their corresponding amino acids.

[b]Borrelidin, furanomycin, and minosine specifically inhibit threonyl-, isoleucyl-, and phenylalanyl-tRNA synthesis respectively.

[c]Klein et al. (1977). Other data are taken from Vázquez (1974; review) and references therein.

Table 1 (continued)

Inhibitors of f-Met-tRNA$_f$ formation		
Inhibitors of N^{10}-formyl-H_4 folate synthesis	Inhibitors depleting the pool of N^{10}-formyl-H_4 folate	Analogs of N^{10}-formyl-H_4 folate
Aminopterin	Hydroxylamine	Pyrimidine analogs
Amethopterin (synonym methotrexate)		Pteridine analogs: tetrahydropteroate
Pteroylaspartic acid		N^5-formyl-H_4 folate
Trimethoprim		N^5-methyl-H_4 folate
6-Chloro-8-aza-9-cyclopentylpurine		tetrahydrohomofolate
		tetrahydrohomopteroate

Other examples:

1. Toyomycin, Sangivamycin - inhit ATP function

   Ref: Mori, M. and Altwerger, L: J. Biol. Chem. **243**, 306 (1968)

2. N-Methyl isatin B-thiosemicarbazone-Cu^{+2} complex-inhibit certain aminoacyl-tRNA synthetases

   Ref: Rhade et al: Biochim. Biophys. Acta. **477**, 102 (1977)

3. Amphetamines - inhibit pyrophosphate exchange

   Ref: Nawak, T.S. and Munro, H.M.: Biochem. Biophys. Res. Commun. **77**, 1280 (1977).

The above tables clearly indicates that most of the inhibitors known for the aminoacylation steps are synthetic analogues of either one of the two substrates, namely the amino acids or the ATP. As is clear, there are very few naturally occurring inhibitors for this step. The few known naturally occurring antibiotic inhibitors are listed above. A few inhibitors of this step, which are proteins in nature (as described earlier) are known.

Scope of the present investigation

It is clear from the foregoing that tRNAs are involved in a wide diversity of cellular functions. This reflects the efficient use by nature of a molecule that arose early in evolution. The key role that tRNA plays in differentiation, development, cancer and many other cellular processes points to the versatility of the molecule.

Biochemical taxonomy has become an important tool in evolution studies. As mentioned earlier tRNA molecules make a very interesting subject for the study of biological evolution (96). Among the different types of nucleic aicds, considerable information is available on 5S RNA which has been sequenced from a large number of species (157). This work has helped in clarifying and confirming certain crucial aspects in the existing phylogenetic tree. A lot of information on the primary structure of tRNA is available from prokaryotes, eukaryotes and organelles (45). A systematic analysis of the available data has shown that tRNAs ae much more conserved during evolution. However, in the literature there is only limited information available in the area of invertebrates and the present thesis represents an initial attempt in this direction.

Three different invertebrates representing three different phyla on the evolutionary scale were selected for the present study. They are:

(i) Earthworm (Megascolex sp) - Annelida

(ii) Cockroach (Periplaneta americana) - Arthropoda

(iii) Unio (Lamellidens marginalis) - Mollusca

In all the studies rat liver was used as a standard for comparison. The original plan of work includes:

1. Isolation, purification and quantitation of tRNAs from the above mentioned invertebrates.

2. Isolation and partial purification of total aminoacyl-tRNA synthetases from these animals.

3. Studies on the qualitative and quantitative variations in tRNAs from these animals. This includes:

   (i) Variations in total amino acid acceptor capacity of tRNAs, under homologous and heterologous conditions.

   (ii) The qualitative and quantitative changes in the amino acid acceptor capacities of individual aminoacyl-tRNAs in the three animals under homologous and heterologous conditions.

   (iii) Fractionation of a few aminoacyl-tRNAs to study the evolutionary variations in isoacceptor patterns of these tRNAs.

But the proceedings of the work took an unexpected and interesting turn with the identification of an aminoacylation inhibitor(s) in earthworm and cockroach. The work has been carried out further in this direction and characterization of this naturally occurring aminoacylation inhibitor was attempted. The thesis represents two broad areas of work:

1. Studies on the qualitative and quantitative variation of tRNAs in earthworm, cockroach and unio.

2. Discovery of aminoacylation inhibition in earthworm and cockroach and partial characterization of the inhibitor(s).

CHAPTER - II

MATERIALS AND METHODS

2.1. Animals

2.1.1. Earthworm (Megascolex sp)

The earthworms used in the present study were obtained from a local supplier as and when needed and were processed immediately. Care was taken to see that the worms are always collected from the same location, to minimize environmental variation. Worms ranging in size from 6-10 inches were pooled and used in the different experiments.

2.1.2. Cockroach (Periplaneta americana)

Cockroaches were also supplied by a local supplier. No distinction of sex was made. Adult cockroaches were used in all experiments. Nymphs were excluded. The whole animal after removing the wings were used in different experiments.

2.1.3. Unio (Lamellidens marginalis)

These animals were also obtained from a local supplier as and when needed. They were either used immediately or kept in fresh water for 2-3 days before use. In all experiments the whole animal was used as such after removing the shell.

2.1.4. Albino rats (Rattus norvegicus albinus)

30 week-old female albino rats of Wistar strain were used in different experiments. They were obtained from the rat colony maintained in the department. Ther rats were fed with commercial rat feed obtained locally from Pragati Suppliers, Hyderabad. Tap water was supplied ad libitum. The rats were killed and the liver was removed immediately and used in different experiments.

2.2. Chemicals

DEAE-cellulose (capacity : 0.9 meq/gm), Sephadex G-100 (particle size 40-120 μ), Dowex-50 (H^+ form), *E. coli* W tRNA, β-mercapto-ethanol, ATP, CTP, calf thymus DNA, yeast total RNA, bovine serum albumin, blue dextran, DTT, PPO, POPOP and ribonuclease (type III A from bovine pancreas) were purchased from Sigma Chemical Co., St. Louis, MO, USA. ^{14}C-chlorella protein hydrolysate (42 mCi/m atom C) and ^{14}C amino acids of high specific activity were purchased from Bhabha Atomic Research Centre, Bombay, India. Whatman 1 filter paper sheets were obtained from Whatman, England. Filter paper discs of 2.5 cm diameter were cut out from Whatman 1 filter paper sheets.

All other reagents used were of analytical grade. Double distilled water was used in all the experiments.

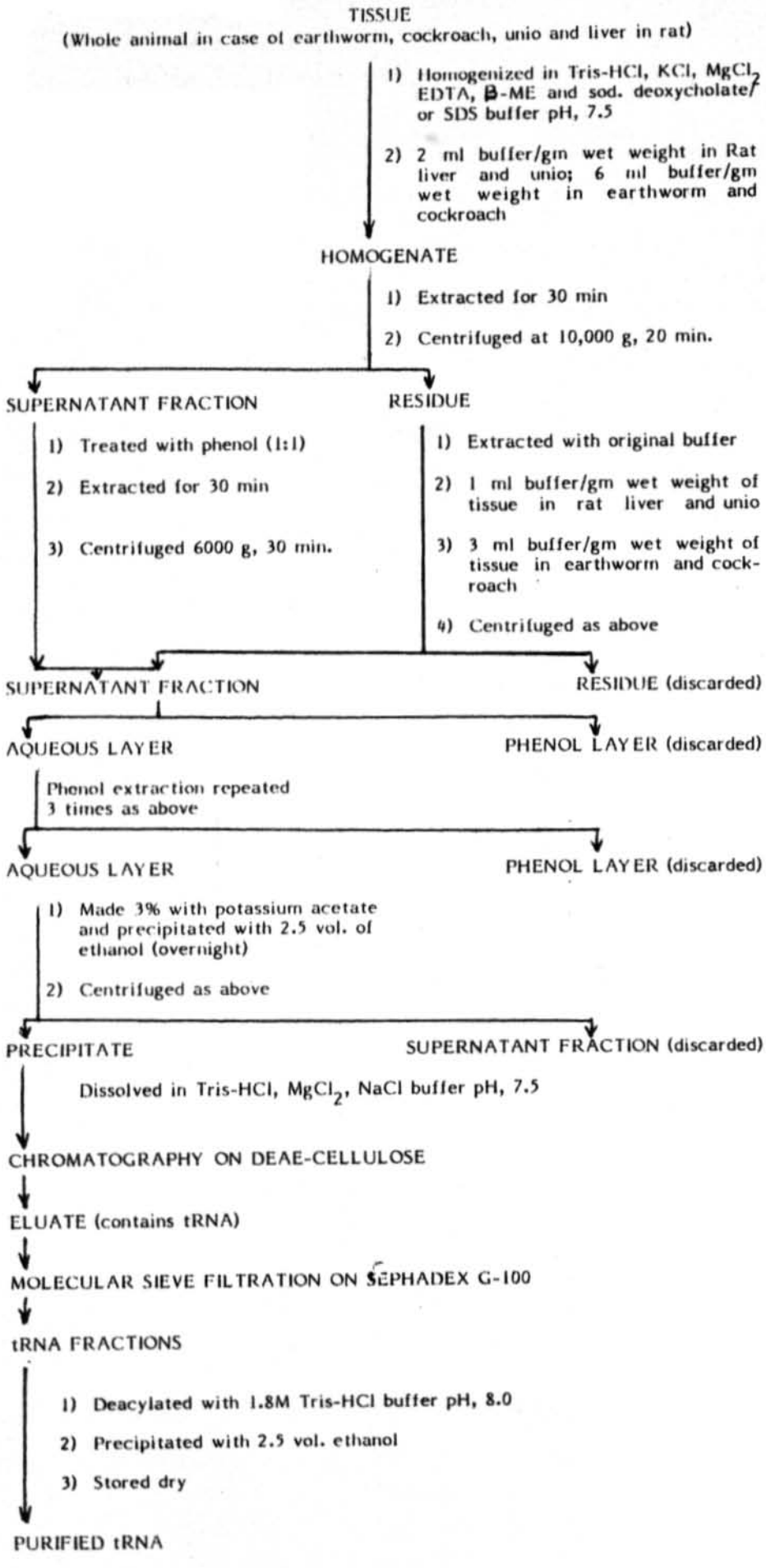
2.3. Methods

2.3.1. Extraction of transfer RNA

Transfer RNA was extracted from the different animals according to the method of Dure (159) with modifications suggested by Manjula et al (160). All the steps were carried out at 0-4°C unless otherwise specified. The tissue was minced and suspended in buffer (A) containing 0.01M Tris-HCl, pH, 7.5, 0.01M KCl, 0.01M $MgCl_2$, 0.01M EDTA and 1% sodium deoxycholate (or 0.5% sodium dodecyl sulfate in the case of earthworm and cockroach) and 0.001M β-mercaptoethanol. 2 ml of buffer per gram wet weight of tissue were used in the case of rat liver and unio, whereas 6 ml buffer were used in the case of earthworm and cockroach. The suspension was homogenized in Waring blender for 5 min. at low speed. The homogenate was stirred well on a magnetic stirrer for 20 min. to extract all the RNA and centrifuged

CHART - 1

PREPARATION OF TOTAL TRANSFER RIBONUCLEIC ACIDS



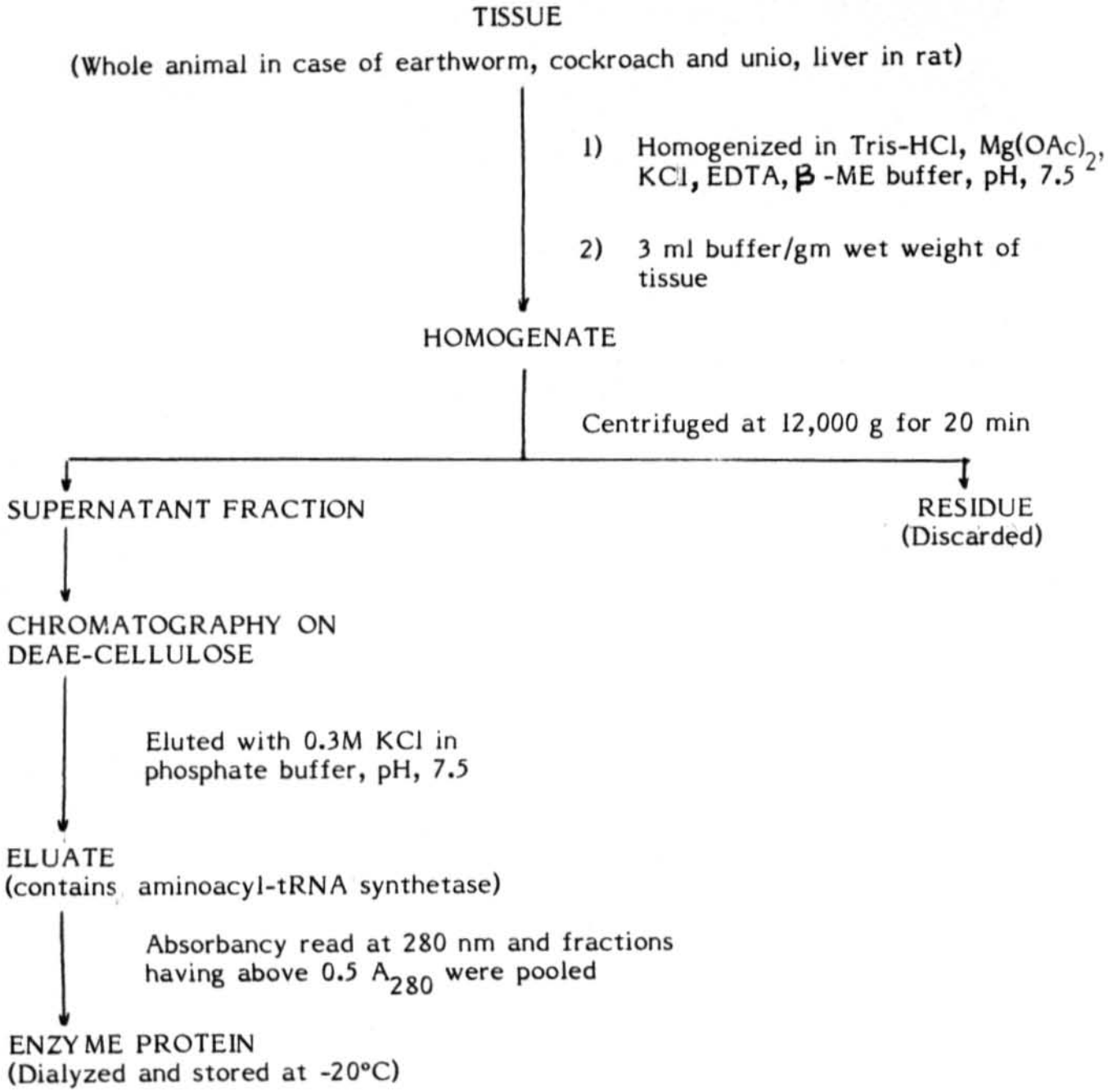
at 10,000 g for 20 min in a Mistrel 6L or Remi C24 model centrifuge. The pellet was re-extracted with half the volume of buffer A originally used and centrifuged as above. The combined supernatant was extracted with an equal volume of prechilled, buffer-saturated phenol for 30 min and centrifuged at 6000 g for 30 min. The aqueous layer was collected and subjected to two more phenol extractions. The combined phenol layer was also re-extracted with buffer A. The final deproteinized, pooled aqueous layer was made 3% with acidic potassium acetate and precipitated overnight with 2.5 volumes of ice cold absolute ethanol. The precipitate was dissolved in a minimum volume of 0.01M Tris-HCl buffer, pH, 7.5 containing 0.01M $MgCl_2$ and 0.25M NaCl (Buffer B). The solution was applied to a DEAE-cellulose column previously equilibrated with buffer B (at room temperature). The column was washed with the same buffer till the eluate was free of absorbancy at 260 nm. The tRNA was eluted out from the column using 1M NaCl in the same buffer and the fractions having A_{260} above 0.2 were pooled. The pooled tRNA was applied onto a Sephadex G-100 column previously equilibrated with the buffer containing 0.01M Tris-HCl, pH, 7.5, 0.01M $MgCl_2$ and 0.4M NaCl, and eluted with the same buffer. For quantitative estimations, the fractions containing tRNA were pooled, the volume noted and the A_{260} was determined. The amount of tRNA present was estimated on the basis of optical density. When expressed on a weight basis, 20 A_{260} units were taken as equivalent to one mg of tRNA. In large scale preparations the purified tRNA after gel-filtration was precipitated with 2.5 volumes of ice-cold absolute ethanol, washed with 75% and 80% ethanol, dried and stored at -20°C. This procedure is shown in Chart 1.

2.3.2. Deacylation of tRNA

Deacylation was carried out according to the method of Dure (159).

CHART - 2

PREPARATION OF AMINOACYL-tRNA SYNTHETASE



The tRNA precipitate was dissolved in 1.8M Tris-HCl buffer, pH, 8.0 and incubated at 37°C for 1 hr. At the end of the incubation period the deacylated tRNA was recovered by precipitation with 2.5 volumes of ethanol. The amino acids were removed by washing the precipitate twice with 75% ethanol. The precipitate was dried and stored at -20°C.

2.3.3. Preparation of aminoacyl-tRNA synthetase

Total aminoacyl-tRNA synthetases were prepared from whole animal in the case of invertebrates and from liver in the case of the rat. It was prepared according to the method of Yang and Novelli (161) with minor modifications. All operations were carried out at 0-4°C.

The tissue was homogenized in a buffer containing 0.01M Tris-HCl pH, 7.5, 0.005M magnesium acetate, 0.01M KCl, 0.005M EDTA and 0.01M β-mercaptoethanol. Three volumes of buffer were used per gram wet weight of tissue. The homogenate was centrifuged at 14,000 g for 20 min. in a Mistrel 6L centrifuge. The supernatant obtained was freed of ribosomes by further centrifugation at 150,000 g for 3 hrs. in MSE-75 ultracentrifuge. The endogenous tRNA and amino acids present in the 150,000 g supernatant were removed by chromatography on DEAE-cellulose, which was earlier equilibrated with 0.01M phosphate buffer, pH 7.5, containing 0.001M $MgCl_2$, 0.002M DTT and 0.005M KCl. The column was washed with the same buffer and the aminoacyl-tRNA synthetases were eluted with the same buffer containing 0.3M KCl. The absorbancy of the fractions at 280 nm was measured and fractions having absorbancy above 0.5 at this wavelength were pooled and used as the enzyme source. The peak region was pooled and used, instead of peak fraction to avoid loss of any particular synthetase. The enzyme was dialyzed against 0.05M Tris-HCl pH, 7.5 containing 0.005M EDTA and 0.002M DTT.

CHART - 3

PREPARATION OF AMINOACYLATION INHIBITOR (FRACTION 'C')

M KCl ELUATE OBTAINED FROM DEAE-CELLULOSE CHROMATOGRAPHY
(for earthworm/cockroach - Chart 2)

↓ Dialysed extensively against double distilled water for 20 hrs with 3 changes, water: sample = 50:1

DIALYSED PROTEIN FRACTION
(Fraction A)

↓ 1) Denatured by heating at 90-95°C for 15 min in boiling water bath

2) Centrifuged at 10,000 g for 10 min to remove denatured proteins

SUPERNATANT FRACTION
(Fraction B)

↓ Dialysed against small volume of double distilled water for 20 hrs water: sample = 6:1

RETENTATE
(Inside bag)

DIALYSATE
(Outer water)

↓ Concentrated to original sample (Fraction B) volume

INHIBITOR FRACTION
(Fraction C)

0.5 ml aliquots were stored frozen at -20°C until use. The enzyme protein was estimated by the method of Lowry et al (162). The procedure for the preparation of aminoacyl-tRNA synthetase is shown in Chart 2.

2.3.4. Preparation of aminoacylation inhibitor

The 0.3M KCl eluate obtained from DEAE-cellulose chromatography by the above mentioned procedure (2.3.3) was used as the source, for the isolation of aminoacylation inhibitor in the case of earthworm and cockroach. This protein fraction was dialysed extensively for 20 hrs. against double distilled water with three changes, using 50 ml of water for every 1 ml of the protein fraction. The dialyzed protein fraction (Fraction A) was heated at 90-95°C for 15 min. in a boiling water bath. The denatured proteins were removed by centrifugation at 10,000 g for 10 min. The supernatant fraction (Fraction B) was dialysed against a small volume of double ditilled water for 20 hrs, without change. (water: sample = 6:1). The dialysate (outer water) was concentrated to a volume equal to the sample (Fraction B) volume and used as the inhibitor fraction (Fraction C) in all experiments. The procedure for the preparation of aminoacylation inhibitor is shown in Chart 3.

2.4. Assays

2.4.1. Amino acid acceptor assay of tRNA

Aminoacylation of tRNA was measured by incorporation of radio-active amino acids into TCA insoluble material using the 'paper disc assay' of Mans and Novelli (163). The reaction mixture contained the following components in a total volume of 0.3 ml.

Tris-HCl, pH, 7.5	100 mM
$MgCl_2$	10 mM

KCl	-	10 mM
NH_4Cl	-	10 mM
β -mercaptoethanol	-	4 mM
ATP pH, 7.0	-	2 mM
CTP pH, 7.0	-	1 mM
^{14}C-algal protein hydrolysate or ^{14}C-amino acids	-	0.2 uCi
Transfer RNA	-	2.0 A_{260}
Enzyme protein	-	0.3 mg

E. coli tRNA was used as a standard in all experiments. A blank was set up for each experiment. The blank consisted of the same components of the reaction mixture except the tRNA. Assays were done in duplicates and the average value was taken.

The reaction mixture was incubated at 37°C for varying time periods. At the end of the incubation period aliquots were taken and applied to Whatman 1 filter paper discs. The reaction was stopped by putting the discs in icecold 10% TCA. The discs were washed once in 10% TCA and twice in 5% TCA for 10 min. each. Finally they were washed in ethanol and dried under an infrared lamp. Radioactivity retained on the filter paper discs was measured in Beckman LS-1800 liquid scintillation spectrometer (Channel 2) in 5 ml of toluene based scintillator containing 0.4% PPO and 0.01% POPOP. Average counting efficiency of 57% for ^{14}C was obtained under these conditions. (Channel 2. limits 400-670).

2.4.2. Assay for the detection of aminoacylation inhibitor (mixed enzyme assay)

The presence of the aminoacylation inhibitor in the different samples

were checked using a 'mixed enzyme assay'. The assay was basically similar to the amino acid acceptor assay for tRNA (2.4.1). Here also the total volume of the reaction mixture was 0.3 ml and the reaction mixture contained the same concentrations of the above mentioned components, and in addition it contained either 0.3 A_{280} units of the earthworm /cockroach enzyme protein (fraction A) or 0.025 A_{260} units of fraction C. The other particulars of the assay were same as in 2.4.1.

2.5. Column chromatography

2.5.1. DEAE-cellulose chromatography

DEAE-cellulose was soaked and washed as recommended by the manufacturers. Before packing the column the ion-exchanger was washed with 1M NaCl to remove any high salt elutable material in the resin followed by repeated washes with distilled water. The washed resin was packed into a column under gravity and equilibrated with 10 bed volumes of the equilibration buffer. In the case of nucleic acid chromatography, for every 50 A_{260} units of the RNA, 1 ml bed volume of the ion-exchanger was used and when proteins were chromatographed, for 10 mg of protein, 1 ml bed volume of the resin was used. The column was packed at room temperature and other operations were continued at room temperature for tRNA purification, whereas in the case of enzyme purification, the column was operated in cold room at 4°C. The nucleic acids were detected by monitoring the A_{260} absorption of the fraction and the protein peaks were located by A_{280} absorption in a Gilford Spectrophotometer.

2.5.2. Dowex-50 (H^+) Chromatography

Dowex-50 (H^+) was soaked in distilled water and the resin was washed successively with 0.5N NaOH, distilled water, 0.5N HCl and finally with

distilled water. The washed ion-exhcnager was packed into a column under gravity and washed thoroughly with distilled water till the effluent was free of any acid and showed a neutral pH. The aminoacylation inhibitor fraction (0.7 ml, fraction C adjusted to pH 2) was loaded on the column. The column was washed with 4 bed volumes of water and eluted with 4 bed volumes of 1N NH_4OH. The effluent and eluate were evaporated to dryness, on a water bath, reextracted with distilled water and evaporated again. This extraction and evaporation was repeated till the extract showed a neutral pH. Then it was assayed for inhibitor activity as described in 2.4.2.

2.5.3. Molecular sieve filtration on Sephadex G-100

The Sephadex G-100 was packed into a 46 ml column taking necessary precautions recommended by the manufacturer and equilibrated with 10 bed volumes of 0.01M Tris-HCl, pH, 7.5 containing 0.01M $MgCl_2$ and 0.4M NaCl. The RNA sample (2 ml: 5 A_{260}/ml) was loaded carefully on top of the gel and eluted with the equilibration buffer at a flow rate of 20 ml/hr. Fractions (1.5 ml) were collected using an LKB Redirac automatic fraction collector. Packing of column and other operations were done at room temperature. The nucleic acids were detected by monitoring the fractions for A_{260} absorption. The column was standardized using a mixture of blue dextran, E. coli tRNA and ATP.

2.6. Estimations

2.6.1. Estimation of protein

Protein was estimated according to the method of Lowry et al (160). Crystalline bovine serum albumin was used to obtain the standard graph.

2.6.2. Estimation of DNA and RNA

DNA was estimated by the diphenylamine method and RNA by orcinol reagent as described by Schneider et al (164). Calf thymus DNA and yeast total RNA were used to obtain the respective standard graphs.

2.7. Other methods

2.7.1. Ashing

1 ml of the dialyzed earthworm/cockroach enzyme preparation (Chart 2) was taken in a silica crucible, evaporated to dryness and was heated above 500°C for 1 hour on an electric bunsen or for 2 hrs in a muffle furnace till the contents became white. The ash was extracted with 1 ml of 1N HCl and it was evaporated to dryness. The extraction and evaporation were repeated 4 times to make sure that all the insoluble oxides were converted into the soluble chlorides. The residue was finally taken in 1 ml of 0.01M Tris-HCl pH, 7.5, containing 0.01M $MgCl_2$ and checked for inhibitor activity.

2.7.2. Nitric acid oxidation

1 ml of the dialyzed earthworm/cockroach enzyme preparation was taken in a silica crucible and evaporated to dryness. To the residue 2 ml of conc. HNO_3 was added and the contents were stirred and evaporated to dryness on an electric bunsen. The acid oxidation was repeated 4 times. The residue so obtained was converted into the chlorides as described under ashing (2.7.1) and checked for inhibitor activity.

CHAPTER - III

STUDIES ON THE QUALITATIVE AND QUANTITATIVE VARIATION OF tRNA IN THE THREE INVERTEBRATES [EARTHWORM (*MEGASCOLEX* SP), COCKROACH (*PERIPLANETA AMERICANA*), UNIO (*LAMELLIDENS MARGINALIS*)]

Studies on the qualitative and quantitative variation of tRNA in three invertebrates [Earthworm (Megascolex sp.), Cockroach (Periplaneta americana) and Unio (Lamellidens marginalis)]

3.1. Extraction of tRNA

The extraction method mentioned under Materials and Methods (2.3.1. and Chart 1) was highly reproducible. In the case of earthworm and cockroach the ratio of buffer to tissue was increased to 6:1, since the normal ratio of 2:1 gave a thick paste like material with very little supernatant fraction on centrifugation. Care was taken at each step of extraction to minimize the loss of tRNA. Re-extraction of the residue and the pooled phenol layer did not give any appreciable amount of tRNAs confirming that there was no loss at these steps. Recovery of tRNAs from the DEAE-cellulose columns were usually quantitative. Since loss was avoided at all stages the tRNA estimated is expected to represent the amount that is present in the tissue.

3.2. Purification of tRNA (Figures 3 and 4)

The tRNA samples obtained after DEAE-cellulose chromatography were further checked for purity by molecular sieve filtration on Sephadex G-100. The column was standardized using blue dextran (MW. 2×10^6), *E. coli* tRNA (MW. 25,000) and ATP (MW. 551). Fig 3 and Fig 4 show typical elution profiles of the tRNA samples from a Sephadex G-100 column. When unio tRNA was gel filtered on this column it gave a single A_{260} peak eluting in the *E. coli* tRNA region. However, in the case of rat liver, cockroach and earthworm tRNA samples two A_{260} peaks were observed, one in the region of blue dextran and the other in *E. coli* tRNA region. The above data clearly indicated that the unio tRNA preparation was free of any high molecular weight nucleic acids, whereas the rat liver fraction was contaminated with high molecular weight nucleic acids to the extent of 15-20% and that of earth-

S. 3
4.

10 A_{260} units (2 ml) of rat liver/unio/cockroach/earthworm tRNA obtained from DEAE-cellulose chromatography was applied onto a Sephadex G-100 column (34.5 x 1.3 cm) equilibrated with 0.4M NaCl, 0.01M $MgCl_2$ in 0.01M Tris-HCl buffer, pH, 7.5 and eluted with the same buffer. 1.5 ml fractions were collected at the rate of 20 ml per hour.

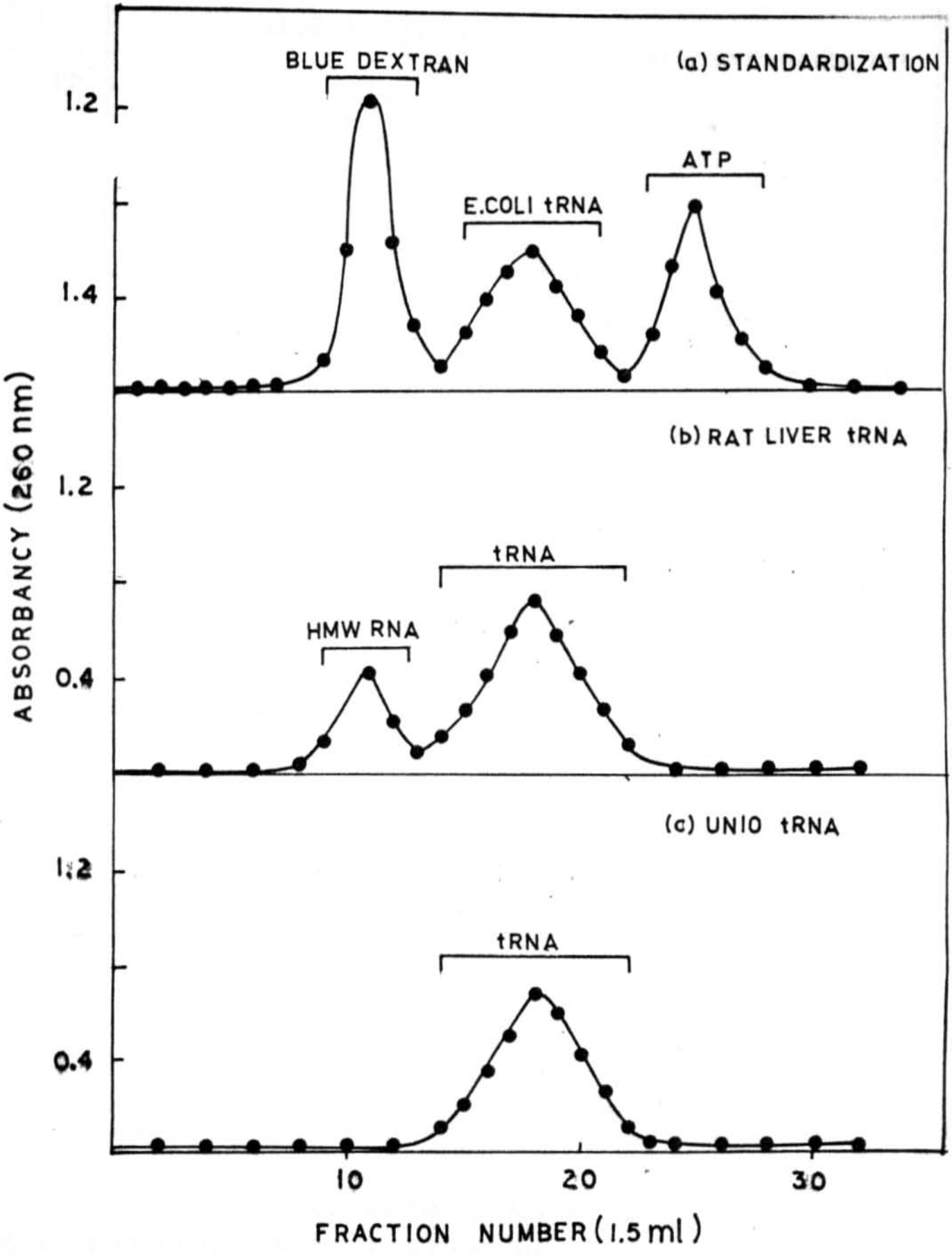


FIG. 3. GELFILTRATION OF RAT LIVER AND UNIO tRNAs ON SEPHADEX G-100.

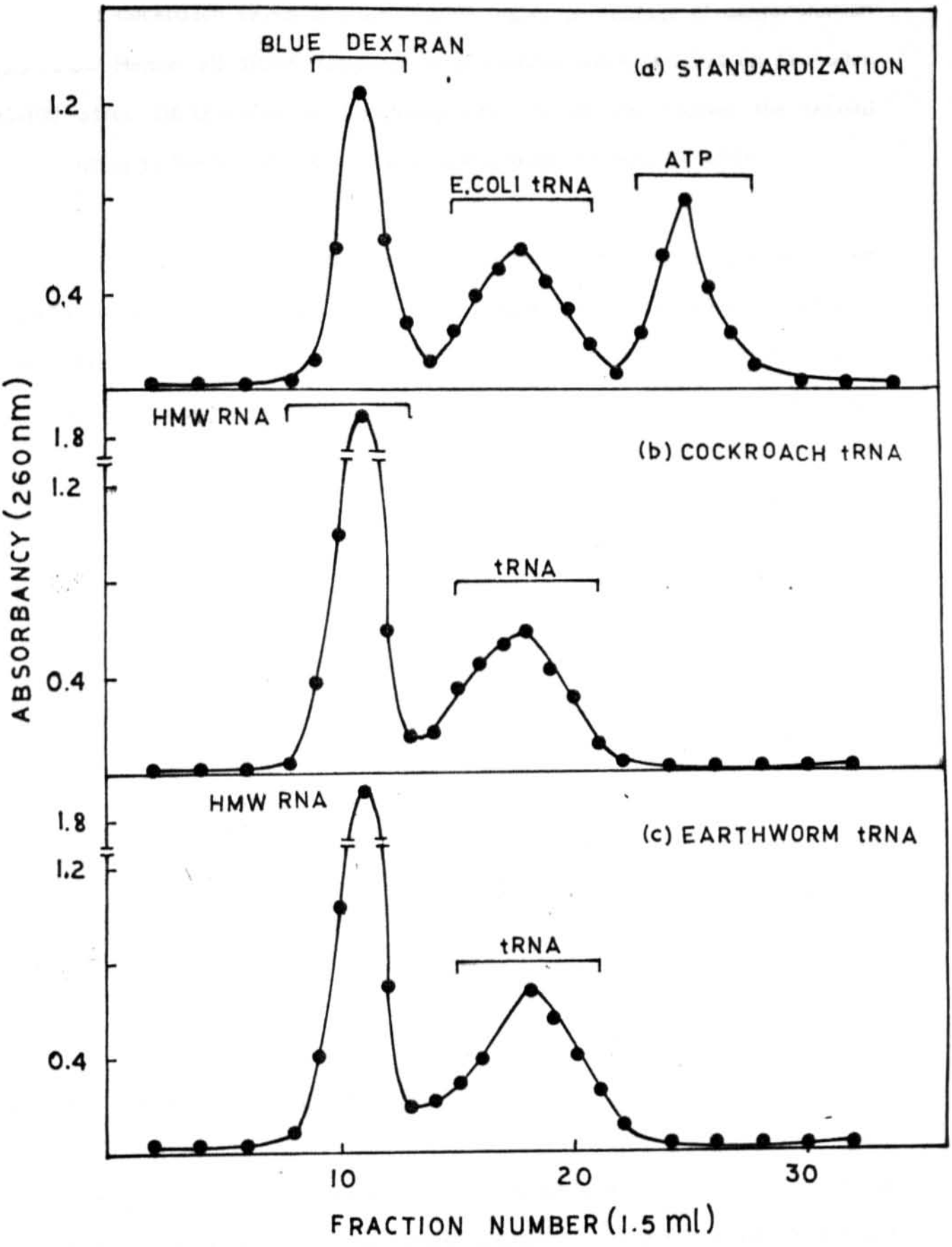


FIG. 4. GELFILTRATION OF EARTHWORM AND COCKROACH tRNAs ON SEPHADEX G-100.

worm and cockroach tRNA fractions had a higher percentage of contamination (70-75%). Hence all tRNA samples, as a routine, were purified on Sephadex G-100 after DEAE-cellulose chromatography. In all the studies the second peak eluting in the E. coli tRNA region was pooled and used as tRNA.

Eventhough the same method was used for the extraction of the tRNAs, it is not clear why the tRNA preparations from different animals have different degree of high molecular weight nucleic acid contaminations. It is possible that in the case of earthworm and cockroach. The high molecular weight nucleic acids are complexed with some other component which makes them elute at a lower salt concentration from the DEAE-cellulose chromatography or the phenol extraction step could be less efficient in denaturing the macromolecules. However, care was taken to purify the tRNAs before use.

3.3. Purity of tRNA

3.3.1. Spectral properties (Figure 5)

Figure 5 shows a typical absorption spectrum given by the purified tRNA fraction. The spectrum is that of a typical nucleic acid (159) showing the absence of any protein contamination. It has $A_{320} = 0$, $A_{280} = 1/2\ A_{260}$, $A_{220} < A_{260}$ and A_{230} in the bottom of the spectral trough.

3.3.2. DNA Contamination (Figure 6)

RNA, in general, is completely degraded to mononucleotides by alkali due to the presence of 2'-hydroxyl on the ribose (159), whereas DNA is stable to alkali. Fig 6 shows a typical elution profile of the purified tRNA and the alkali digest of the same from Sephadex G-100 column. The results indicate that the purified tRNA from rat liver, unio, cockroach and earthworm elutes

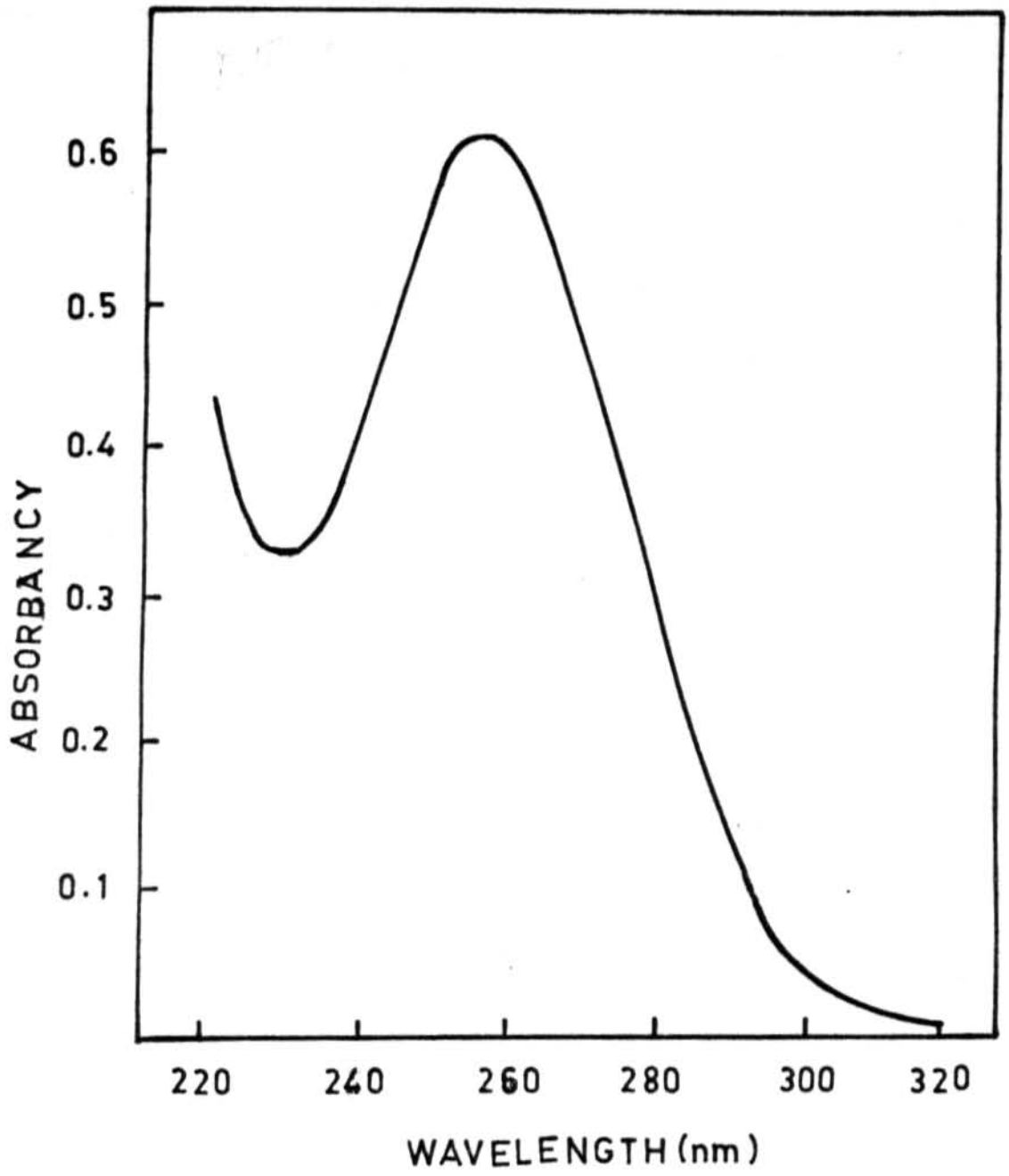


FIG. 5. A TYPICAL ABSORPTION SPECTRUM OF tRNA.

FIG. 6. Purified tRNA (4.0 A_{260} units) was hydrolysed with 0.3M NaOH at 37°C for 18 hrs. The digest was neutralized with HCl and gel filtered on Sephadex G-100 column (34.5 cm x 1.3 cm) as described under legend to Figure 4. 1.5 ml fractions were collected at the rate of 22 ml per hour. Purified tRNA was used as a standard.

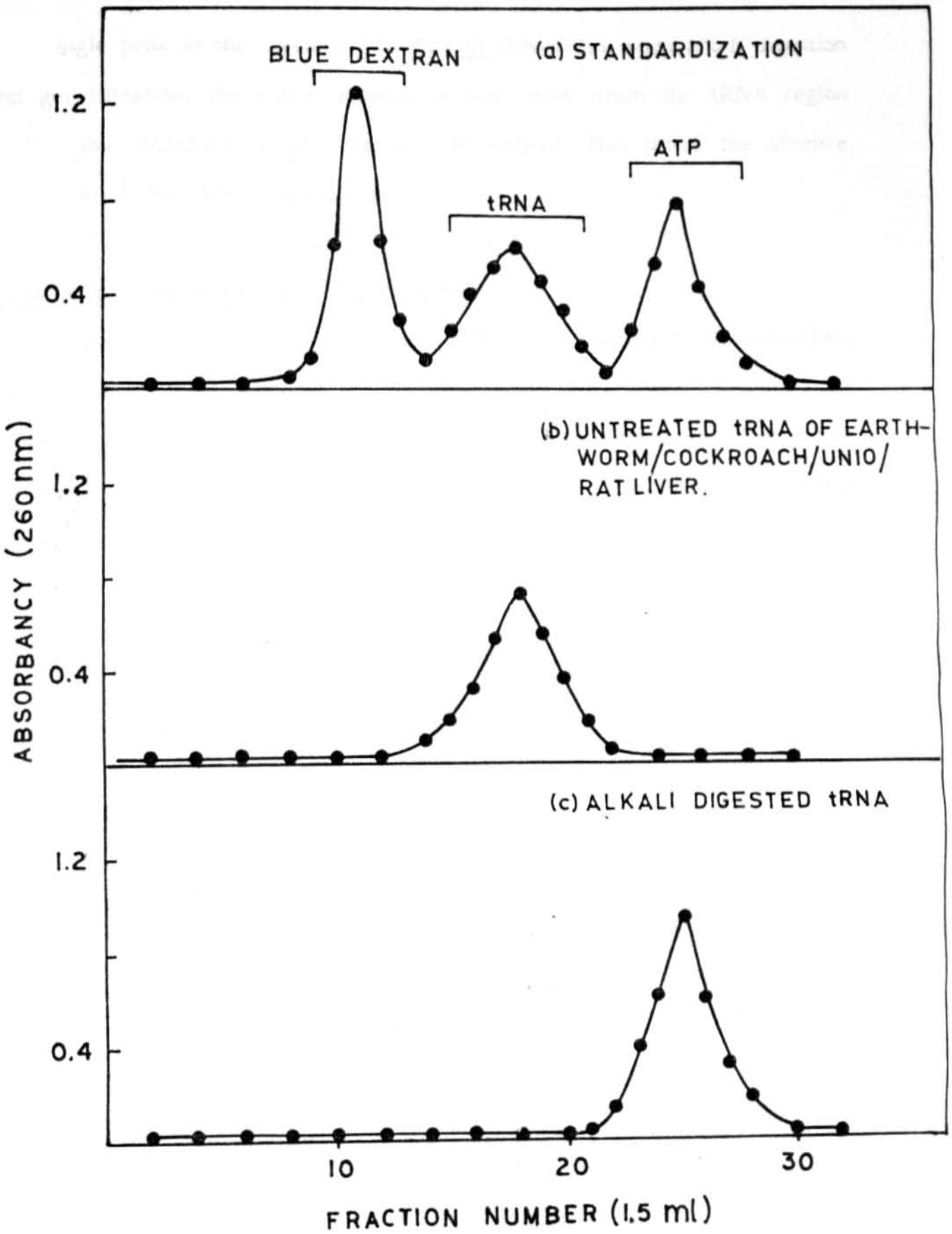


FIG.6. GELFILTRATION OF ALKALI DIGEST OF tRNA ON SEPHADEX G-100.

TABLE - 2

VARIATION OF TOTAL tRNA CONTENT IN THE DIFFERENT INVERTEBRATES AND RAT LIVER

Unit of measurement	Earthworm	Cockroach	Unio	Rat liver
ugms of tRNA/gm wet weight	53.2 ± 10.10*	58.8 ± 10.33*	95.00 ± 15.5*	469.0 ± 21.57*
ugms of tRNA/mg DNA	21.77 ± 1.24	17.15 ± 1.31	45.24 ± 2.52	87.77 ± 4.52
ugms of tRNA/ gm protein	465.0 ± 31.08	358.0 ± 46.00	730.7 ± 38.50	1934.6 ± 62.3
ugms of tRNA/ mg RNA	5.10 ± 0.88	6.26 ± 0.69	5.13 ± 0.50	22.35 ± 2.28
mg DNA/gm wet weight of tissue	2.48 ± 0.49	1.62 ± 0.38	2.10 ± 0.30	5.35 ± 0.20
mg protein/gm wet weight of tissue	101.0 ± 12.9	156.0 ± 7.02	130.0 ± 9.05	242.3 ± 8.02
mg RNA/gm wet weight of tissue	11.11 ± 1.59	9.31 ± 0.37	18.50 ± 0.15	23.78 ± 4.97

*Mean value ± Standard deviation

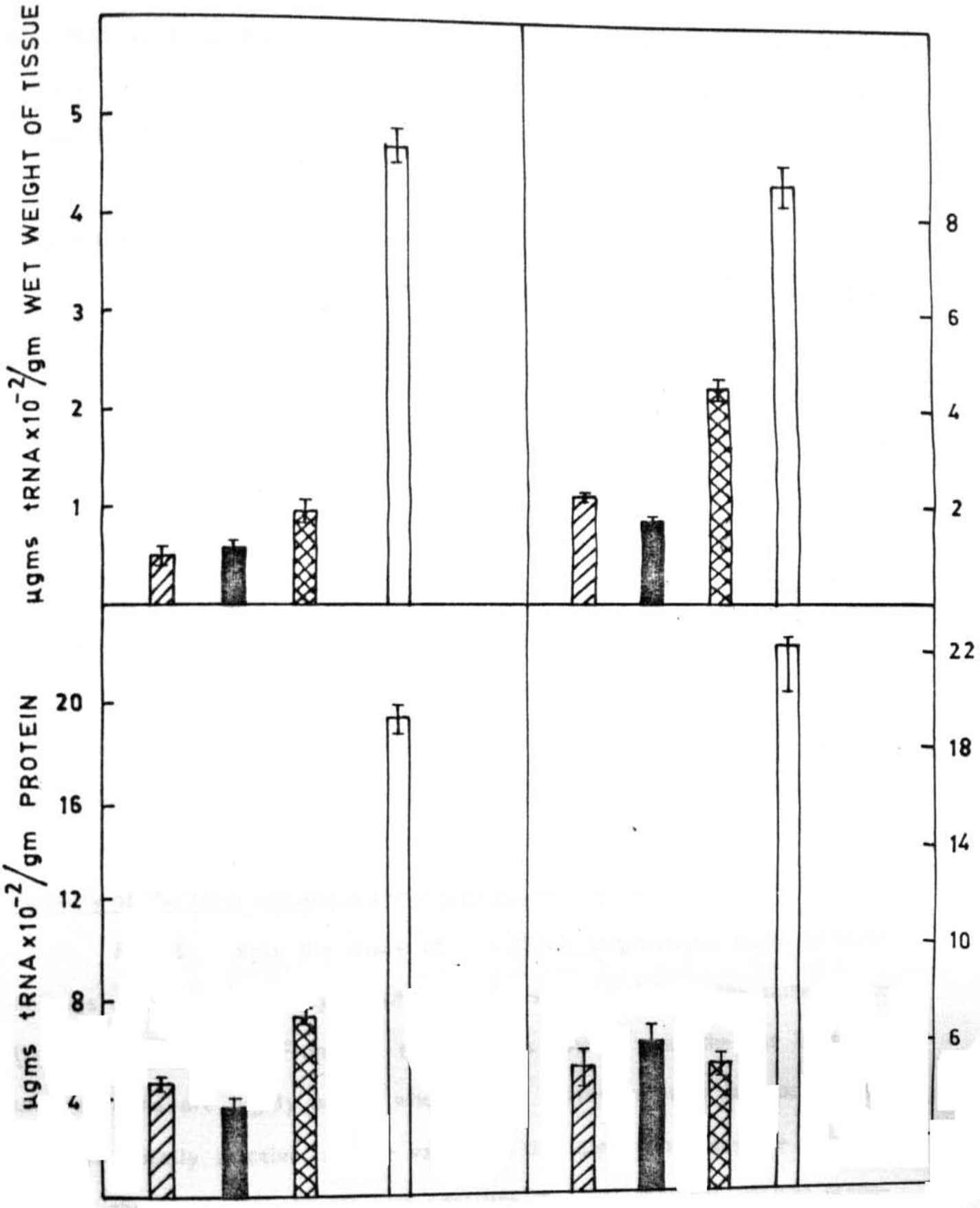


FIG.7: VARIATION OF TOTAL tRNA CONTENT OF EARTHWORM, COCKROACH, UNIO AND RATLIVER.

(160,166). It is possible that in the case of invertebrates also the rate of protein synthesis may be much slower compared to a metabolically active tissue like the rat liver.

5. Preparation of total aminoacyl-tRNA synthetase

The total aminoacyl-tRNA synthetases were prepared according to the method mentioned in materials and methods (2.5.3. and Chart 2). This method gave an enzyme preparation which had a protein concentration of 5-7 mg/ml. This was high enough to store the rat liver and unio enzyme as such without further concentration and the enzyme thus stored was found to be active for at least 15 days. Storing the enzyme in aliquots also helped in avoiding loss due to denaturation by repeated thawing and freezing. Inclusion of the DEAE-cellulose chromatography step in the preparation of the enzyme helped in purifying the enzyme from the endogenous tRNA and free amino acids. Preferential loss of certain synthetases was avoided by pooling the entire A_{280} absorbancy peak ($A_{280} > 0.5$) rather than the peak fractions.

6. Activity of the total aminoacyl-tRNA synthetases (Figure 8)

Fig. 8 depicts the assay of the tRNA preparations from different animals using the aminoacyl-tRNA synthetases prepared from the same source (Homologous system). From the results it is obvious that the rat liver and unio systems are highly active whereas the earthworm and cockroach systems are totally inactive, as the values in the later two cases are equal to that of the blank. The Figure also confirms the fact that the enzyme preparations from rat liver and unio are free of endogenous tRNA as indicated by the blank values.

These results can indicate two possibilities i.e. either the tRNA

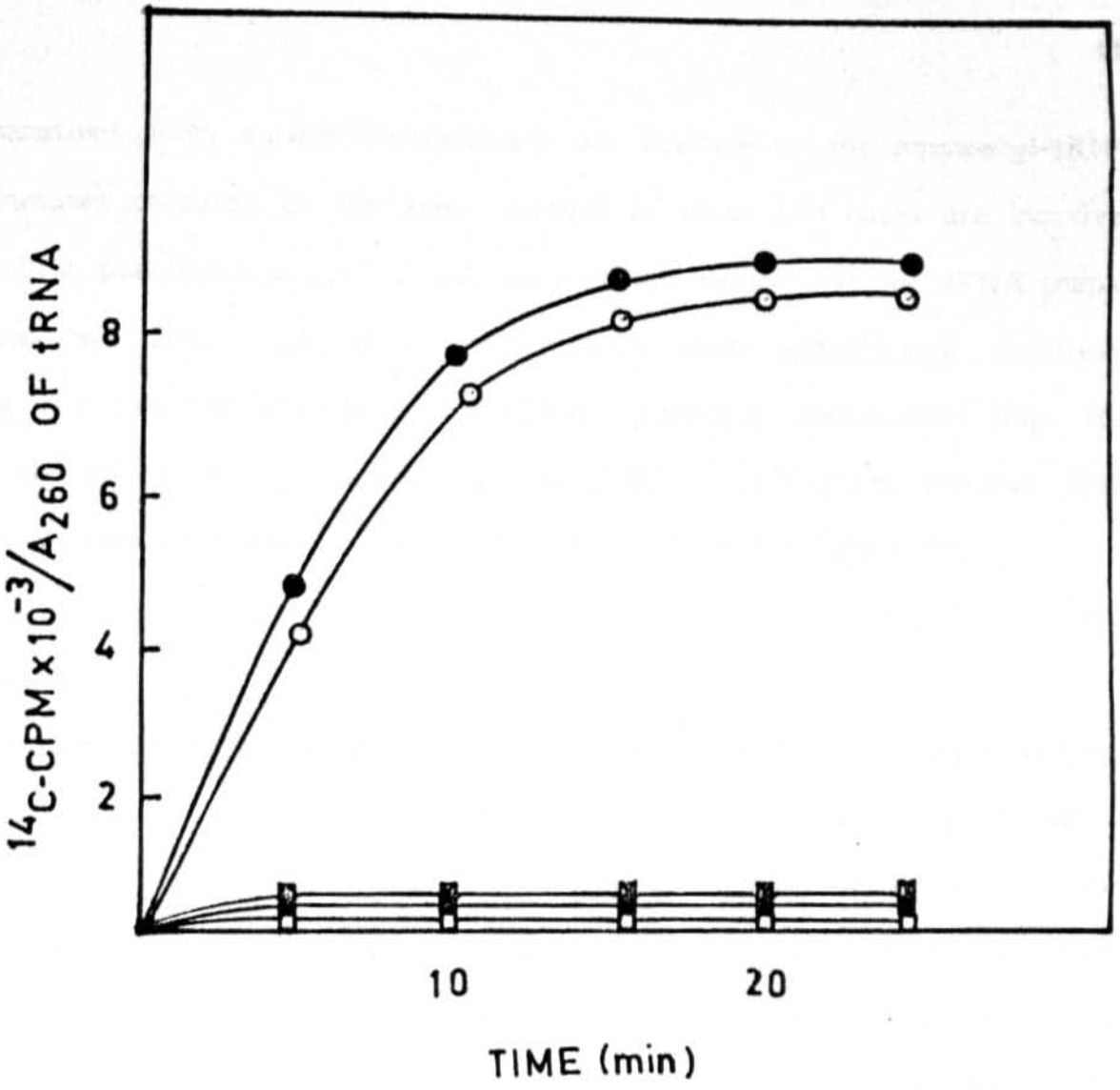


FIG.8. AMINO ACID ACCEPTOR ACTIVITIES OF THE tRNAs FROM UNIO, RATLIVER, COCKROACH AND EARTHWORM.

Legend: The aminoacylation was carried out as described under materials and methods (2.4.1). ^{14}C algal protein hydrolysate was used as the source of amino acids.

(●—●) represents the aminoacylation of unio tRNA with unio enzyme.

(○—○) represents the aminoacylation of rat liver tRNA with rat liver enzyme.

(⊠—⊠) represents the aminoacylation of earthworm tRNA with earthworm enzyme.

(■—■) represents the aminoacylation of cockroach tRNA with cockroach enzyme.

(□—□) represents blank i.e., without tRNA

preparations from earthworm/cockroach are inactive or the aminoacyl-tRNA synthetases prepared by the above method in these two cases are inactive. The first possibility was ruled out since it was shown that the tRNA preparations are active (partially) when assayed under heterologous conditions using rat liver or unio aminoacyl-tRNA synthetase preparations (Fig. 10). The second possibility that the aminoacyl-tRNA synthetases prepared from these animals were inactive was confirmed as will be discussed later.

7. Aminoacylation reaction

In the aminoacylation reaction, to get optimal acylation, it is advisable to chose the minimum amount of enzyme, and the minimum time of incubation required; otherwise nucleases which are the common contaminants of the enzyme preparation will degrade the tRNA leading to confusing results. Therefore, before assaying the different tRNA preparations, these parameters were studied and standardized. For these standardization experiments ^{14}C-algal protein hydrolysate was used as the source of total radioactive amino acids and homologous charging systems from rat liver and unio were used. The algal protein hydrolysate provided almost 16-18 amino acids which gave a fair measure of the total acceptor activity.

3.7.1. Time-course of the aminoacylation reaction (Figure 9a)

Fig. 9, right half (9a) shows the time-course of the aminoacylation reaction in unio and rat liver homologous systems. In the unio system acylation reaction keeps on increasing upto 25 min and then plateaues off and remains constant upto 60 min. This shows the absence of significant amount of nucleases in the unio enzyme preparation. A similar pattern follows in the case of rat liver system. From the above data 25 min. was chosen as the incubation period in all further work.

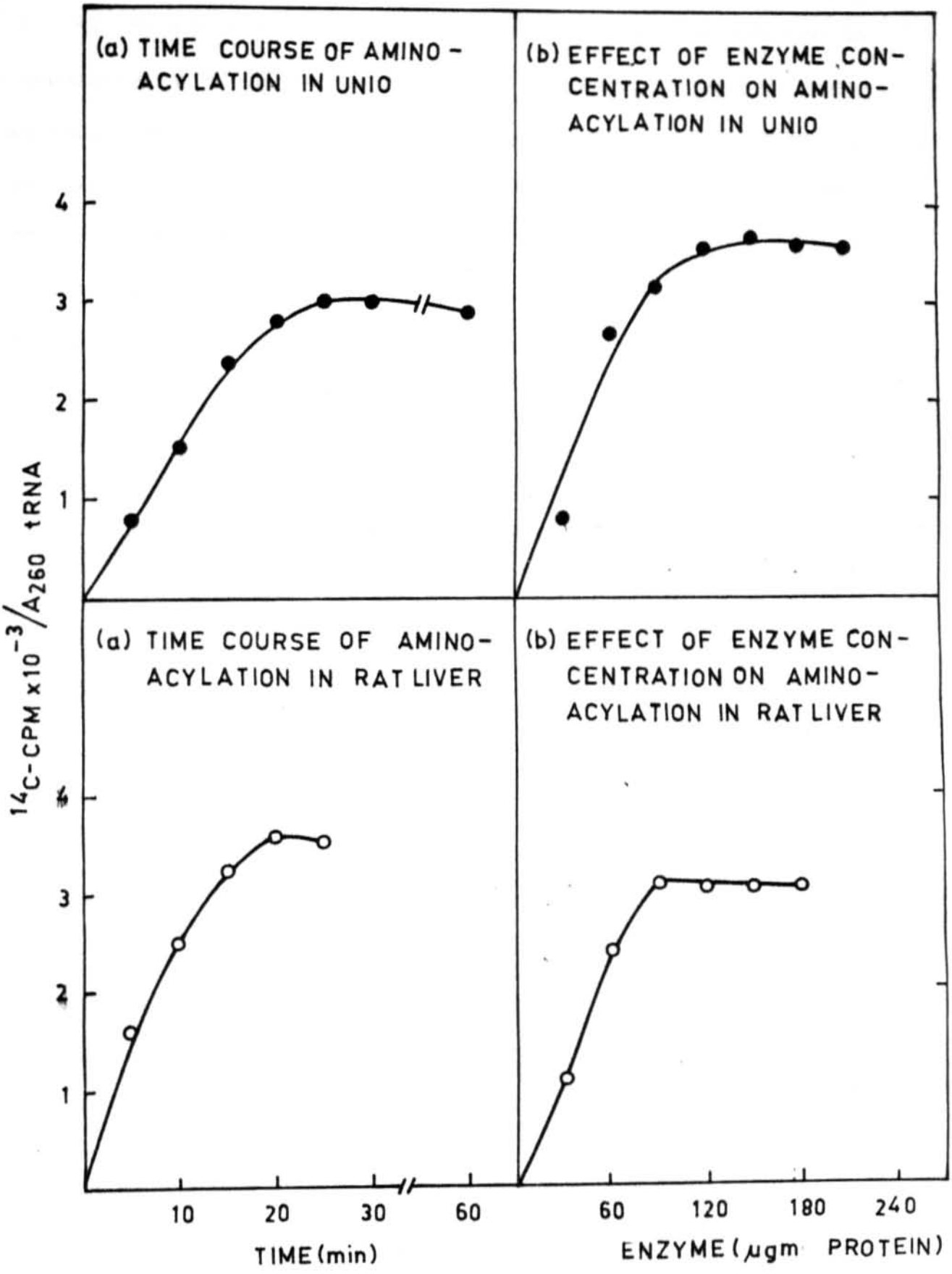


FIG. 9. STANDARDIZATION OF AMINOACYLATION REACTION IN UNIO AND RAT LIVER.

7.2. Effect of enzyme concentration (Figure 9b)

Fig. 9, left half (9b) shows the effect of enzyme concentration on the aminoacylation of unio and rat liver tRNA (homologous system). It can be seen that the acylation keeps on increasing with increasing enzyme concentration upto 90-120 ugs of enzyme protein per A_{260} of tRNA and then remains a constant. Increasing the enzyme concentration further does not increase the aminoacylation. These results indicate the absence of significant amounts of nucleases in the enzyme preparation and also indicate that 150 ugm of enzyme protein is adequate to bring about complete aminoacylation of one A_{260} unit of tRNA in 25 min.

3. Total amino acid acceptor activity of the tRNAs (Table 3, Figure 10)

Table 3 and Fig. 10 represent the total amino acid acceptor capacity of the tRNAs extracted from the four animals; rat liver, unio, cockroach and earthworm. These tRNAs were aminoacylated using the rat liver and unio aminoacyl-tRNA synthetases, since the enzyme preparations from the other two animals were inactive as indicated in Fig. 8.

From the results it is obvious that the unio and rat liver tRNAs are highly active in both homologous and heterologous systems, whereas the tRNAs from earthworm and cockraoch show a very low activity on a unit weight basis. The earthworm and cockraoch tRNA show on an average, only about 20-25% of the activity as compared to rat liver or unio tRNA under similar conditions.

It is not clear at present why the earthworm and cockroach tRNAs show a low amino acid acceptor activity. It is obviously not because they are assayed under heterologous conditions, since unio which is closely related

TABLE - 3

TOTAL AMINO ACID ACCEPTOR ACTIVITIES OF DIFFERENT tRNAs

Source of tRNA	Source of aminoacyl-tRNA synthetase	
	Rat liver	Unio
	^{14}C-cpm/mg tRNA	
Earthworm	64513 ± 5970*	85735 ± 10005*
Cockroach	71650 ± 6510	52570 ± 5225
Unio	292595 ± 33150	276000 ± 25000 (Homologous)
Rat liver	249443 ± 30000 (Homologous)	220850 ± 10000

*Mean value ± S.D.

FIG. 10. 2.0 A_{260} units of tRNAs prepared from earthworm (▨), cockroach (■), unio (▩) and rat liver (□) were amino-acylated using aminoacyl-tRNA synthetase preparations from (A) Unio and (B) Rat liver. ^{14}C algal protein hydrolysate was used as the source of total amino acids. Other comonents of the reaction mixture were the same as described under materials and methods (2.4.1). The reaction was carried out at 37°C for 25 min. 100λ aliquots were processed in duplicate and the acid precipitable counts were estimated. The average value was taken.

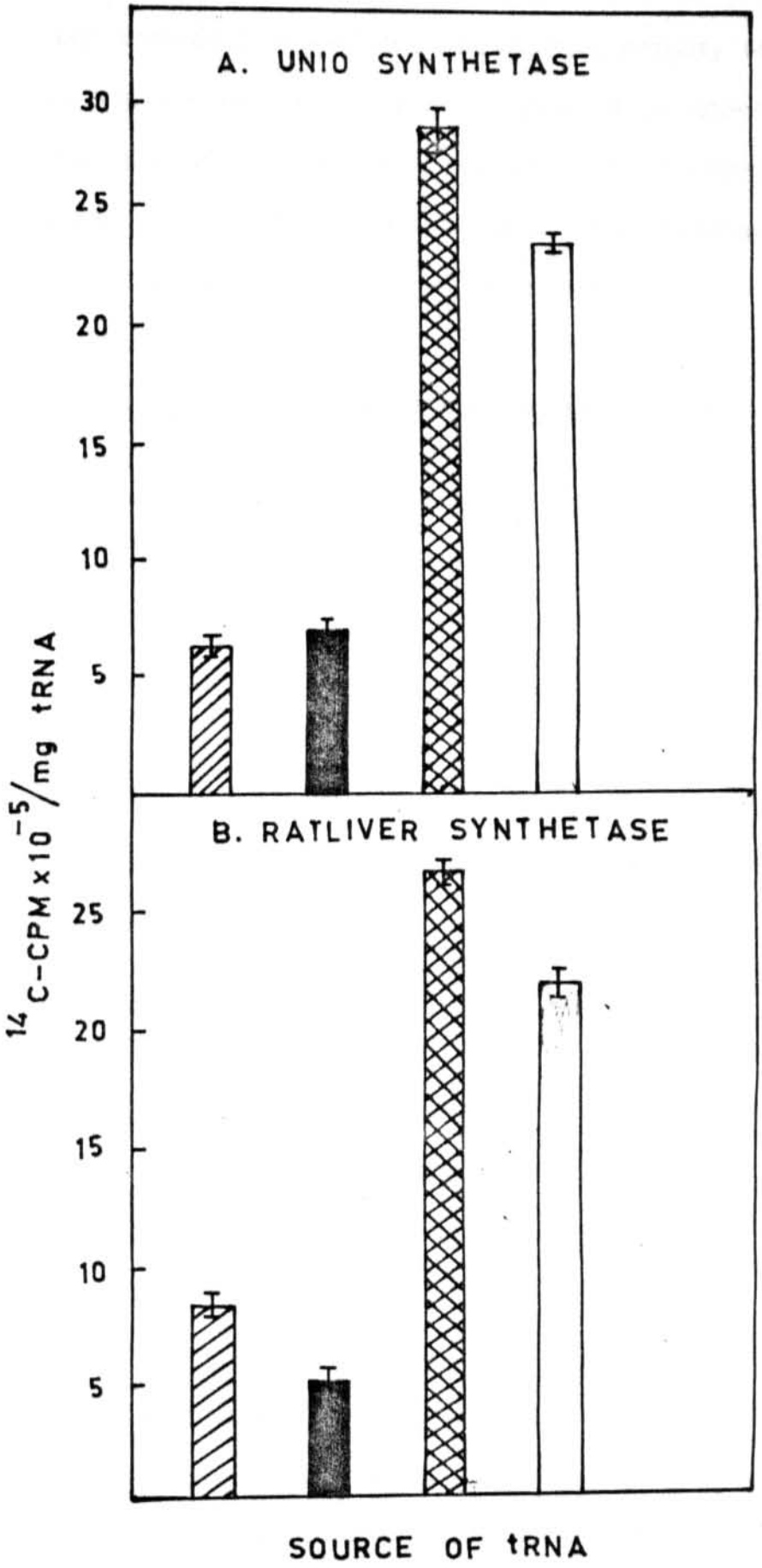


FIG.10: TOTAL AMINO ACID ACCEPTOR ACTIVITIES OF DIFFERENT tRNAs

to earthworm and cockroach in evolution shows equal activity when assayed with homologous enzyme or the rat liver enzyme. It is rather farfetched to think that the unio enzyme is closely related to the mammalian enzyme and is very much different from the earthworm and cockroach enzymes. The possible reasons for the variation will be discussed later.

9. Amino acid acceptor activity of the tRNA samples with individual amino acids (Tables 4a, 4b; Figures 11a, 11b)

Tables 4a and 4b and Figures 11a and 11b show the results obtained on the amino acid acceptor activity of the tRNA samples with 10 different ^{14}C-amino acids. The amino acids chosen for these studies were arginine and leucine (wth six codons), alanine, proline and valine (with four codons), and aspartic acid, glutamic acid, lysine, phenylalanine and tyrosine (with 2 codons). The top channel shows the acceptor activity of the tRNAs for different amino acids when acylated using rat liver aminoacyl-tRNA synthetase and the bottom channel the same data when unio enzyme was used for acylation. As mentioned in the earlier section the tRNAs from earthworm and cockroach in general show a low acceptor activity for most of the amino acids studied. In spite of this low activity the earthworm tRNA shows a marked higher acceptor activity with respect to leucine and alanine in both the heterologous systems.

9.1. Rat liver tRNA

There is a quantitative variation in different individual aminoacyl-tRNAs. The tRNAs for valine and arginine are found in higher concentration followed by leucyl, prolyl, lysyl, aspartyl, glutamyl and alanyl-tRNAs. The tRNAs for aromatic amino acids, phenylalanine and tyrosine, are found in much lower concentration.

In mammals, liver is known to be a metabolically active organ synthesizing different types of proteins and also rich in enzymes. Hence, it is very difficult to correlate the variations in the levels of aminoacyl-tRNAs with the synthesis of any particular protein. In general, it can be concluded that 30 week old rat liver proteins are rich in arginine and valine and poor in aromatic amino acids like phenylalanine and tyrosine.

.9.2. Unio tRNA

The unio tRNAs show a different relative abundancy pattern. It has high concentration of leucyl, arginyl and valyl-tRNAs. The tRNAs for the amino acids alanine, proline, phenylalanine, tyrosine and lysine are found at moderate concentrations and those for the acidic amino acids, aspartic acid and glutamic acid, are found at a very low concentration.

Here again the relative abundance of the aminoacyl-tRNAs cannot be correlated with the synthesis of any particular protein in the unio since no data is available on the type and amounts of proteins in the unio.

TABLE - 4a

AMINO ACID ACCEPTOR ACTIVITIES OF tRNAs FROM EARTHWORM, COCKROACH, UNIO AND RAT LIVER USING ^{14}C-AMINO ACIDS AND AMINOACYL-tRNA SYNTHETASE FROM RAT LIVER

Amino Acid	Source of tRNA			
	PICOMOLES OF ^{14}C-AMINO ACID/A_{260} OF tRNA			
	Earthworm	Cockroach	Unio	Rat liver (Homologous)
Arginine	3.75 ± 0.95*	3.5 ± 0.5*	25.5 ± 2.12*	39 ± 2.5
Leucine	33.0 ± 9.6	7.5 ± 0.25	24.0 ± 3.0	20.5 ± 0.5
Alanine	11.0 ± 0.5	2.5 ± 0.5	22.0 ± 3.5	14.0 ± 0.25
Proline	2.0 ± 0.5	3.0 ± 1.0	20.75 ± 2.4	21.5 ± 1.25
Valine	3.0 ± 0.5	8.0 ± 1.0	22.5 ± 2.5	45.0 ± 2.5
Aspartic acid	2.0 ± 0.7	4.0 ± 1.3	18.75 ± 1.06	17.0 ± 2.5
Glutamic acid	2.5 ± 0.25	4.0 ± 0.5	22.0 ± 2.0	14.0 ± 1.0
Lysine	2.5 ± 0.5	2.5 ± 0.12	19.75 ± 4.9	20.5 ± 0.5
Phenylalanine	2.16 ± 0.76	2.25 ± 0.2	10.0 ± 2.5	9.0 ± 0.75
Tyrosine	1.12 ± 0.34	2.5 ± 0.5	10.5 ± 3.5	7.0 ± 0.5

*Mean value ± S.D.

TABLE - 4b

AMINO ACID ACCEPTOR ACTIVITIES OF tRNAs FROM EARTHWORM, COCKOACH, UNIO AND RAT LIVER USING ^{14}C-AMINO ACIDS AND AMINOACYL-tRNA SYNTHETASE FROM UNIO

Amino Acid	Source of tRNA: PICOMOLES OF ^{14}C-AMINO ACID/A_{260} OF tRNA			
	Earthworm	Cockroach	Unio (Homologous)	Rat Liver
Arginine	3.16 ± 0.28*	6.3 ± 1.04*	25.0 ± 0.707*	25.0 ± 4.5*
Leucine	20.25 ± 1.0	7.5 ± 1.5	32.15 ± 1.15	28.0 ± 5.5
Alanine	9.8 ± 2.57	3.5 ± 0.86	17.0 ± 1.4	10.0 ± 3.0
Proline	2.0 ± 1.0	2.0 ± 0.25	13.0 ± 0.7	18.0 ± 2.5
Valine	6.1 ± 0.25	5.8 ± 0.76	26.25 ± 1.06	20.0 ± 2.25
Aśpartic acid	1.33 ± 0.28	3.0 ± 0.25	2.25 ± 0.35	5.5 ± 1.25
Glutamic acid	2.0 ± 0.25	1.75 ± 0.25	2.75 ± 0.35	10.0 ± 1.75
Lysine	1.33 ± 0.28	2.37 ± 0.48	9.0 ± 1.41	10.0 ± 1.5
Phenylalanine	7.75 ± 0.25	3.75 ± 0.25	17.75 ± 0.25	8.0 ± 1.25
Tyrosine	2.75 ± 0.35	2.5 ± 0.61	10.0 ± 0.5	7.5 ± 0.5

*Mean value ± S.D.

FIGURES 11a, 11b

Transfer RNAs from earthworm, cockroach, unio and rat liver were acylated with ^{14}C amino acids. 2.0 A_{260} units of tRNA were used for each reaction. The reaction was carried out at 37°C for 25 min. Other conditions were the same as described under materials and methods (2.4.1.).

The top channel of the Figure shows the amino acid acceptor activities of different tRNAs using rat liver aminoacyl-tRNA synthetases. The lower channel shows the same when unio aminoacyl-tRNA synthetase was used. Specific radioactivities (mCi/mmole) of the ^{14}C amino acids were as follows; arginine (246), leucine (156), alanine (120), proline (175), valine (190), aspartic acid (152), glutamic acid (190), lysine (288), phenyl-alanine (234) and tyrosine (360)

(▨) represents earthworm; (■) - cockroach; (▩) -unio and (□) - rat liver.

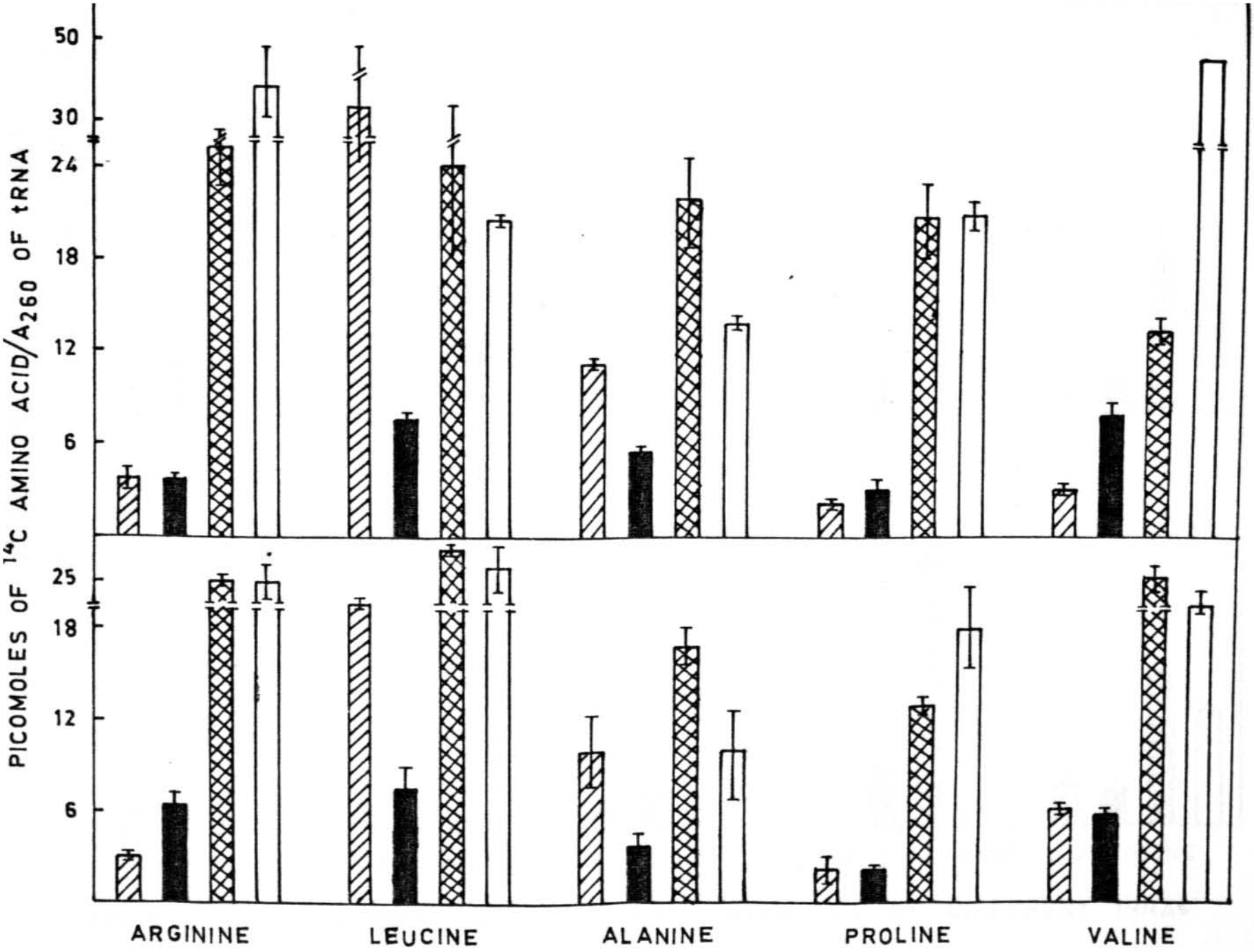


FIG.11a: INDIVIDUAL AMINO ACID ACCEPTOR ACTIVITIES OF DIFFERENT tRNAs

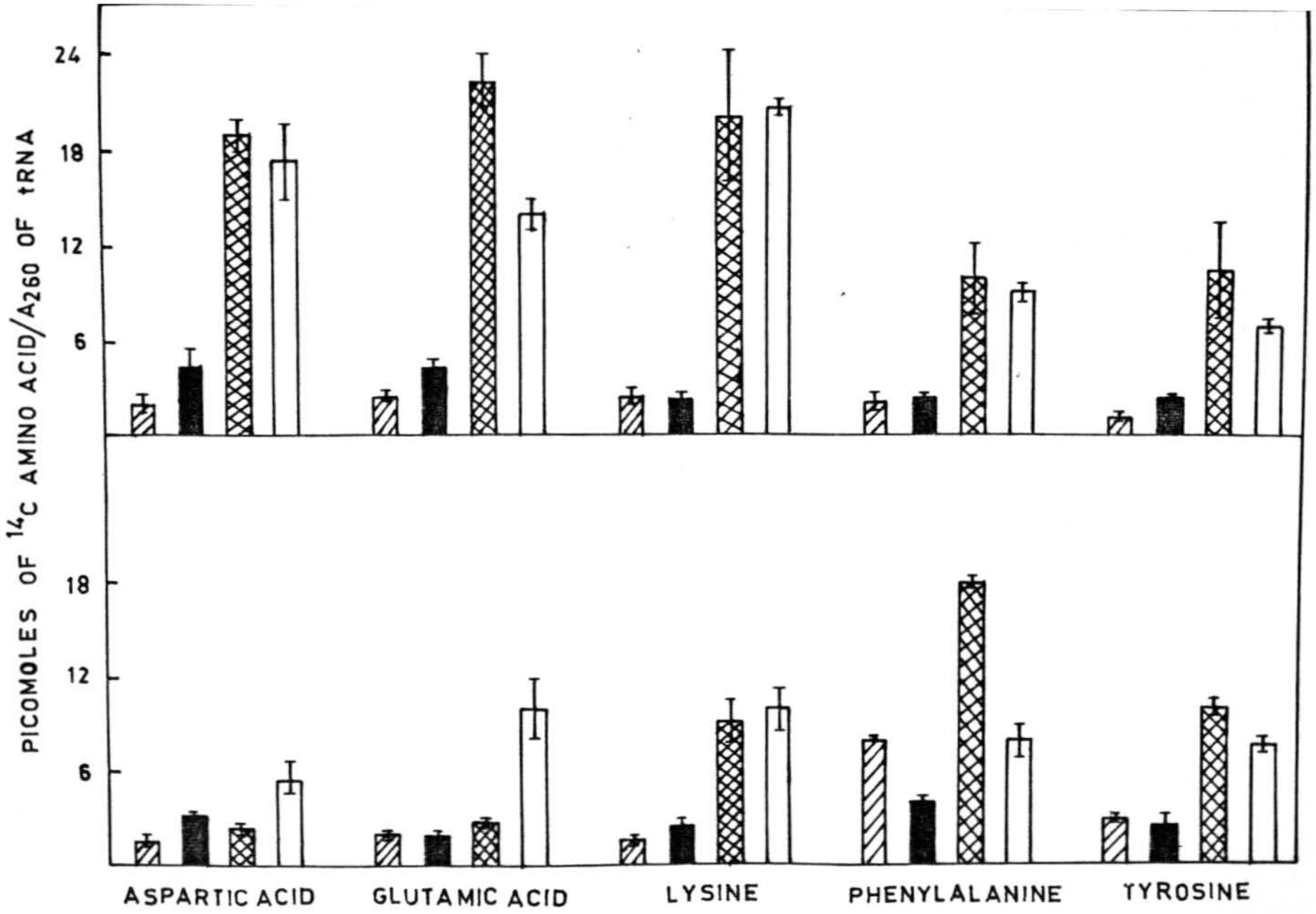


FIG.11b: INDIVIDUAL AMINO ACID ACCEPTOR ACTIVITIES OF DIFFERENT tRNAs

CHAPTER - IV

INHIBITION OF AMINOACYLATION IN CERTAIN INVERTEBRATES

Inhibition of aminoacylation in certain invertebrates

The results of the previous chapter threw light on the fact that aminoacyl-tRNA synthetases from rat liver and unio were highly active, whereas those prepared from earthworm and cockroach were inactive (3.6, Fig. 8). It is well known from literature that aminoacyl-tRNA synthetase complexes are highly unstable and get inactivated rather easily during the course of purification (167). Hence, various methods known to stabilize the enzyme, like inclusion of glycerol in the extraction buffer, use of very mild homogenizing conditions etc., were tried in vain. In many cases synthetases have been shown to be membrane bound (168). Solubilizing the enzyme using detergents like Triton X-100, sodium-deoxycholate etc., were tried with no success.

.1. Effect of earthworm enzyme preparation on aminoacylation in rat liver and unio homologous systems (Fig. 12)

Since all attempts to stabilize and obtain an active enzyme preparation from earthworm and cockroach failed, it was suspected that the enzyme preparation from these sources may contain an aminoacylation inhibitor(s). This was checked using a 'mixed enzyme assay' as described under materials and methods (2.4.2). Figure 12 shows the time-course of aminoacylation reaction, in the presence and absence of the earthworm enzyme preparation (Inhibitor/s). The data clearly indicate that the rat liver and unio homologous systems are highly active in the absence of the earthworm enzyme preparation but in its presence the aminoacylation is completely inhibited in both the otherwise active systems. This suggests the presence of a potent, endogenous aminoacylation inhibitor(s) in the earthworm enzyme preparation.

.2. Effect of different aminoacyl-tRNA synthetases on the homologous rat liver system (Figure. 13)

The total inhibition of aminoacylation shown by the earthworm enzyme

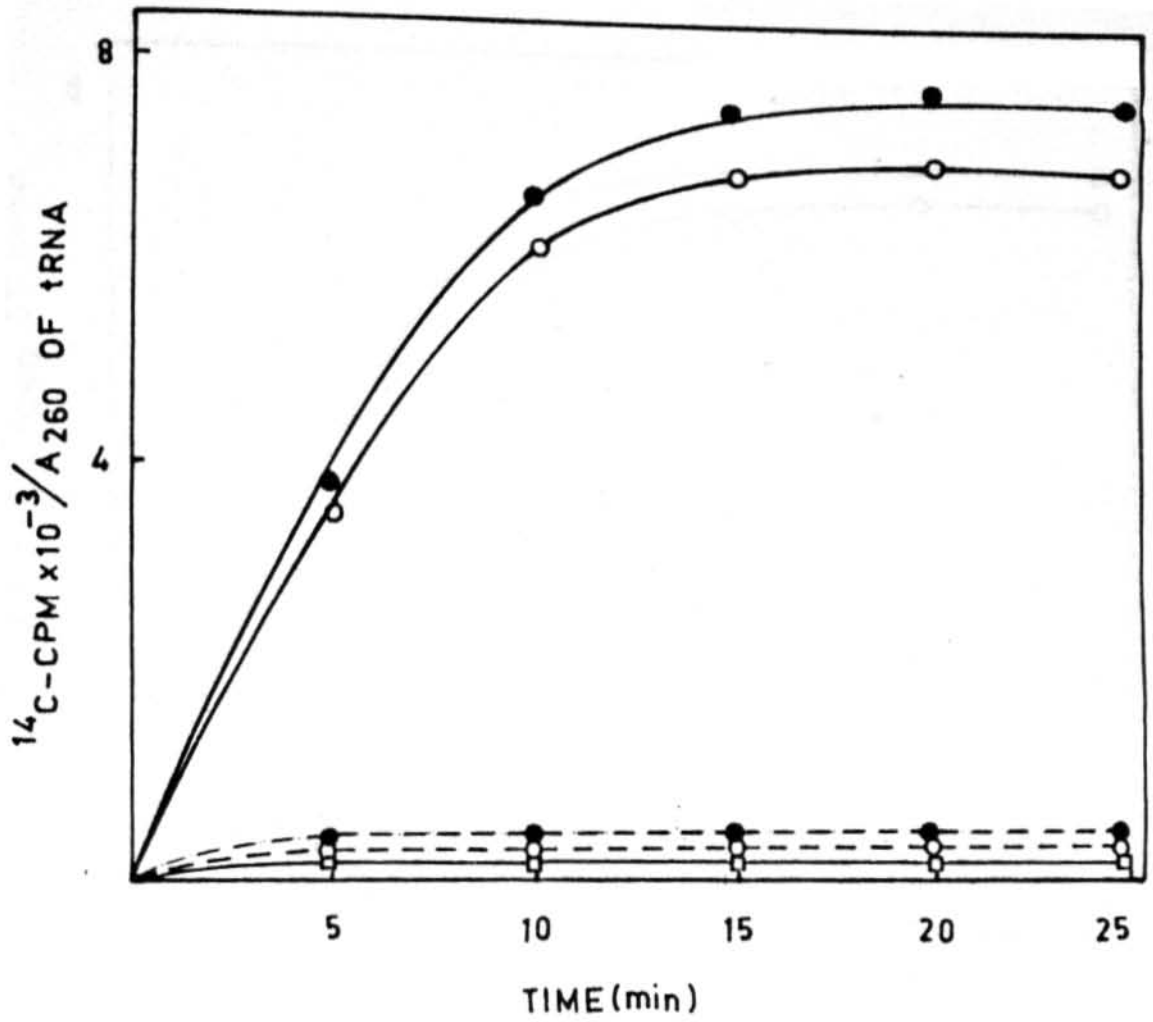


FIG.12. EFFECT OF EARTHWORM ENZYME PREPARATION ON HOMOLOGOUS RATLIVER AND UNIO SYSTEMS

Legend: Aminoacylation and 'mixed enzyme assay' were carried out as described under materials and methods (2.4.1 and 2.4.2). 2.0 A_{260} unit's of tRNA were aminoacylated using the enzyme from the same source. ^{14}C-algal protein hydrolysate was used as the source of total amino acids.

(●–●) - represents unio tRNA aminoacylated with unio enzyme.

(○–○) - rat liver tRNA aminoacylated with rat liver enzyme.

(●···●) - unio tRNA aminoacylated with unio enzyme in presence of earthworm enzyme.

(○···○) - rat liver tRNA aminoacylated with rat liver enzyme in presence of earthworm enzyme.

(□–□) - represents blank i.e., without tRNA.

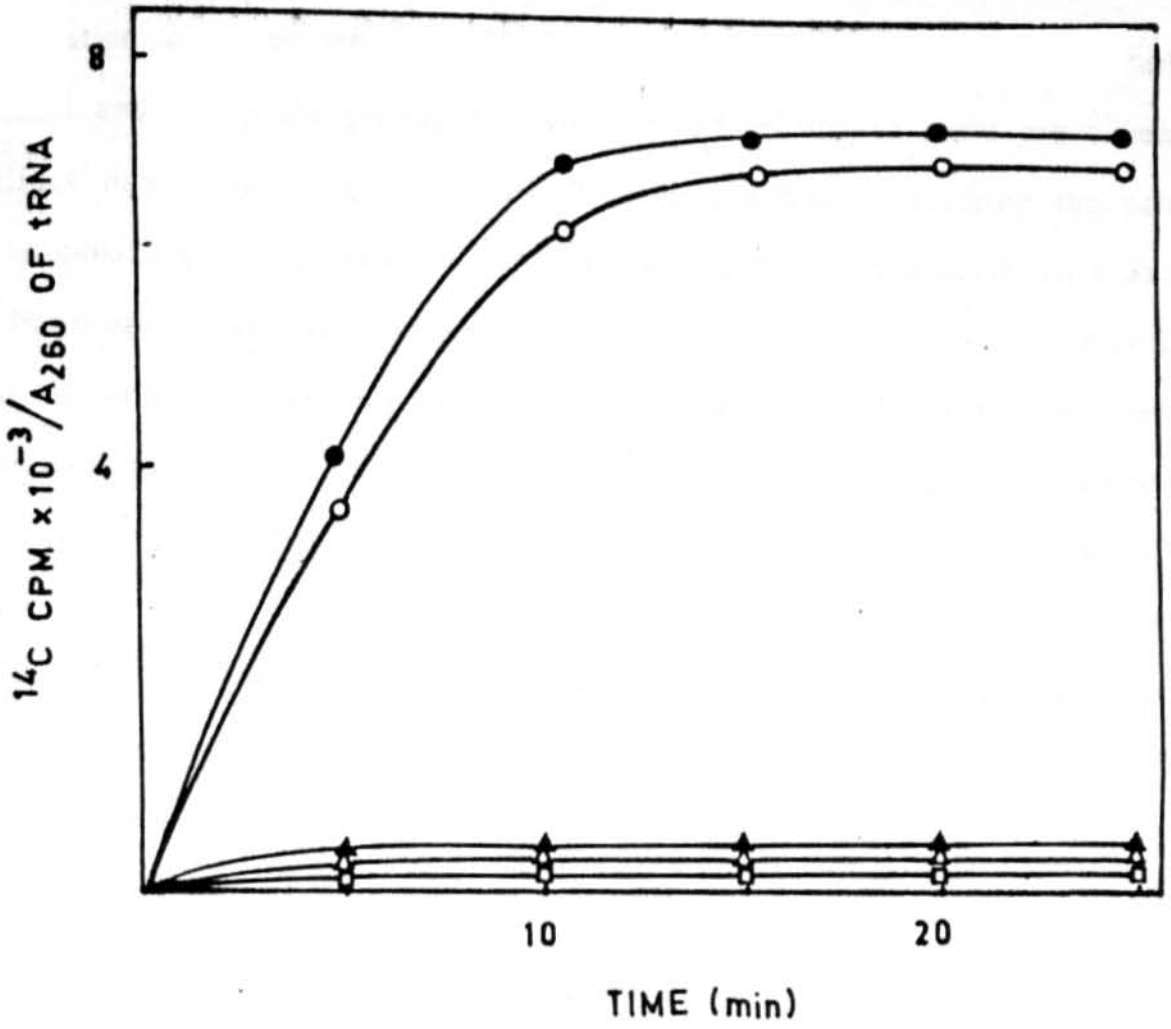


FIG.13. EFFECT OF DIFFERENT ENZYME PREPARATIONS ON RAT LIVER HOMOLOGOUS SYSTEM.

Legend: Aminoacylation and 'mixed enzyme assay' were carried out as mentioned under materials and methods.

(○–○) - represents aminoacylation of rat liver tRNA with rat liver synthetase.

(●–●) - aminoacylation of rat liver tRNA with rat liver synthetase in presence of unio enzyme.

(▲–▲) - aminoacylation of rat liver tRNA with rat liver enzyme in presence of cockroach enzyme.

(△–△) - aminoacylation of rat liver tRNA with rat liver enzyme in presence of earthworm enzyme.

(□–□) - blank i.e., without tRNA.

preparation on both the rat liver and unio-homologous systems was rather unusual and hence the possibility that it could be due to some experimental artifact had to be first ruled out. This was checked by studying the effect of aminoacyl-tRNA synthetases from different sources on the acylation capacity in homologous rat liver system (Figure. 13). It is evident from the results that the unio enzyme has no inhibitory action on the rat liver system whereas complete inhibition of aminoacylation is observed when cockroach or earthworm enzyme preparations are used under similar experimental conditions. The above observation rules out the possibility that the inhibition is a general phenomenon observed in 'mixed enzyme assay' and also rules out that it could be an experimental artifact and it reconfirms the presence of a potent, endogeneous aminoacylation inhibitor in the earthworm and cockroach enzyme preparations. In all future experiments the results obtained with earthworm system alone are given to avoid repetition since similar results were also obtained with cockroach enzyme preparations unless and otherwise specifically stated.

.3. Effect of earthworm enzyme preparation on heterologous charging systems (Figure. 14)

It was of interest to ascertain, whether the endogenous inhibitor blocks the aminoacylation only in the homologous systems or whether it is a general potent inhibitor which will knock off the acylation activity in any tRNA-synthetase recognition reaction (heterologous system). These results are depicted in Figure 14 and it throws light on the effect of the endogenous inhibitor(s) on heterologous acylation systems. It is obvious from the results that the rat liver enzyme recognizes equally well the E. coli tRNA and unio tRNA, but this aminoacylation activity is completely inhibited in both the heterologous systems in the presence of earthworm enzyme preparation. The inhi

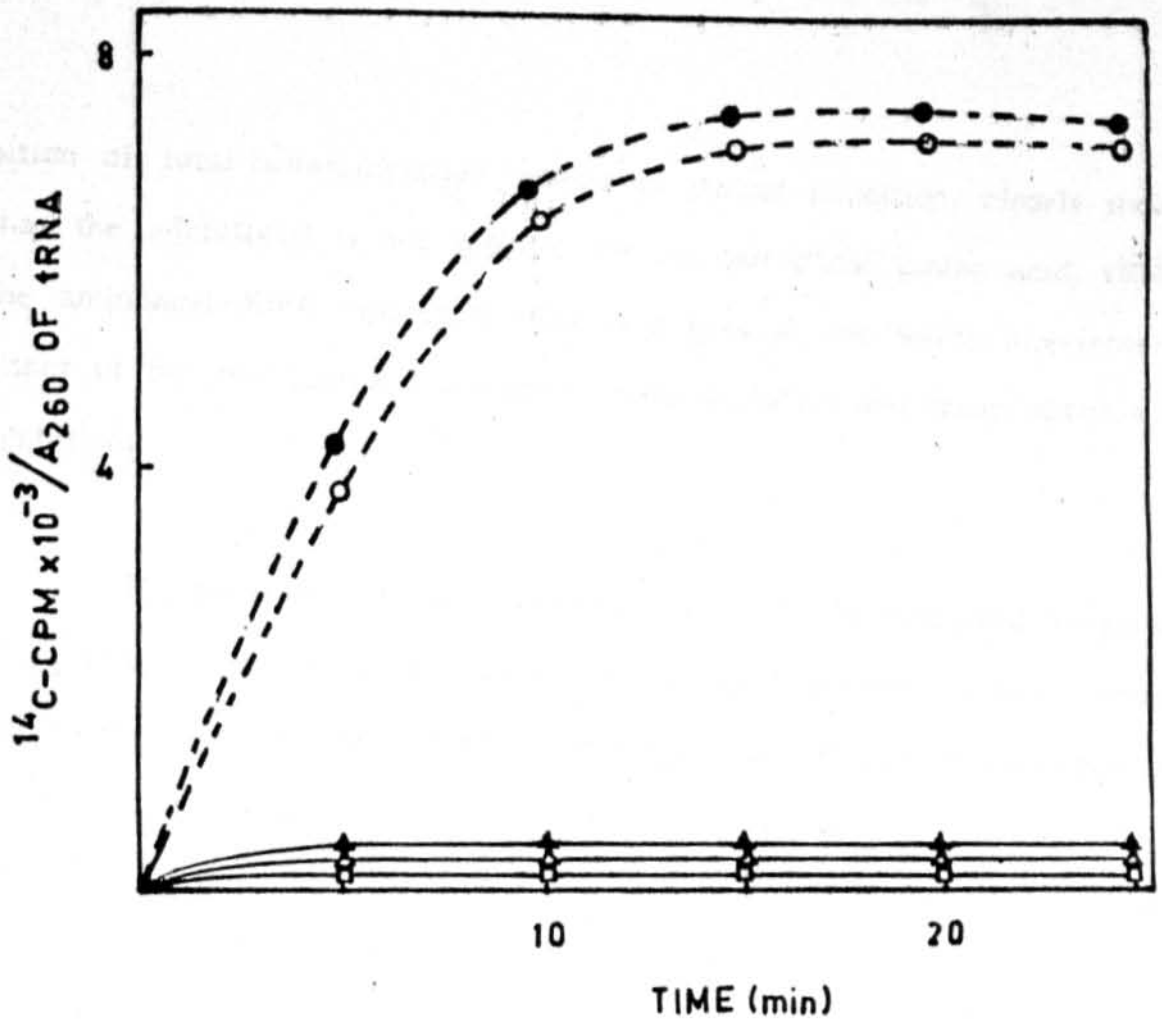


FIG.14. EFFECT OF EARTHWORM ENZYME PREPARATION ON DIFFERENT HETEROLOGOUS AMINOACYLATION SYSTEMS.

Legend: The aminoacylation and 'mixed enzyme assay' were carried out as mentioned under materials and methods.

(o-o) - represents E. coli tRNA aminoacylated with rat liver enzyme.

(●-●) - unio tRNA aminoacylated with rat liver enzyme.

(△-△) - E. coli tRNA aminoacylated with rat liver enzyme in the presence of earthworm enzyme preparation.

(▲-▲) - unio tRNA aminoacylated with rat liver enzyme in presence of earthworm enzyme preparation.

(□-□) - blank i.e., without tRNA.

bition of total aminoacylation instead of partial inhibition, clearly indicates that the inhibitor(s) is not specific for any particular amino acid, tRNA or the aminoacyl-tRNA synthetase, but is a general one which interferes with either of the two partial reactions of aminoacylation and brings about a total inhibition.

The above mentioned findings (4.1, 4.2 and 4.3) confirmed the presence of a potent, aminoacylation inhibitor in certain invertebrates like earthworm and cockroach and it also ruled out the possibility that it is an experimental artifact since another invertebrate i.e., the unio was free of this inhibitor (4.3). This very interesting finding regarding the existence of a naturally occurring aminoacylation inhibitor prompted further study on this aspect.

It was observed that the inhibitor(s) copurified with the total aminoacyl-tRNA synthetases from a DEAE-cellulose column followed by dialysis. This initially suggested that it may be a macromolecule, most probably a protein. Usually, the crude synthetase preparations are contaminated with ribonuclease which can degrade the tRNA and inhibit the aminoacylation. Although such a possibility is very unlikely in this case since the inhibition was extremely striking for the amount of the total protein used in the assay and still this possibility was checked.

.4. Effect of earthworm enzyme preparation on precharged aminoacyl-tRNA (Figure. 15)

The experiment was done as mentioned under the legend to Figure 15. From the graph it is obvious that the earthworm enzyme preparation had no effect on the precharged tRNA and behaved identical to the buffer control, under similar conditions the pancreatic ribonuclease completely degraded

Legend: Aminoacylation was carried out in a total volume of 1.2 ml, maintaining the molarity of each component as mentioned under materials and methods (2.4.1). 8.0 A_{260} units of rat liver tRNA were aminoacylated using the enzyme preparation from the same source. ^{14}C-algal protein hydrolysate was used as the source of total amino acids. 50 λ aliquots were processed at different time intervals and the acid precipitable counts were estimated. After 25 min. of incubation the reaction mixture was divided into three parts of 300 λ each. To one tube 0.3 A_{280} units of earthworm enzyme, to the second an equal volume of buffer containing 50mM Tris-HCl, pH, 7.5. 5mM EDTA and 2mM β-ME and to the third tube an equal volume of RNAase IIIA (18 units; from bovine pancreas) were added. The reaction was continued. 50 λ aliquots were taken at regular intervals and the acid precipitable counts were estimated.

(0-0) - represents aminoacylation of rat liver tRNA with rat liver enzyme.

(▮···▮) - the effect of buffer on the precharged rat liver tRNA.

(▲-▲) - the effect of earthworm enzyme preparation on the precharged rat liver tRNA.

(△···△) - the effect of RNAase IIIA on the precharged rat liver tRNA.

(□-□) - blank i.e, without tRNA.

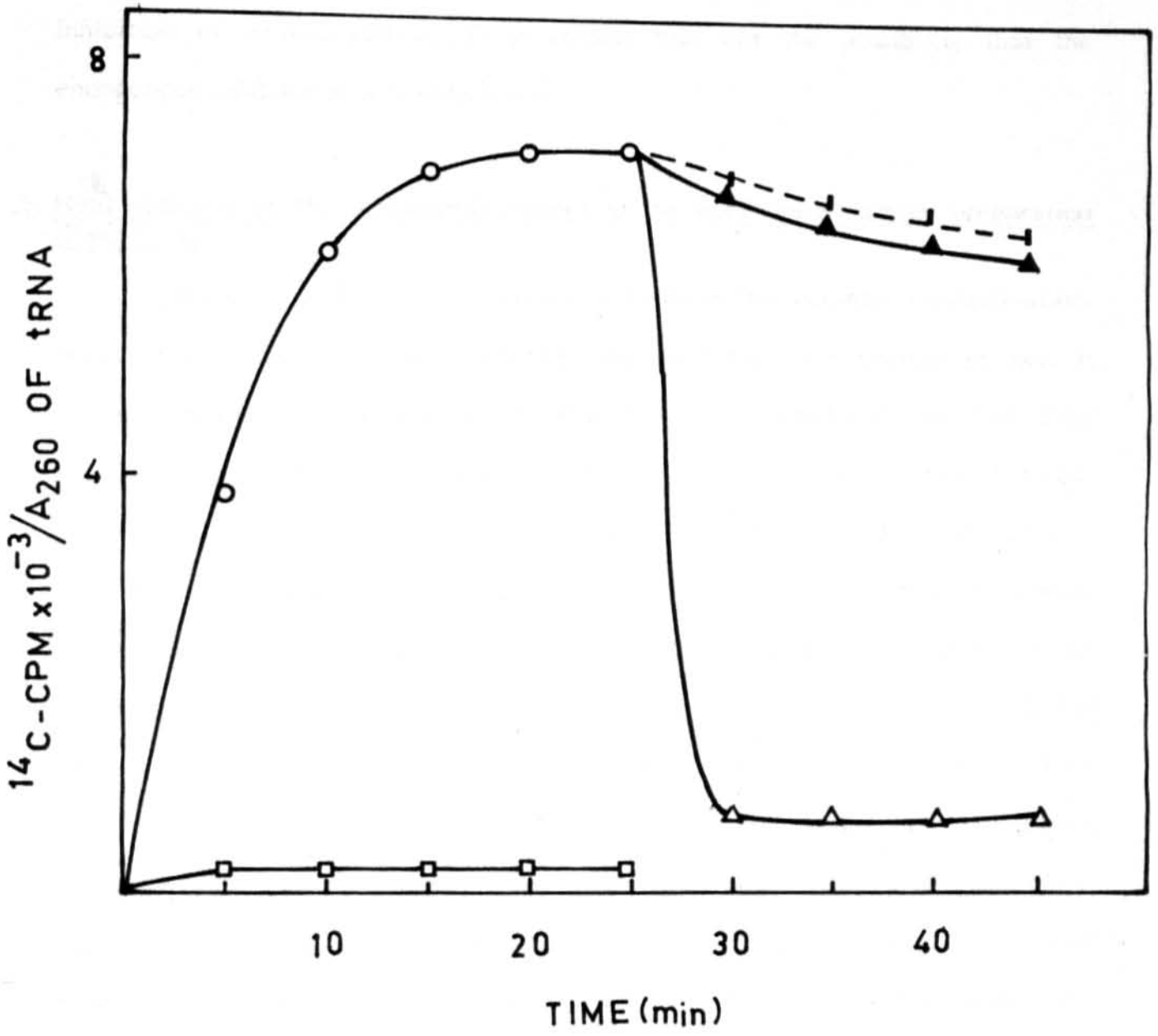


FIG. 15. EFFECT OF EARTHWORM ENZYME PREPARATIO ON PRECHARGED RAT LIVER tRNA.

the tRNA and solubilized all the acid precipitable counts. Care was taken to ascertain that the same amount of earthworm enzyme protein per A_{260} unit of tRNA was used in the assay which normally brings about complete inhibition of aminoacylation. These results rule out the possibility that the endogenous inhibitor is a ribonuclease.

.5. Heat stability of the inhibitor(s) present in the earthworm enzyme preparation (Figure. 16)

The fact that the inhibition was not due to any nuclease contamination, made it essential to confirm whether the inhibitor is a protein or not. It is well known that proteins, in general, get heat denatured and lose their activity. Fig. 17 shows the effect of heat denatured earthworm enzyme preparation on aminoacylation in rat liver system. The supernatant fraction obtained after heat denaturation and centrifugation of the earthworm enzyme preparation still retained the inhibitor activity. But this inhibition was lost on dialysis of the supernatant fraction i.e., the dialysed supernatant fraction inhibited the acylation reaction, only by about 15%. To confirm that this loss of inhibitory activity was due to removal of the inhibitor, the supernatant fraction was dialysed against a small volume of buffer without change and the dialysate (outer buffer) was concentrated and used in the assay. This fraction completely inhibited the assay thereby showing that the inhibitor was dialysable and could be recovered from the outer buffer. This indicated that the inhibitor is a small molecular weight compound which is non-covalently adsorbed to a macromolecule and upon heat denaturation of the macromolecule, it gets released.

4.6. Preparation of the inhibitor(s) fraction

The observation that the inhibitor is a heat stable, dialysable compound

Legend: Aminoacylation and 'mixed enzyme assay' were carried carried out as mentioned in materials and methods.

(○–○) - represents the aminoacylation of rat liver tRNA with rat liver enzyme.

(▲–▲) - represents the effect of earthworm enzyme on rat liver homologous system.

The earthworm enzyme preparation was heated for 15 min at 95°C in a boiling water bath. The denatured proteins were removed by centrifugation at 10,000 g for 15 min. The supernatant fraction was used in the 'mixed enzyme assay'.

(■–■) - represents the effect of the heat supernatant on rat liver aminoacylation.

The supernatant (obtained above) was dialyzed against 0.05M Tris-HCl, pH , 7.5 containing 0.005M EDTA and 0.002M β-ME with 2 changes (sample: Buffer = 1.50). The solution in the bag (retentate) was used in the 'mixed enzyme assay'.

(●–●) - represents the effect of the retentate on the rat liver aminoacylation.

The supernatant fraction was dialyzed against a small volume of buffer (sample: buffer = 1:6) for 20 hrs. without change. The outer buffer was concentrated to the volume of heat supernatant and used in the 'mixed enzyme assay'.

(△–△) - the effect of concentrated dialysate on rat liver aminoacylation.

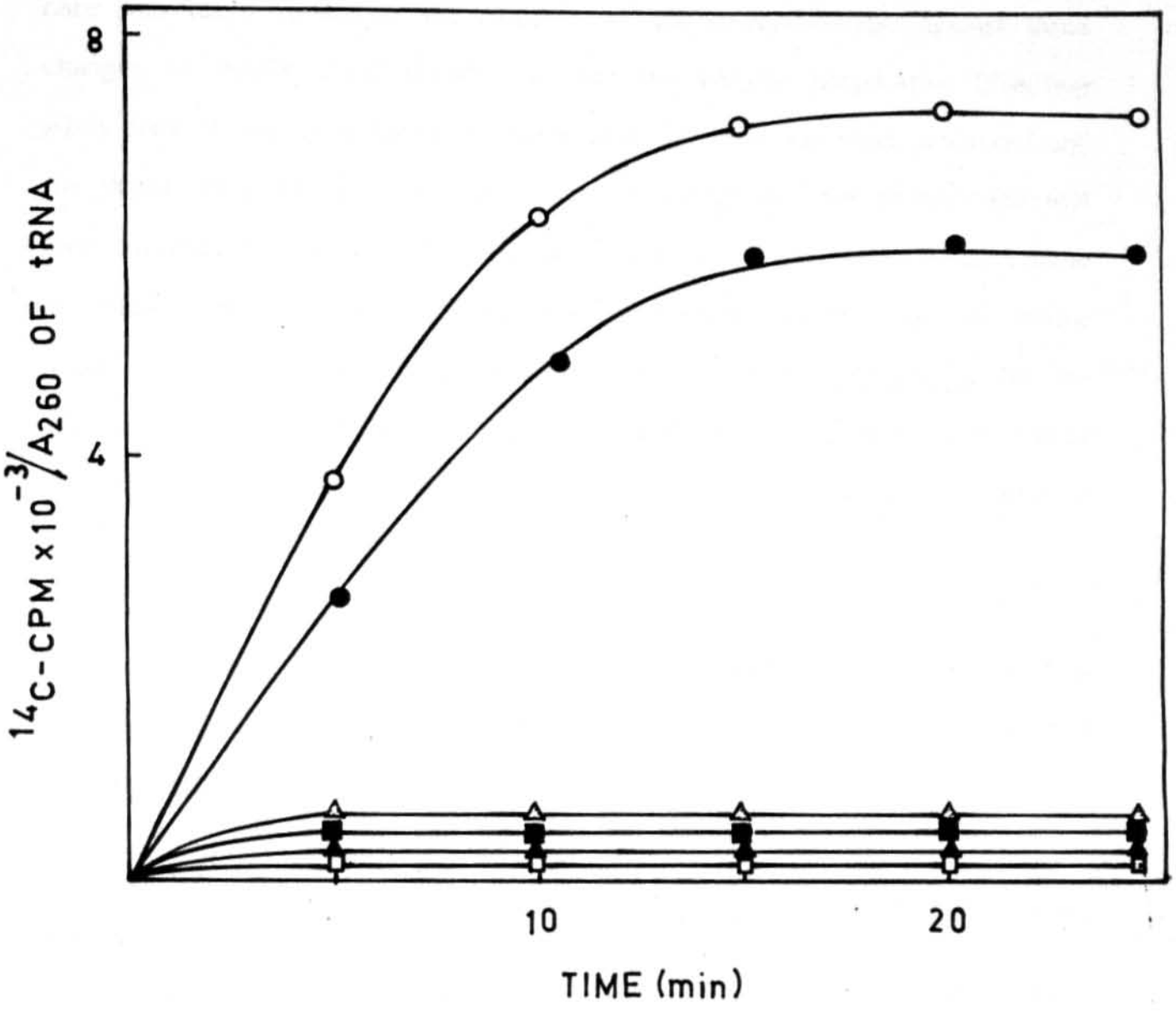


FIG. 16. EFFECT OF HEAT ON THE AMINOACYLATION INHIBITOR PRESENT IN EARTHWORM ENZYME.

helped in standardizing a procedure for the preparation of the inhibitor fraction. This is depicted in Chart 3 under materials and methods. In this procedure care was taken to dialyse the eluate from the DEAE-cellulose against three changes of double distilled water so that the enzyme preparation (Fraction A) is free of any salt. The salt free protein fraction was heat denatured and the denatured proteins were removed by centrifugation. The protein and salt free supernatant fraction (Fraction B) was dialysed against a small volume of double distilled water and this was concentrated to the required volume which gave an inhibitor preparation free of any salt (Fraction C). All the experiments mentioned earlier were repeated with Fraction C and similar results were obtained. In all further experiments Fraction C was used as the source of the inhibitor.

Sulfhydryl enzymes are known to be inhibited by heavy metal ions. Amioacyl-tRNA synthetases being sulfhydryl enzymes it was speculated that the heat stable, dialysable inhibitor(s) could be a heavy metal ion.

.7. Effect of different divalent cations on aminoacylation in rat liver system (Figure. 17)

A study was undertaken to ascertain the action of divalent cations on the activity of the aminoacyl-tRNA synthetases. Figure 17 shows the effect of different divalent cations on the aminoacylation reaction catalyzed by rat liver synthetase. It is clear from the results that different metals inhibit the enzyme to a different extent at 3.3mM concentration. Out of the various metal ions tested only zinc and copper ions completely inhibited the enzyme under the conditions used. The concentration of metal ions needed to bring about complete inhibition was standardized earlier using copper ions.

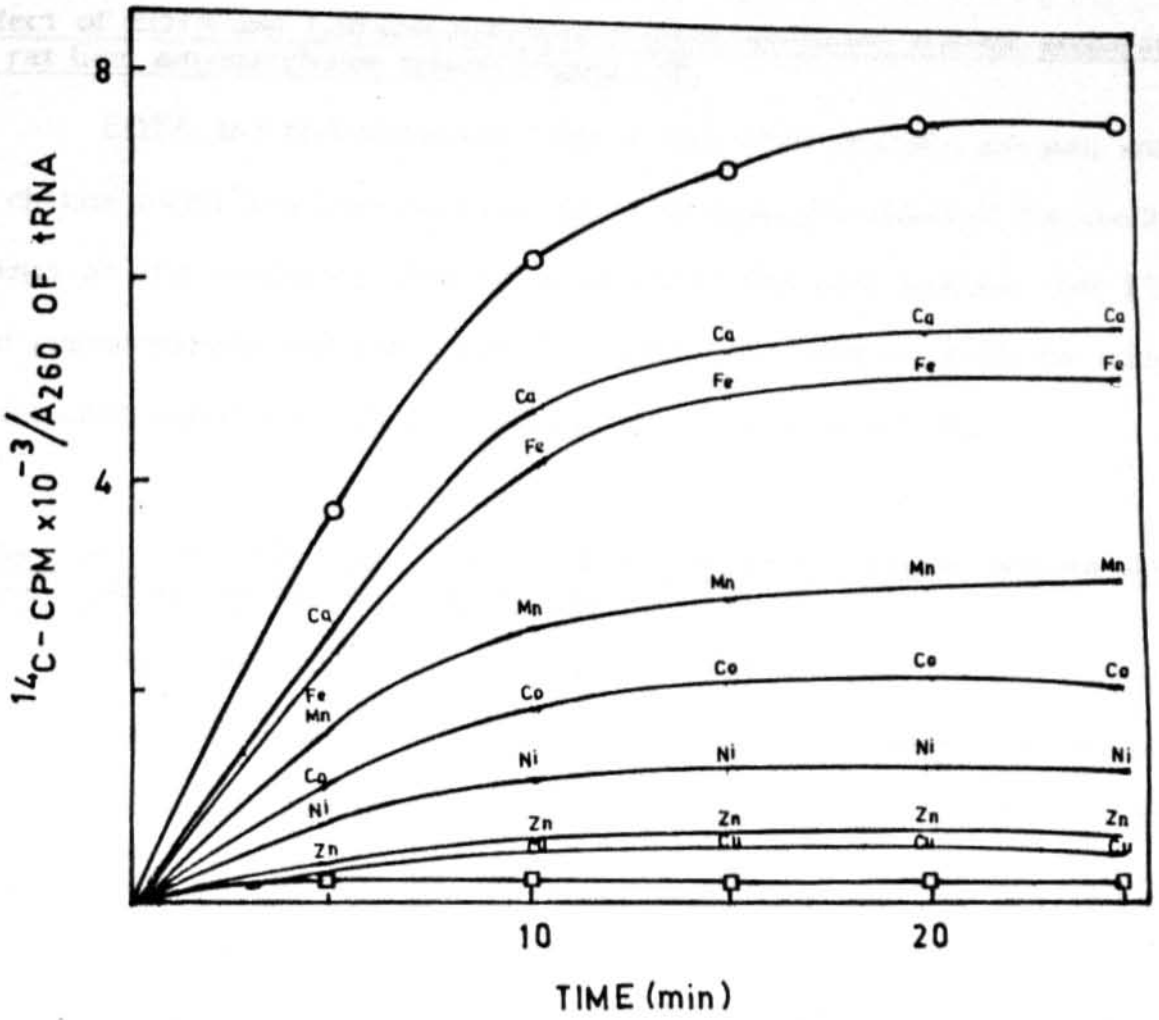


FIG. 17. EFFECT OF DIFFERENT DIVALENT METAL IONS ON AMINOACYLATION IN RAT LIVER HOMOLOGOUS SYSTEM.

Legend: Aminoacylation and 'mixed enzyme assay' were carried out as mentioned under materials and methods.

(0-0) - represents the aminoacylation of rat liver tRNA with rat liver enzyme.

(□-□) - represents blank i.e., without tRNA

The effects of different metal ions at 3.3mM concentration are shown by their respective symbols.

.8. Effect of EDTA-and 1,10-phenanthroline-treated earthworm enzyme preparation on rat liver aminoacylation system (Figure. 18)

EDTA and phenanthroline (169) at high concentrations are well known to chelate metal ions from proteins. Figure 18 shows the effect of the chelating agents on the earthworm enzyme preparation. The data indicate that EDTA and phenanthroline treatment does not remove the inhibitor from the enzyme preparation suggesting that the inhibitor may not be a metal ion.

.9. Effect of ashed and nitric acid oxidized earthworm enzyme preparation on aminoacylation reaction in rat liver system (Figure. 19)

Ashing and nitric acid oxidation procedures are well known to destroy all organic compounds present in a sample. The ones which survive ashing and acid oxidation are the inorganic metal ions. Figure 19 shows the effect of ashing and acid oxidation on the earthworm enzyme preparation. The experiment was carried out using either the enzyme preparation or Fraction C as mentioned under materials and methods (2.7.1; 2.7.2). The results clearly indicate that upon ashing or nitric acid oxidation the inhibitor is completely destroyed, and there is no more inhibition of aminoacylation. This clearly ruled out the possibility that the inhibitor(s) is a metal ion and confirmed the organic nature of the compound.

The foregoing data confirmed the occurrence of a potent, endogenous aminoacylation inhibitor(s) in earthworm and cockroach and ruled out the possibility that it is an experimental artifact. The inhibitor was shown to be an organic compound which is heat stable and of small molecular weight. The results also indicated that the inhibitor as it is prepared is non-covalently associated with a macromolecule. The total inhibition of aminoacylation instead of partial inhibition suggested that the inhibitor is not specific for any particular amino acid, tRNA or aminoacyl-tRNA synthetase but a general one

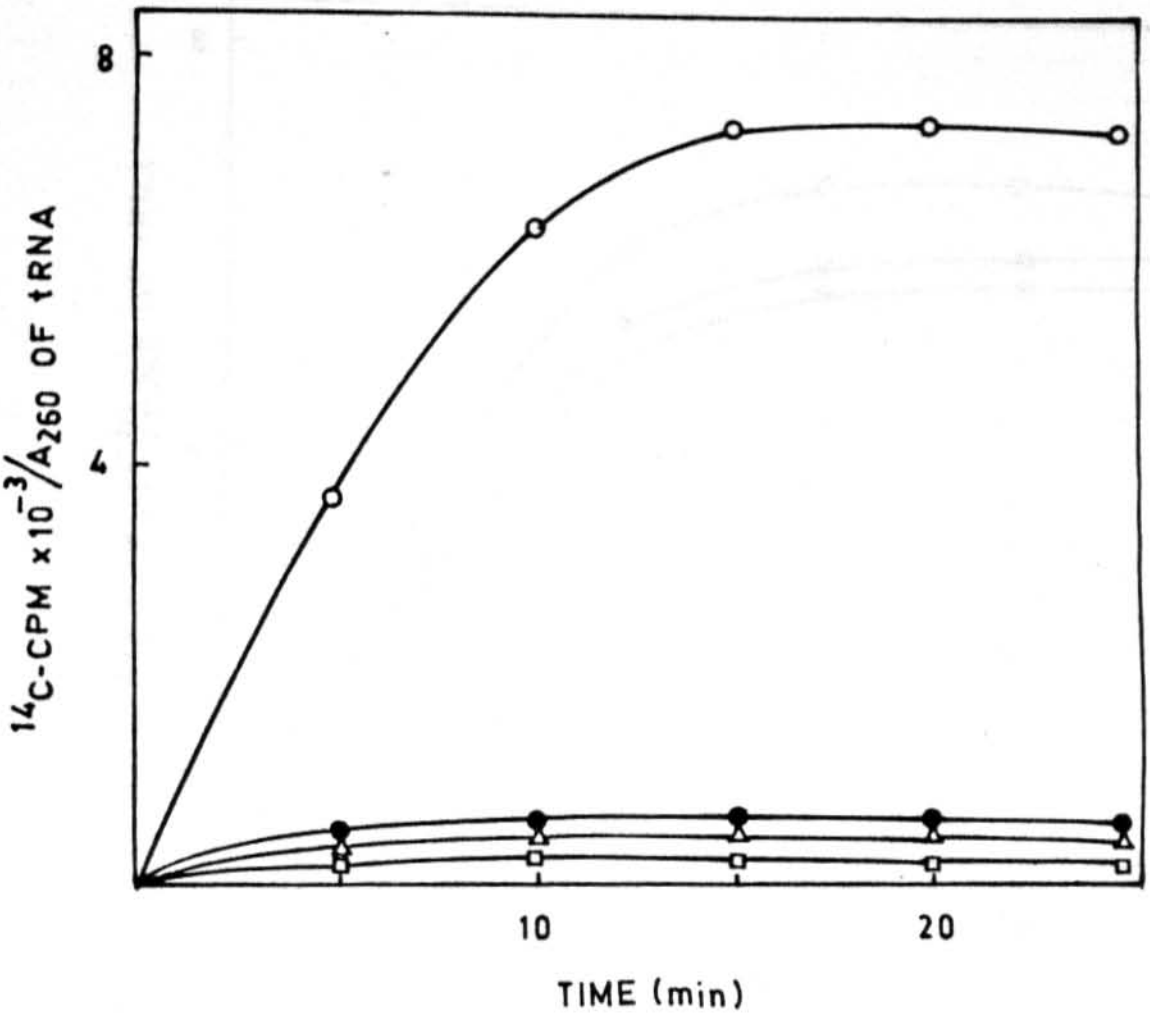


FIG.18. EFFECT OF EDTA AND 1,10 PHENANTHROLINE ON EARTHWORM ENZYME PREPARATION.

Legend: Earthworm enzyme preparation was made 10mM with respect to EDTA or 1,10-phenanthroline and left in ice for 10 min. The solution was dialysed against a buffer containing 0.05M Tris-HCl, pH, 7.5, 0.002M β-mercapto-ethanol for 16 hrs with two changes (buffer: sample=50:1). The aminoacylation reaction and the 'mixed enzyme assay' were carried out as mentioned under materials and methods (2.4.1 and 2.4.2).

(0-0) - represents the aminoacylation of rat liver tRNA with rat liver enzyme.

(●-●) - represents the effect of EDTA treated earthworm enzyme preparation on rat liver aminoacylation system.

(△-△) - represents the effect of 1,10-phenanthroline treated earthworm enzyme preparation on rat liver aminoacylation system.

(□-□) - represents blank, i.e, without tRNA.

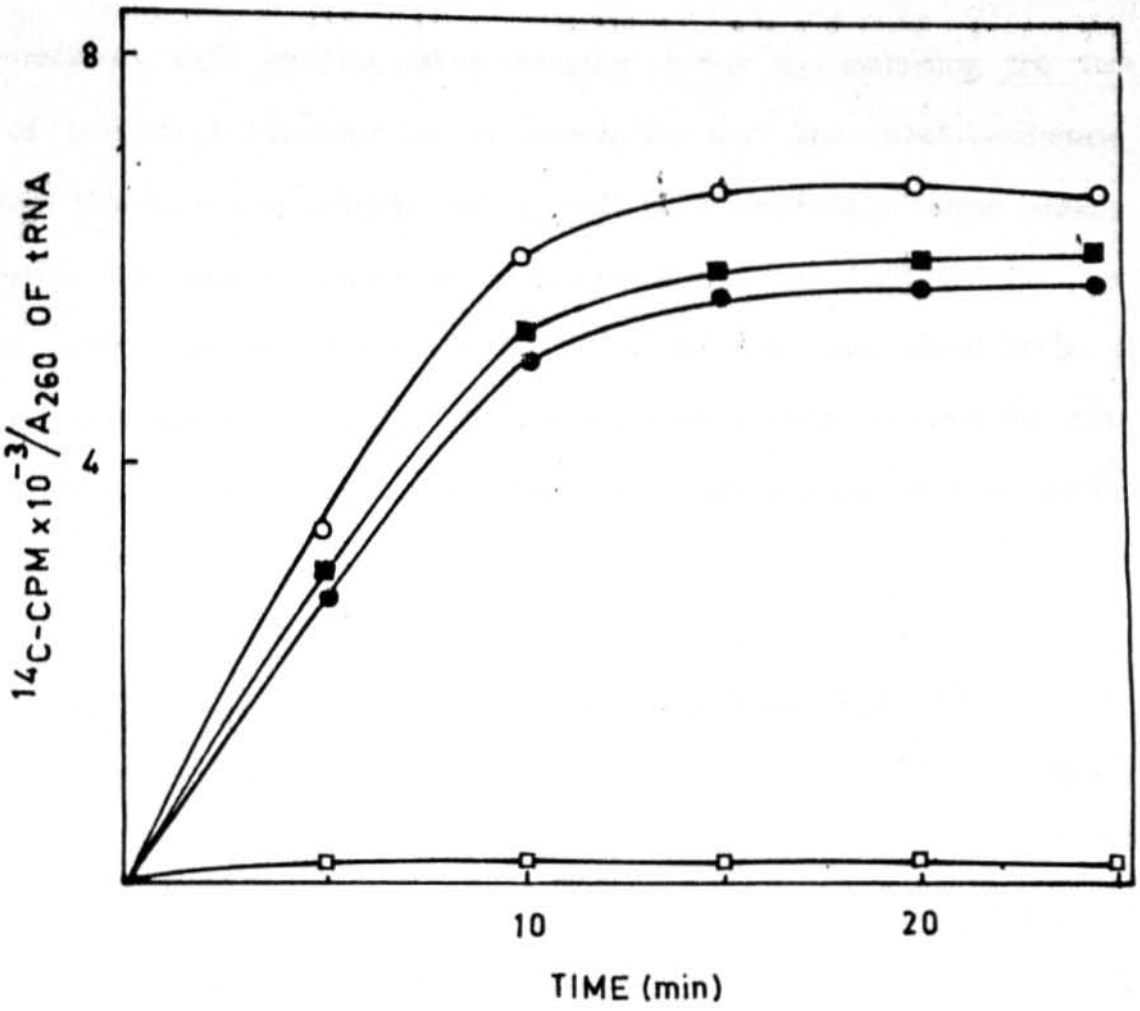


FIG. 19. EFFECT OF ASHING AND NITRIC ACID OXIDATION ON EARTHWORM ENZYME PREPARATION.

Legend : 1 ml of earthworm enzyme preparation or fraction C was subjected to ashing or conc. HNO_3 oxidation as described under materials and methods (2.7.1; 2.7.2). The aminoacylation and 'mixed enzyme assay' were carried out as described.

(○–○) - represents the aminoacylation of rat liver tRNA with rat liver synthetase.

(■–■) - represents the effect of ashed earthworm enzyme preparation of rat liver aminoacylation system.

(●–●) - represents the effect of nitric acid oxidized earthworm enzyme on rat liver aminoacylation system.

(□–□) - blank i.e., without tRNA.

which interferes with aminoacylation reaction either by inhibiting the formation of aminoacyl adenylate or by interfering with the tRNA-synthetase interaction. Hence, it was thought that it could be a substrate analogue namely a nucleotide, an oligonucleotide or a peptide which has high affinity, for either the tRNA or the synthetase. Since the inhibitor was found to be a small molecular weight molecule, methods were designed to remove the inhibitor and obtain an active aminoacyl-tRNA synthetase preparation from earthworm and cockroach.

0. Action of KSCN and KI on earthworm enzyme preparation (Figure 20)

Chaotropic reagents like KSCN and KI are known to remove tightly bound coenzymes from proteins (170). Hence this technique was employed to free the aminoacyl-tRNA synthetase from the inhibitor. The results depicted in Figure 20 indicates that these chaotropic reagents have no effect on the removal of the bound inhibitors from the enzyme.

1. Molecular sieve chromatography on Sephadex G-100 (Figure 21)

Zanakis et al (171) have reported the removal of substances of MW less than 20,000 from the enzyme which affect the post-translational addition of amino acids to proteins, by gel filtration on Sephacryl S-200. In line with this the earthworm enzyme preparation was subjected to gel filtration on a Sephadex G-100 column, as described under Figure 21. Rat liver aminoacyl-tRNA synthetase preparation was used as a standard. It is clear from the data that the aminoacyl-tRNA synthetase preparation from rat liver elutes as a single peak in the void volume (MW $\geq$ 100,000). On the other hand, the earthworm enzyme preparation, surprisingly, fractionated into three peaks; one eluting in the void volume, the second in the E. coli tRNA region (MW: 25,000) and the third in the small molecular weight region (ATP). The inhibitor

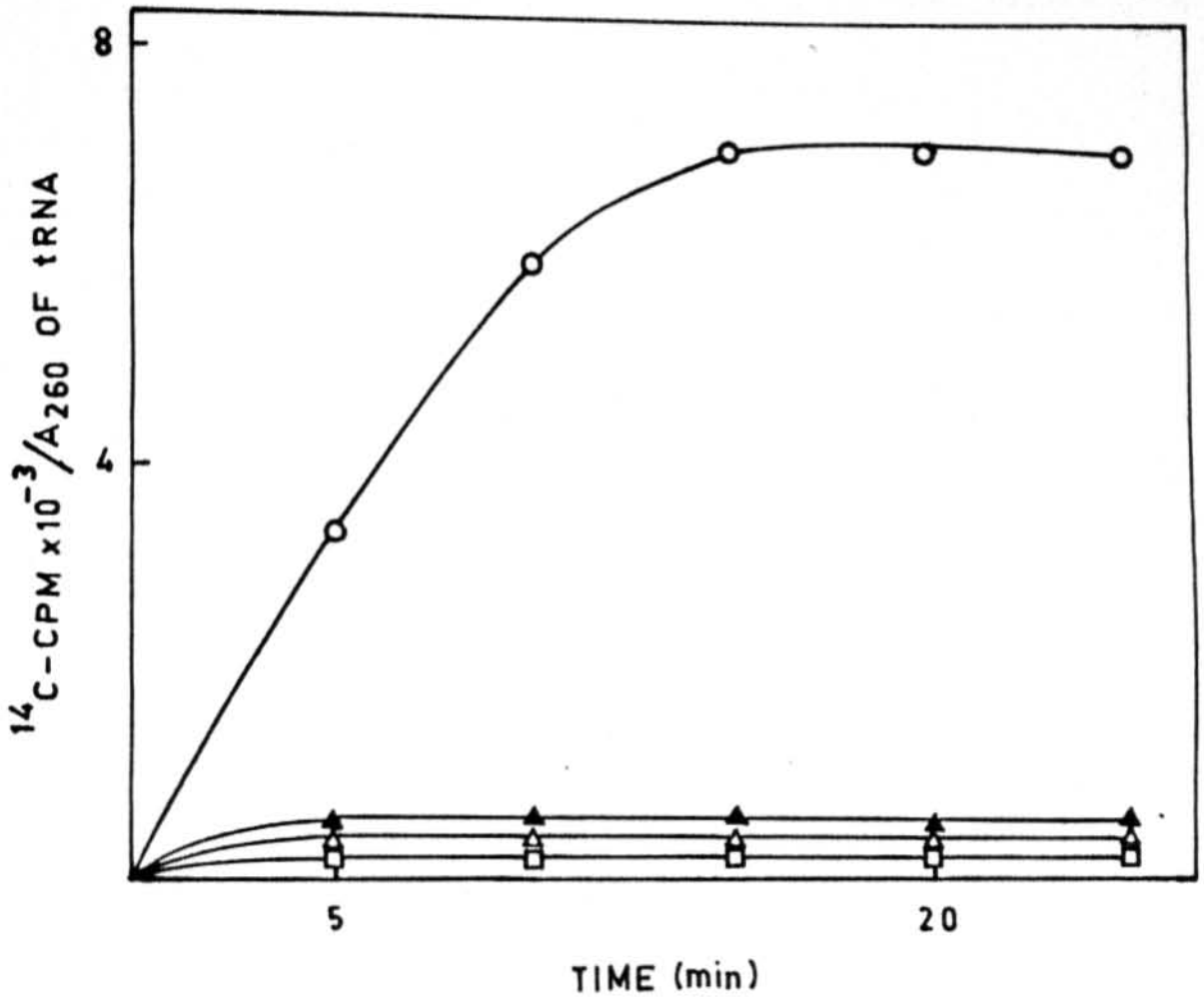


FIG. 20. EFFECT OF KSCN AND KI ON EARTHWORM ENZYME PREPARATION

Legend : The earthworm enzyme preparation (Chart 2) was made 0.3M with respect to KSCN or KI and incubated for 24 hrs. at 37°C or overnight at 0-4°C. The contents were dialyzed overnight with 2 changes. The retentate (inside bag) was used in the assay. The aminoacylation and 'mixed enzyme assay' were carried out as mentioned under materials and methods.

(0–0) - represents the aminoacylation of rat liver tRNA with rat liver enzyme.

(▲–▲) - the effect of KSCN treated earthworm enzyme preparation on the aminoacylation of rat liver tRNA with rat liver enzyme.

(Δ–Δ) - the effect of KI treated earthworm enzyme preparation on the aminoacylation fo rat liver tRNA with rat liver enzyme.

(□–□) - blank i.e., without tRNA.

FIG. 21: 8.0 A_{280} units of rat liver aminoacyl-tRNA synthetase preparation or 6.0 A_{280} units of earthworm enzyme preparation (2 ml) obtained from DEAE-cellulose chromatography and dialysis (2.3.3, Chart 2) were loaded on to a Sephadex G-100 column (45 cmxl.3 cm) equilibrated with 0.005M EDTA, 0.002M -mercaptoethanol in 0.01M Tris-HCl buffer, pH, 7.5 and eluted with the same buffer. The column was operated in cold at 4°C. 1.5 ml fractions were collected at a flow rate of 20 ml/hr and the absorbancy at 280 nm was recorded for all the fractions.

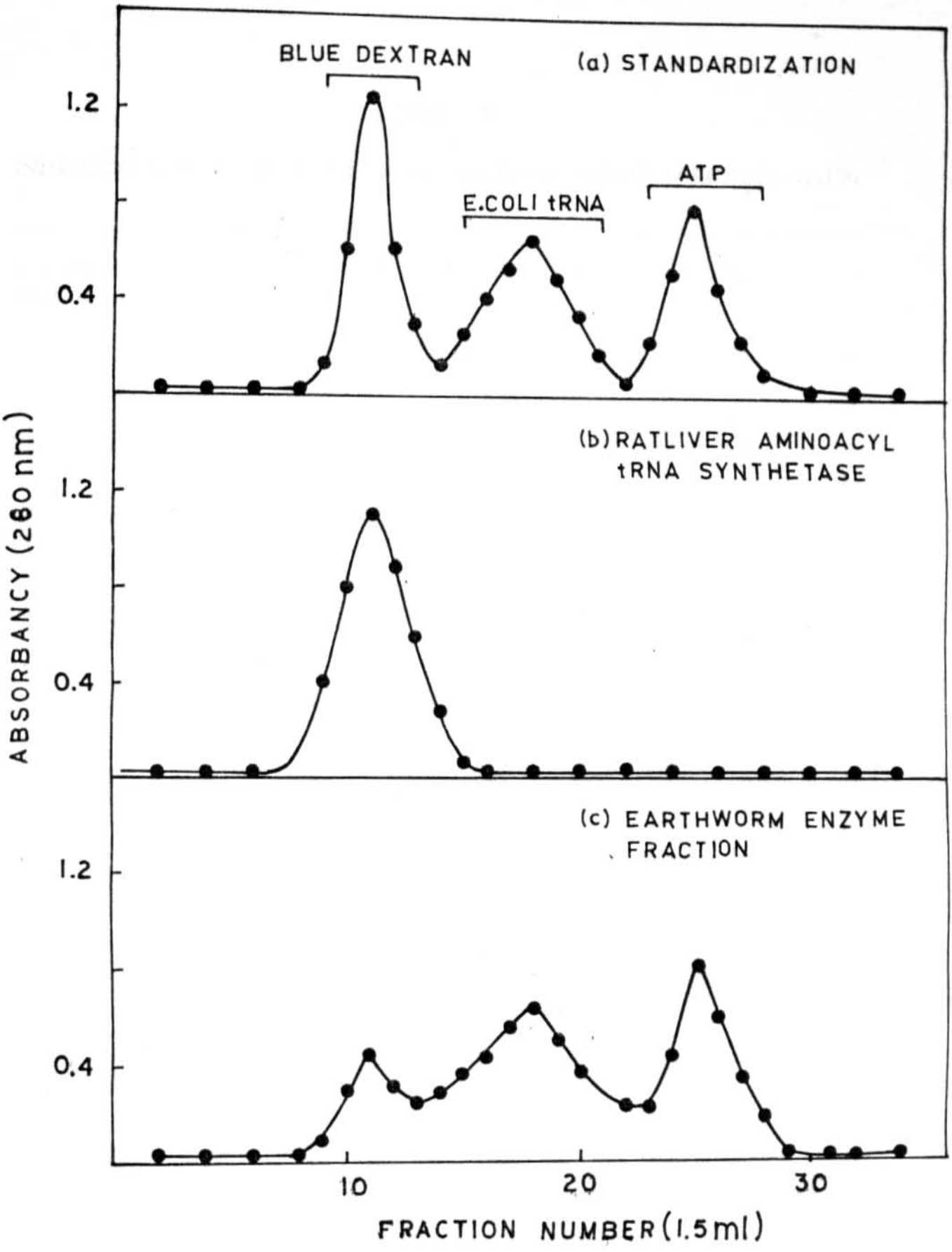


FIG. 21. GELFILTRATION OF RATLIVER AND EARTHWORM ENZY PREPARATIONS ON SEPHADEX G-100.

TABLE - 5

INHIBITOR ACTIVITIES OF THE PEAKS OBTAINED FROM SEPHADEX G-100

Sephadex G-100 Fraction	$\frac{A_{280}}{A_{260}}$	A_{280} units used in the assay	% Inhibition
Peak I (Fractions 10-12)	1.15	0.05	65
Peak II (Fractions 13-20)	1.25	0.05	84
Peak III (Fractions 23-29)	0.51	0.05	85

Legend: The earthworm enzyme preparation was gel filtered as mentioned under Fig. 20. The peak I (10-12), peak II (13-20) and peak III (23-29) were pooled, concentrated and checked for inhibitory activity. Aminoacylation and mixed enzyme assay were carried out as described under materials and methods. 0.05 A_{280} units of peak I, peak II or peak III were used in the assay. The reaction was carried out for 25 min at 37°C and the acid precipitable counts were estimated. The rat liver tRNA aminoacylated with rat liver enzyme was used as the control (0% inhibition).

activates and the A_{280}/A_{260} ratio of these three peaks are given in Table 5. The A_{280}/A_{260} ratio of the different peaks indicate that the protein fractions in peak I and peak II are contaminated with nucleic acids since a pure protein gives a ratio of about 1.6 to 1.75 (172). Whereas the peak III shows a higher A_{260}. The data also indicate that the inhibitor is present in all the three peaks showing its affinity for the components of all the three peaks and suggesting the difficulty in removing the inhibitor from the protein/macromolecule. In addition, it is interesting to observe that the earthworm enzyme preparation which has been purified by DEAE-cellulose chromatography and dialysis is still contaminated with low molecular weight compounds. This observation suggests that during gel filtration, the inhibitor which is a low molecular weight compound adsorbed to a macromolecule may be released very slowly from the macromolecule which accounts for the third peak. The high affinity of the inhibitor for various macromolecules and the difficulty involved in removing the inhibitor from the enzyme preparation except by heat denaturation posed a problem of obtaining an active aminoacyl-tRNA synthetase preparation. Hence, attempts were first made to characterise the inhibitor, which will help in designing methods to purify the enzyme. Some preliminary results are available in this direction.

.12. Preliminary data on the inhibitor(s) present in earthworm enzyme preparation (Fraction C)

4.12.1. Solubility (Table 6)

Table 6 shows the solubility of Fraction C in different organic solvents. Since the amount of the inhibitor(s) present in Fraction C was too low to see any visible residue on evaporation, the procedure given under legend to Table 6 was used to ascertain the solubility. It is clear from the table that it is insoluble in ether, chloroform etc., and is soluble only in highly

TABLE - 6

SOLUBILITY OF INHIBITOR (FRACTION C)

S.No.	SOLVENT	SOLUBILITY
1.	Ether	Insoluble
2.	Chloroform	Insoluble
3.	Ethanol	Soluble
4.	Methanol	Soluble
5.	Water	Soluble

1 ml of Fraction C was evaporated to dryness. 1 ml of the solvent was added and the contents were stirred well and centrifuged at 10,000 g for 10 min. The supernatant was removed, evaporated to dryness and taken in 1 ml of water and checked for inhibition in the 'mixed enzyme assay' (2.4.2.)

polar solvents like ethanol, methanol and water, suggesting that the compound is highly polar in nature.

4.12.2. Chromatography on Dowex-50 H^+ (Figure 22)

Fraction C was subjected to Dowex-50 (H^+) chromatography to identify the nature of charge present on the molecule. It is obvious from the data that Fraction C is held on the Dowex-50(H^+) column at acidic pH since the effluent does not inhibit the acylation reaction. But the inhibitor(s) is eluted with 1N NH_4OH as shown by the inhibitory activity of the eluate. This data indicate that there is a net positive charge on the compound(s) (Fraction C).

4.12.3. Absorption spectrum (Figure 23)

Figure 23 shows the UV absorption spectrum of Fraction C against a water blank. It shows two characteristic absorption peaks one in the region of 210-215 nm and the other at 260-270 nm. There is no clear trough although the minimum absorption falls between 245-250 nm.

4.12.4. Ninhydrin reaction

Since Fraction C gave an absorption peak at 210-215 nm region which is generally observed for peptides (210 nm), its reaction with ninhydrin was checked. It developed a deep blue colour showing the presence of primary amino group containing compound(s). It also gave a deep pink color when analysed for peptides with copper-ninhydrin reagent (173) indicating the presence of peptides.

4.12.5. Paper chromatography of Fraction C (Figure 24)

Fraction C, as mentioned earlier, gave a positive reaction with nin-

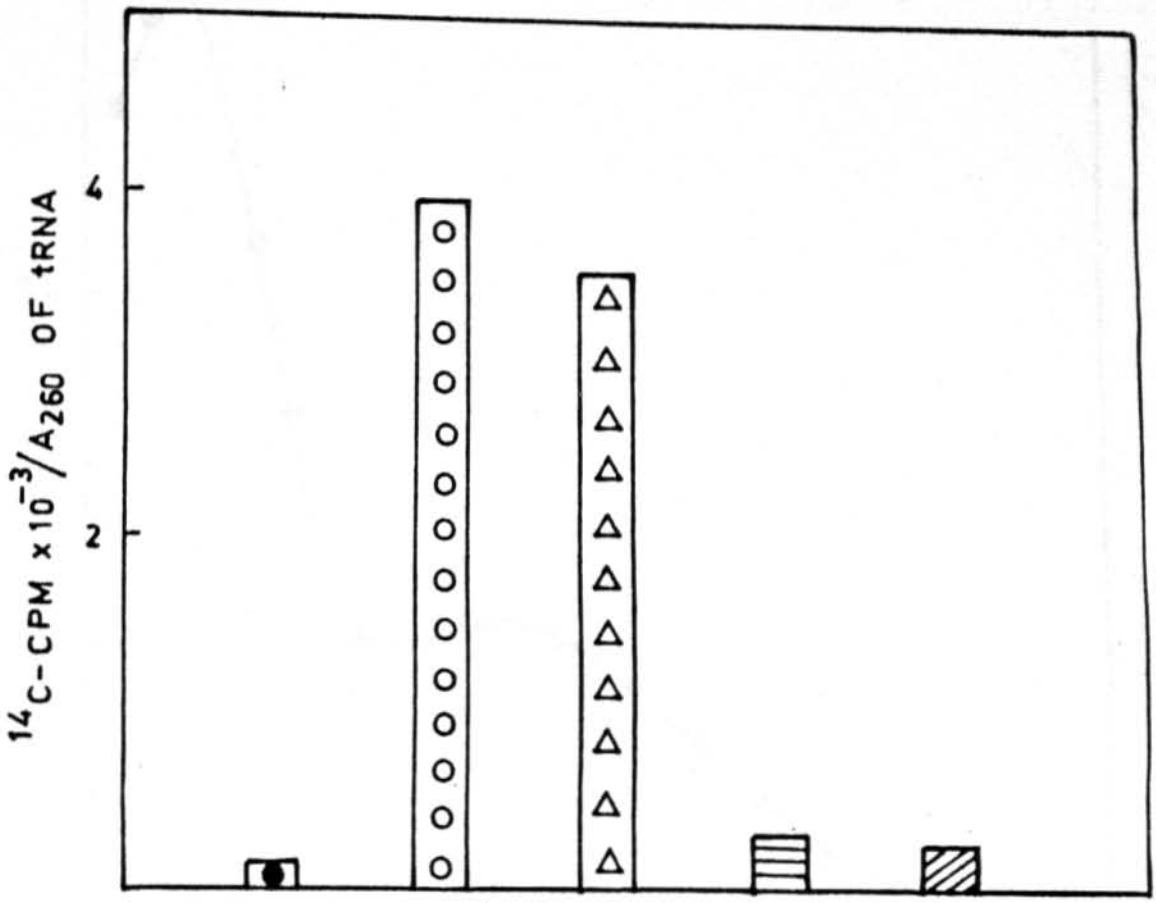


FIG.22. INHIBITORY ACTIVITIES OF DOWEX-50(H^+) COLUMN FRACTIONS.

Legend: Dowex-50 (H^+) column chromatography of fraction C was carried out as described under methods (2.5.2). The aminoacylation and 'mixed enzyme assay' were carried out as described under materials and methods. The reaction was carried out at 37°C for 25 min and the acid precipitable counts were estimated.

- (▣) - represents blank i.e., without tRNA.
- (▣) - represents aminoacylation of rat liver tRNA with rat liver enzyme.
- (▣) - aminoacylation of rat liver tRNA with rat liver enzyme in presence of Dowex-50 (H^+) effluent.
- (▣) - aminoacylation of rat liver tRNA with rat liver enzyme in presence of Dowex-50 (H^+) eluate.
- (▣) - aminoacylation of rat liver tRNA in presence of earthworm fraction C.

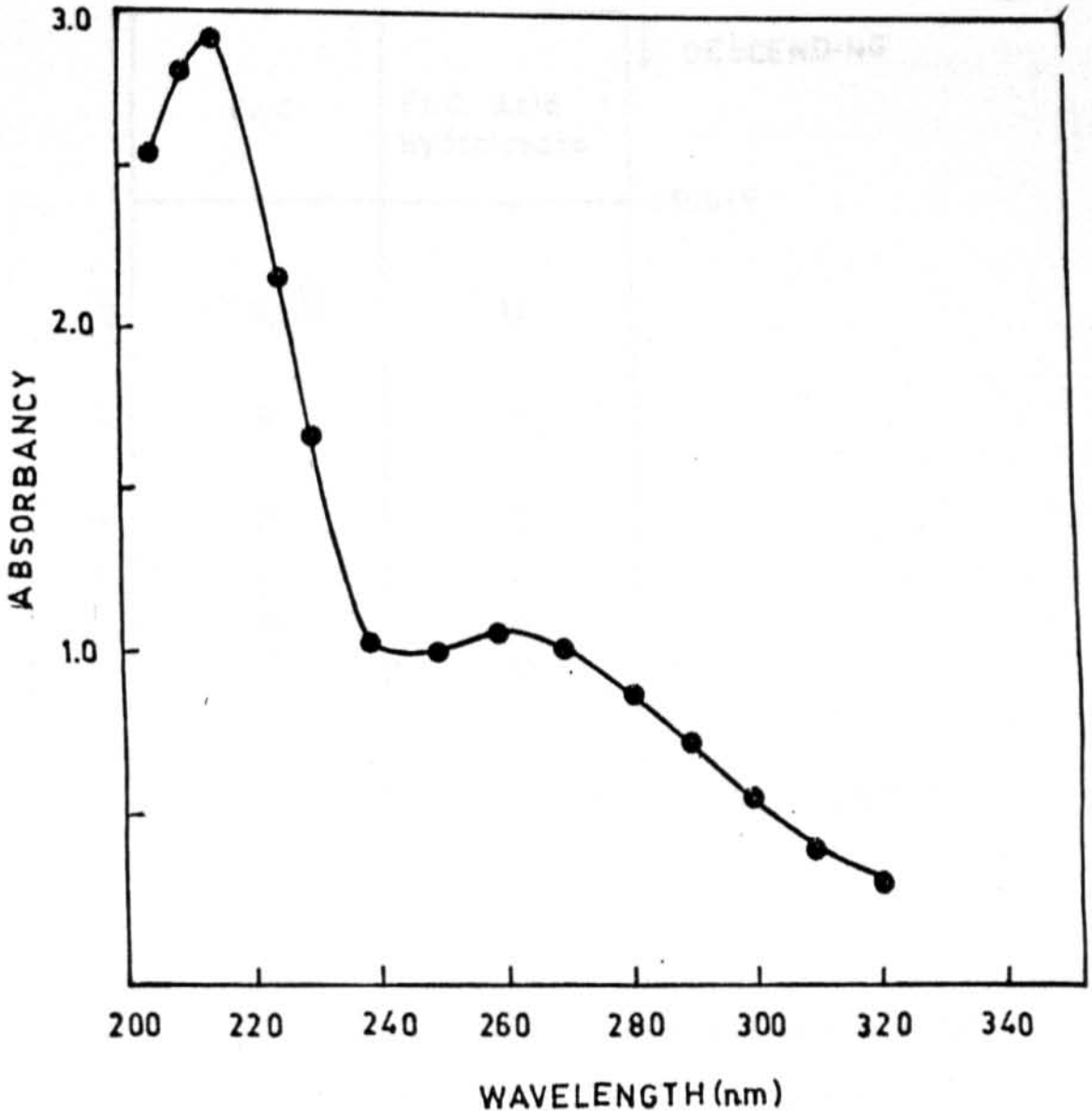


FIG.23. ABSORPTION SPECTRUM OF FRACTION C FROM EARTHWORM.

Legend: Fraction C from earthworm was prepared as described under materials and methods (2.3.3, Chart 3). The UV absorption spectrum was recorded in a Gilford spectrophotometer against double distilled water.

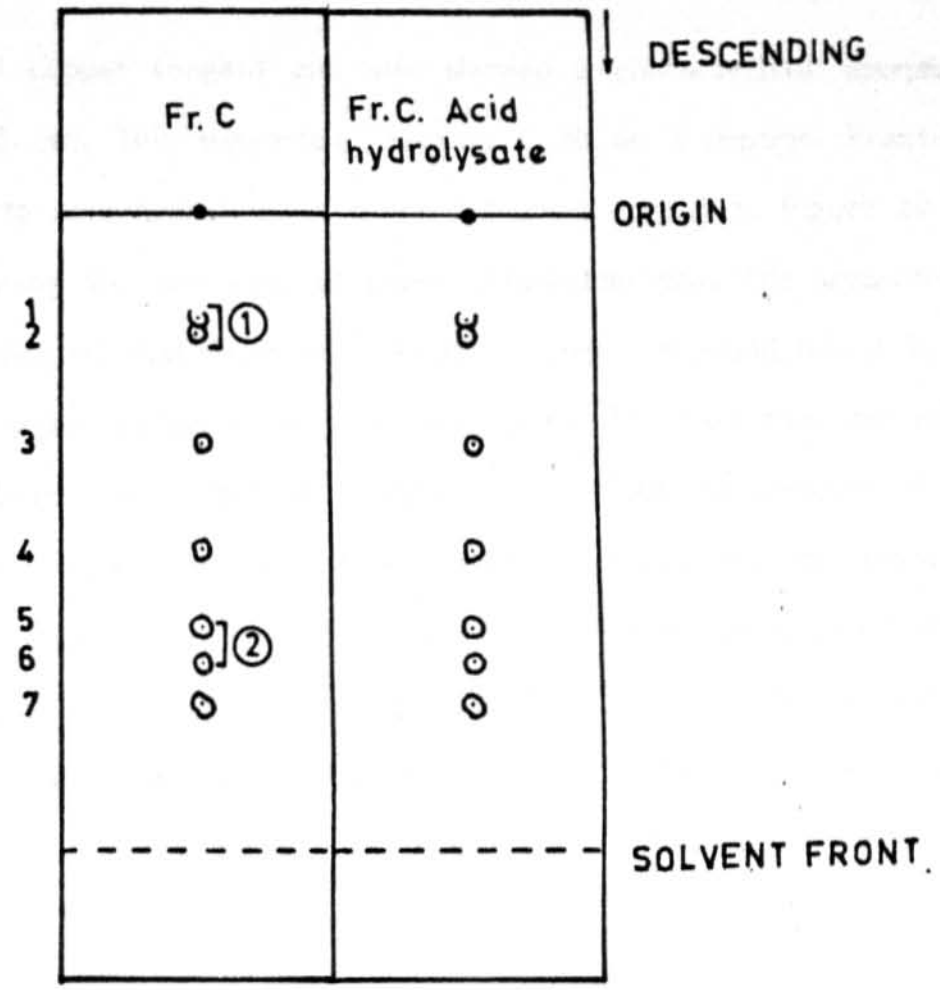


FIG. 24. DESCENDING PAPERCHROMATOGRAPHY OF EARTHWORM FRACTION C (DIAGRAMATIC REPRESENTATION)

Legend: Fraction C was prepared from earthworm as described under materials and methods (2.3.3. Chart 3). It was subjected to acid hydrolysis with 6N HCl, at 110°C under vacuum for 16 hrs. The acid was removed by repeated evaporation. 100 λ of fraction C or the acid hydrolysate taken in water was applied on a Whatman No-1 chromatographic sheet. The chromatogram was developed for 17 hrs, in a solvent system containing butanol, acetic acid and water (4:1:2). The spots were identified by ninhydrin in 80% ethanol, containing 1 ml of pyridine.

hydrin and copper reagent and also showed a characteristic absorption peak at 210-215 nm. This suggested Fraction C to be a peptide. Fraction C was subjected to acid hydrolysis as described under legend to Figure 24 and fractionated using the technique of paper chromatography. The separation pattern clearly indicated that Fraction C is not a pure compound but it is a mixture of about seven ninhydrin positive compounds. The fact that the pattern was identical before and after acid hydrolysis ruled out the presence of a peptide. The inhibitor activities of different spots were checked and found that only two zones, marked (1) and (2) in the figure, showed inhibition (50% and 60% respectively). These results suggest that Fraction C is not a pure compound and is a mixture of at least seven ninhydrin positive compounds all of which are not inhibitory. At present, it is not clear whether the ninhydrin positive compound and the inhibitor are one and the same.

4.12.6. Competition of inhibitor(s) with tRNA (Figure 25)

Since the inhibitor(s) was found to be a general one rather than a specific substrate analogue it was of interest to ascertain its affinity to the different substrate molecules. The aminoacylation reaction mixture contains all components in saturating concentrations except the tRNA which is in limiting concentrations. Hence the possibility that the inhibitor could be binding to the tRNA and making it unavailable for acylation was checked. Figure 27 shows the competition of the inhibitor with increasing amounts of tRNA. It is evident from the data that increasing the concentration of tRNA from 2.0 to 100 A_{260} units relieves the inhibition proportionately and a 50-fold excess of tRNA in the assay completely removed the inhibition. The activity obtained with 100 A_{260} units of tRNA in the presence of the inhibitor is equal to the activity obtained with 2 A_{260} units of tRNA in the absence

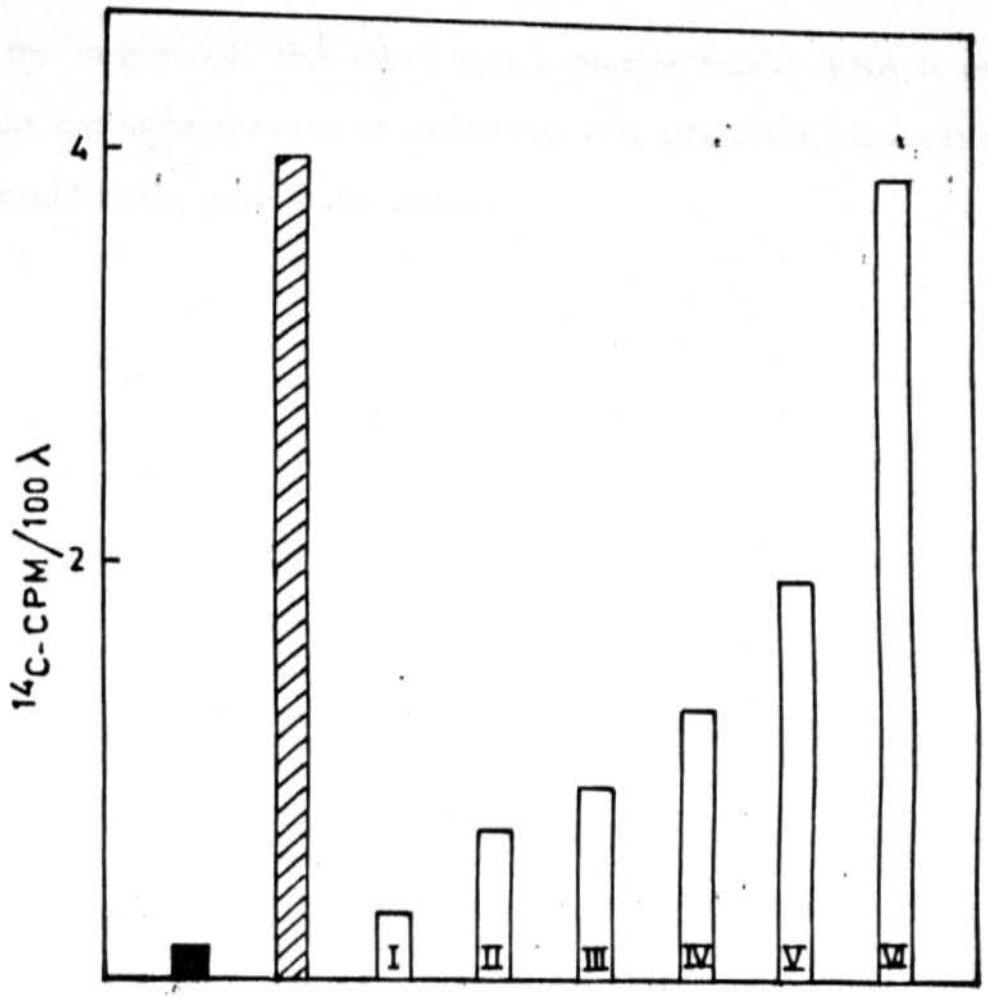


FIG.25. EFFECT OF INCREASING t-RNA CONCENTRATION ON THE INHIBITION BY FRACTION C. (tRNA COMPETETION).

Legend: Aminoacylation reaction was carried out as mentioned under materials and methods (2.4.1), using E. coli tRNA and rat liver aminoacyl-tRNA synthetase. ^{14}C algal protein hydrolysate was used as the source of total amino acids. (■) represents blank with no. tRNA (▨)- control with 2.0 A_{260} units of E. coli tRNA and rat liver aminoacyl-tRNA synthetase. The assay for inhibitor was carried out as mentioned, with varied amounts of E.coli tRNA; 2.0 (I), 5(II), 10 (III), 20 (IV), 40 (V) and 100 (VI) A_{260} units. The reaction was carried out at 37°C for 25 min. 100 λ aliquots were taken and the acid precipitable counts were estimated.

of the inhibitor(s). This shows that a 50-fold excess tRNA is needed to bring about complete removal of inhibition. This interesting observation is discussed in detail in the general discussion.

CHAPTER - V

GENERAL DISCUSSION AND CONCLUDING REMARKS

Aminoacylation is the first and most important step in protein biosynthesis, where the amino acids are activated by acylation to tRNA, a reaction catalyzed by the enzyme aminoacyl-tRNA synthetase (17). In prokaryotes with short lived messenger RNAs, gene expression and protein synthesis are probably controlled mainly at the level of transcription. In eukaryotic cells, on the other hand, gene expression is probably controlled not only at transcriptional, but also at translational level. Relatively little information is presently available regarding the translational control of gene expression in eukaryotic cells. it is conceivable that such a control may be exerted by inhibitors and/or activators of protein synthesis. There are reports in recent years describing the presence, in eukaryotic cells, of inhibitors which appear to block either the initiation or elongation steps in protein synthesis (174-178). One of the best understood regulation at translation level is the control of globin synthesis in reticulocytes, by the level of heme, the prosthetic group of hemoglobin (175). Oligonucleotides from eukaryotic cell extracts have been implicated in translational control. Bogdonsky et al (179) reported that the activity of an initiation factor containing rabbit reticulocyte ribosomal salt wash, lost upon dialysis, was largely restored by oligonucleotides from dialysate.Adelman et al (180) described an inhibitor of poly (U) directed polyphenylalanine synthesis in the high speed supernatant fraction of zoospore cytoplasm. Ribosomes isolated with 0.01M KCl-buffer from ungerminated spores are inactive in poly (U) directed Poly(phe) synthesis. The non-functional zoospore nuclear cap ribosomes became active when washed with 0.5M KCl-buffer. This inhibition was shown to be due to a low molecular weight compound but not due to a protein on nucleic acid. The presence of such a translation inhibitor suggested that this substance could function in significant biochemical control mechanisms such as the deactivation and subsequent protection of part of ribosomal pool from degradation prior to and during nuclear cap formation.

It is proposed that the inhibitor is produced in response to the same environmental trigger which induces plants to differentiate into sporangia to produce zoospores. Metafora et al (181) have shown that salt washing of sea urchin egg ribosomes activated them for in vitro protein synthesis and liberated an inhibitor of protein synthesis which was shown to be a protein. Bacon et al (182) described a self inhibitor of Dictiostelium discodium spores and have shown it to be N,N'-dimethyl guanosine which blocks protein synthesis in vitro probably by acting as a specific inhibitor for GTP. Lee-Huang et al (183) described the presence of both an activator and an inhibitor oligonucleotides in the dormant and developing embryos of Artemia salina embryos, the coordination of which controls the dormancy and development of these embryos.

The inhibitors described in the literature primarily act at the ribosomal level of translation. Very little information is available regarding the control mechanisms that operate at the very first step of protein synthesis i.e., aminoacylation. It is rather logical to think that regulation, at this stage itself, would be beneficial to the organism because of the high energy involved in the formation of the aminoacyl-tRNA (142). Activation of the peptide chain elongation reaction in the uterine ribosomes of ovariectomized immature rats is hormone specific. Estradiol seems to exert this effect by removing a ribosome-associated protein factor which inhibits the reaction (153). This heat-labile protein factor induces certain modifications in tRNA making them unable to bind to aminoacyl-tRNA synthetase and thus inhibiting aminoacylation. Malathi et al (184) have shown the presence of a heat stable, low molecular weight inhibitor in the cytoplasmic extracts of 3T3 and SV 101 cells. This inhibitor primarily acts at the level of tRNA charging, and the synthesis of phe-tRNA and lys-tRNA have been shown to be impaired in its presence.

During our studies on the aminoacylation in a few selected species of invertebrates, it was noticed that this reaction could not be demonstrated at all in earthworm and cockroach using standardized procedures. Subsequently, this was traced to the presence of an endogenous inhibitor(s) in the enzyme preparations from earthworm and cockroach using a mixed enzyme assay (2.4.2). More detailed work was restricted to earthworm in the further investigation on the nature of the inhibition.

The endogenous inhibitor(s) of aminoacylation present in earthworm and cockroach is not specific for any particular amino acid, tRNA or aminoacyl-tRNA synthetase, since the presence of this inhibitor completely knocks off the total aminoacylation. The inhibitory effect exerted by the inhibitor is not due to product (pyrophosphate) inhibition (105) since the effect is seen equally well on homologous and heterologous charging systems. Preliminary results from our laboratory have shown that incubation of inhibitor fraction with rat liver aminoacyl-tRNA synthetase, followed by dialysis, does not cause any significant decrease in the activity of the enzyme, suggesting that the inhibitor may not bind directly to enzyme. The very interesting observation that the inhibition is competed out by increasing concentrations of the tRNA makes one to speculate whether the inhibitor actually binds to the tRNA at a crucial site needed for synthetase recognition and knocking off the total aminoacylation activity. Thus, the primary action of the inhibitor seems to be on the tRNA rather than the enzyme. The inhibitor could bring about this action either by binding to the common C_pC_pA sequence at the 3' end of the tRNA or it may bind to a crucial nucleotide sequence on the inside the L of the tRNA needed for synthetase recognition in general.

The molecular sieve chromatography of the earthworm enzyme

(Figure 21) on Sephadex G-100 clearly indicates the heterogeneity in size. This may be due to either of the following reasons. It is possible that the aminoacyl-tRNA synthetases from earthworm and cockroach are of heterogeneous molecular weight ranging from about 30,000 to > 100,000. Or it is also possible that the affinity of the inhibitor for the nucleic acids (particularly to tRNA) makes them elute at a low concentration from the DEAE-cellulose column and get fractionated on the Sephadex G-100.

These observations on complete inhibition of aminoacylation in in vitro systems makes one wonder as to what happens under in vivo conditions. If there is complete block of aminoacylation how does the organism survive and carry out its essential, basic protein synthesis. It is possible that under in vivo conditions the inhibitor is compartmentalized and does not interfere with normal protein synthesis and is released from the organelle only when its presence is needed for regulation of protein synthesis. Preliminary results obtained with subcellular fractionation studies failed to localize the inhibitor in any particular organelle. The inhibitor was distributed in several/all fractions indicating that this could be an artifact arising out of homogenization. For the purpose of regulation in vivo it is not essential to have physical compartmentalization, but specific association with macromolecules and release of the inhibitor to suit the cellular requirements would offer an adequate mode of control. Whatever be the mechanism by which the inhibitor is maintained in in vivo conditions, under in vitro conditions it seems to have a strong affinity for macromolecules (most probably nucleic acids) and released from them only on drastic treatments like heat denaturation.

Since the presence of this inhibitor(s) has been noted only in representatives of the phyla annelida and arthropoda and not in mollusca, it would

be of great interest to see at what stage during evolution, the presence of such an inhibitor came into existence and whether it is present in still lower invertebrates like nematodes, planarians etc.

In view of the small quantities of the inhibitor fraction available, a rigorous chemical characterization was not possible in the present work, but obviously an inhibitor at the level of translation and at the very first step would be of great interest for further research which would include chemical characterization. Further studies on the mechanism of action of this inhibitor would make it a good system to study the regulation of protein synthesis at translational level and the inhibitor would enable us to understand the mechanism of tRNA-synthetase recognition. This is currently being undertaken in our laboratory by preparing the inhibitor in large quantities and characterizing it using various physical techniques.

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