STUDIES ON THE LOW MOLECULAR WEIGHT RNA
BOUND TO E. COLI RIBOSOMES

by

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We accept this thesis as conforming to the required
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ABSTRACT

Ribosomes were readily prepared by various procedures from E. coli B cells grown to the mid-log or late log phase. The ability of these ribosomes to support protein synthesis varied with the method of preparation. The low molecular weight RNA (LMWRNA) bound to these ribosomes was studied and it, too, varied with the type of preparation. After these initial studies, the procedure which entailed seven extractions with 0.5-1.0 M NH₄Cl in the presence of varying levels of magnesium concentration was used in all further experiments for the preparation of ribosomes (WRib).

Attempts were made to remove all the tRNA bound to WRib. Treatment with HIO₄ resulted in a complete disruption of ribosomal structure and was abandoned. Puromycin (PM) treatment failed to remove all the bound tRNA but resulted in a more active preparation. Incubation of these PM-treated WRib with 0.1 mM Mg⁺⁺ not only removed all the bound tRNA but also resulted in inactive preparations. Treatment of the WRib with 0.1 mM Mg⁺⁺ alone gave identical results. Ribosomal subunits were found to be devoid of bound tRNA. Attempts to substitute these subunits in a protein-synthesizing system in place of whole ribosomes (WRib) failed.

The LMWRNA bound to WRib was fractionated by a number of techniques - Sephadex G-100 chromatography, DEAE-Sephadex A-50, and preparative gel electrophoresis. The optical density patterns of the fractionations showed only small differences, however, acrylamide gel electrophoresis studies
of the peak fractions indicated that chromatography on Sephadex G-100 was the method of choice for the separation of LMW-RNA.

The tRNA bound to WRib was fully characterized. *E. coli* WRib have bound tRNA which has acceptor activity for all the amino acids tested. The amount of charging varied from one tRNA species to another - those for tryptophan and methionine were bound in the largest amounts. The significance of these results cannot, as yet, be explained. The total amount of tRNA bound to WRib amounted to approximately 1.3-1.7% of the total ribosomal RNA (rRNA). Similarly the amount of 5S RNA bound represented 1.5-2.8% of the total rRNA. These percentages are equivalent to 0.9-1.3 molecules of tRNA bound per molecule of 5S RNA. Another species of rRNA, 4.5S RNA was found bound to the WRib but in small amounts. Most of the 5S RNA preparations contained this RNA.

The tRNA bound to WRib, prepared from a pyrimidine-requiring mutant (ATCC13135) grown in the presence of $^3$H-uracil, was exchanged with unlabeled tRNA. After exchange 5.7 molecules of tRNA were bound per molecule of 5S RNA and virtually all of the labeled tRNA had been removed. These exchanged WRib were no longer active in a protein-synthesizing system. The tRNA from these WRib was fractionated on a BD-cellulose column. The radioactivity was evenly spread throughout the salt gradient but a peak was isolated in the ethanol gradient. The radioactivity in this peak region may be accounted for by the presence of 4.5S RNA. The results
suggest that all tRNAs are bound to the WRib to the same degree and indirectly supports the results reported in the literature of the non-existence of a specific chain-terminating tRNA.
ACKNOWLEDGEMENTS

I wish to thank my research supervisor Dr. Gordon M. Tener for enthusiastic support, valuable suggestions and discussions during the course of this work. My associations with the members of this laboratory have been most helpful and I wish to acknowledge the debt I owe to them.
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# ABBREVIATIONS

All abbreviations and terminology used in this thesis are in accordance with those normally accepted by the *J. Biol. Chem.*, 246, 4 (1971) and only those abbreviations not listed in this journal are shown below.

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<tr>
<td>fMet</td>
<td>formylmethionine</td>
</tr>
<tr>
<td>aa-tRNA</td>
<td>Aminoacyl-tRNA</td>
</tr>
<tr>
<td>tRNA&lt;sub&gt;Met&lt;/sub&gt; m or f</td>
<td>A specific member of the group of tRNAs which accept methionine.</td>
</tr>
<tr>
<td>DF</td>
<td>Dissociation Factor</td>
</tr>
<tr>
<td>R factor</td>
<td>Release Factor</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>WRib</td>
<td>Washed Ribosomes</td>
</tr>
<tr>
<td>PM</td>
<td>Puromycin</td>
</tr>
<tr>
<td>ATA</td>
<td>Aurintricarboxylic Acid</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>dimethyl-POPOP</td>
<td>1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene</td>
</tr>
<tr>
<td>DMAPN</td>
<td>3'-dimethylaminopropionitrile</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>A&lt;sub&gt;260&lt;/sub&gt;</td>
<td>Absorbance at 260 nm</td>
</tr>
<tr>
<td>A&lt;sub&gt;260&lt;/sub&gt; unit</td>
<td>One A&lt;sub&gt;260&lt;/sub&gt; unit is that amount of material which when dissolved in one ml of solvent will give an absorbance of one in a cell with a light path of 1 cm.</td>
</tr>
<tr>
<td>Bis</td>
<td>N,N'-Methylenebisacrylamide</td>
</tr>
<tr>
<td>Mg(OAc)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>magnesium acetate</td>
</tr>
<tr>
<td>BD-cellulose</td>
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### LIST OF BUFFERS

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<td>10mM Tris, 10mM Mg(OAc)$_2$, 10mM NH$_4$Cl, 10mM mercapto-ethanol, pH 7.8</td>
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<td>10mM Tris, 0.1mM Mg(OAc)$_2$, 0.5-1.0M NH$_4$Cl, pH 7.4</td>
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</tr>
<tr>
<td><strong>G</strong></td>
<td>10mM Tris, 0.2M KCl, pH 7.4</td>
</tr>
<tr>
<td><strong>H</strong></td>
<td>20mM Tris, 10mM cysteine, pH 7.4</td>
</tr>
<tr>
<td><strong>I</strong></td>
<td>10mM Tris, 0.1mM Mg(OAc)$_2$, pH 7.6</td>
</tr>
<tr>
<td><strong>K</strong></td>
<td>5mM Tris, 0.1mM Mg(OAc)$_2$, pH 7.3</td>
</tr>
<tr>
<td><strong>L</strong></td>
<td>10mM Tris, 10mM Mg(OAc)$_2$, 0.5% sodium dodecyl sulfate, pH 7.6</td>
</tr>
<tr>
<td><strong>M</strong></td>
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</tr>
<tr>
<td><strong>N</strong></td>
<td>10mM Tris, 10mM Mg(OAc)$_2$, 1.0M NH$_4$Cl, pH 7.8</td>
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DEDICATION

To my parents who instilled me with the drive and incentive, to my dearest Bev who gave me encouragement, confidence and love and to Stephanie for priority lessons.
INTRODUCTION

The amino acid sequence of a particular protein is specified by the sequence of nucleotides in a particular segment of the deoxyribonucleic acid (DNA). In the transcription step the DNA is transcribed into a ribonucleic acid (RNA) intermediate called messenger RNA (mRNA), which has a ribonucleotide sequence complementary to that of the deoxyribonucleotide sequence of one of the strands of the DNA serving as template (64). The translation steps follows. The mRNA becomes attached to cytoplasmic ribonucleoprotein particles (ribosomes) which are the sites of protein synthesis, and there it determines the order of linkage of amino acids into a specific protein (65-67). During translation a group of three adjacent nucleotides in the mRNA (codon) specifies which amino acid is to be linked to the growing peptide chain. There are 64 possible codon triplets and 61 of these specify a particular amino acid (68). The other three codons, UAA, UAG and UGA are called nonsense codons since they act as chain terminators which signal the end of a genetic message. Each amino acid is joined specifically to one of the codon-recognizing molecules known as transfer RNA (tRNA). Each tRNA molecule has its own triplet of bases, called an anticodon, that recognizes the relevant codon on the mRNA by pairing bases with it.

The Ribosome

Zamecnik and his co-workers established the central role
of ribosomes in protein synthesis (although earlier cytological studies had shown ribosomes to be the site of protein synthesis), and in addition, discovered most of the components involved in in vitro protein synthesizing systems, such as tRNAs and aminoacyl-tRNA synthetases (1). However, mRNA had not been discovered and it was thought that ribosomal RNAs (rRNAs) were the templates for the proteins synthesized on the ribosomes. Thus it was hoped that studies on the structure of ribosomes and rRNA would give some clue as to the mechanism of information transfer from genes to proteins. About 1957 the first systematic studies on the isolation and characterization of ribosomes were initiated (2-5). These studies were done on ribosomes from E. coli and established the following basic information: (a) Ribosomes isolated and purified in the presence of 10 mM Mg$^{++}$ have a sedimentation coefficient of 70S. After treatment with puromycin which removes nascent peptides, these ribosomes dissociate into 30S and 50S subunits upon lowering Mg$^{++}$ concentration (1 mM or less) (2,6); however, if nascent peptide is present, complete removal of Mg$^{++}$ with EDTA is required (69). (b) The 50S and 30S ribosomal subunits have a particle weight of 1.8 x 10$^6$ and 0.85 x 10$^6$ daltons respectively (6). (c) Both 50S and 30S subunits contain about 2/3 RNA and 1/3 protein. (d) The 50S subunit contains one molecule of RNA, 23S RNA (The presence of one molecule of 5S RNA was discovered later (55)), and the 30S subunit contains one molecule of RNA, 16S RNA (7).
Subsequent progress in the study of ribosomes, however, lagged far behind progress in other areas of molecular biology. This was partly due to the discovery of mRNA which diverted attention from ribosomes, and partly due to the difficulties caused by the structural complexity of ribosomes. For example, 30S subunits contain about 20 different proteins and 50S subunits contain about 30 to 35 different proteins. However, it was soon evident that the detailed mechanism of protein synthesis could not be elucidated without knowledge of the structure and function of ribosomes. Moreover, an active role for ribosomes in the codon-anticodon recognition process was suggested (8). Thus serious interest in the ribosome was revived and the last several years have witnessed rapid progress in the study of them.

Ribosomal RNA

Current evidence suggests that the 30S ribosomal subunit contains one molecule of 16S RNA with a molecular weight of $5.5 \times 10^5$ while the 50S ribosomal subunit contains one molecule of 23S RNA with a molecular weight of $1.1 \times 10^6$ and one molecule of 5S RNA with a molecular weight of $4 \times 10^4$ (7, 42, 44). Since the molecular weight of 23S RNA is about twice that of the 16S RNA, there have been frequent claims that the 23S RNA is a dimer of a "16S" RNA molecule which is identical or very similar to the 16S RNA molecule found in 30S ribosomal subunits. Several observations originally supported this idea (7, 45-47).
One can now exclude this claim. The 23S and 16S rRNAs have been shown to be different in base composition (44), base sequence as judged by DNA-RNA hybridization (48), oligonucleotide patterns obtained after enzymatic digestion (49), methylated oligonucleotides obtained after T₁ RNase digestion (47) and in their 5'-terminal sequences (50). It was also found that the "16S" RNA prepared from 23S RNA according to the method of Midgley (45) could not replace 16S rRNA in the reconstitution of 30S ribosomal subunits. Thus, it was unlikely that the 23S rRNA was formed by simple dimerization of two 16S rRNA molecules.

The claim that the 50S ribosomal subunit contains two RNA chains is also difficult to accept. First, the conversion of 23S RNA into smaller RNAs which had been claimed by several workers could not be observed under conditions minimizing nuclease contamination (51). Second, careful studies done by Stanley and Bock (127) revealed no non-covalent bond in the 23S rRNA molecule. Finally Leppla (52) measured the number of chain terminal bases in the 23S rRNA molecule and obtained results which were consistent with one chain terminus per 23S rRNA. It therefore appears that the 23S rRNA is a single polynucleotide chain with a molecular weight of \(1.1 \times 10^6\).

Although the 23S rRNA is a single polynucleotide chain, the work of Fellner and Sanger (47) strongly indicated that the molecule was made up of two sections which may be identical or very similar in their base sequence. One of their
suggestions was that the 23S rRNA cistron had arisen by a "gene duplication" mechanism during evolution. Whether the possible existence of two identical or similar parts is related to the functions of 23S rRNA (53) is not clear. A related subject is the problem of sequence homology between 16S and 23S rRNAs. Despite clear-cut evidence for a sequence difference between *E. coli* 16S and 23S rRNAs, DNA-RNA hybridization experiments have shown that 16S and 23S rRNAs compete for the same DNA sites to a great extent (54). This suggests that DNA cistrons for both 16S and 23S RNA have evolved by gene duplication starting from a common gene. Alternatively, partial sequence homology may reflect a common (unknown) function performed by parts of both 16S and 23S RNAs. On the other hand, DNA-RNA hybridization experiments done with *Bacillus megaterium* and *Bacillus subtilis* showed a complete lack of sequence homology (48). It is not clear if the observed discrepancy was due to a difference in techniques used or to the difference in bacterial species.

Maturation of rRNAs in prokaryotes and eukaryotes has a common feature; the process seems to involve the cleavage of precursor molecules larger than the mature rRNAs but there are differences. In eukaryotes the two rRNAs are produced by splitting a single large precursor molecule whereas in bacteria the two rRNAs are derived from two discrete precursors each only slightly larger than the mature species (206).
The 50S ribosomal subunit contains one molecule of 5S RNA in addition to 23S rRNA (55). The 5S RNA does not accept amino acids and is thus different from tRNA. It is not a random breakdown product of 16S or 23S rRNA but appears to be a genuine ribosomal component present in all 50S subunits of various origins. To date little, if any is known of the functional role of 5S RNA although Kirtikar and Kaji (128) have shown that the addition of 5S RNA stimulated incorporation of amino acids into protein directed by RNA from phage MS-2. Siddiqui and Hosokawa (131) have suggested that 5S rRNA may have a role in the specific binding of tRNA to ribosomes. Raacke (132) has proposed a cloverleaf conformation for 5S RNAs. The model proposes three functions for 5S RNA: (1) the binding of 5S RNAs to 50S ribosomes by means of a unique and universal base sequence, (2) the joining of 30S to 50S ribosomes in a species-specific manner, (3) the binding of tRNA through specific base pairing and Mg$^{++}$ bridges.

Recently Jordan (248) has found that the most exposed part of the E. coli 5S RNA (as determined by T$_1$ RNase cleavage) has a sequence complementary to the -GTUC$^A_G$ sequences found in all tRNAs thus far sequenced. Although this would appear to strengthen the case for a function of 5S RNA involving interaction with tRNA in the ribosome, this sequence in tRNA is in the most protected loop. Therefore in order for interaction to occur the tRNA would have to undergo a conformational change which would involve ring opening. Other models have also been proposed (42, 133-135). The 5S RNA from E. coli
consists of 120 nucleotides and lacks methylated or unusual bases in contrast to other rRNAs or tRNAs (42). The nucleotide sequence of 5S RNA from *E. coli* (42) and from mammalian KB cells (129) has been established, and it is interesting to note that a part of the nucleotide sequence is duplicated in each of the two 5S RNA species. Preliminary studies also indicate that the sequence of 5S rRNA from two mouse cell lines is the same as that found in human KB cells (136).

Recently the nucleotide sequence of *Pseudomonas fluorescens* 5S RNA has been elucidated and found to contain many similarities to the *E. coli* 5S RNA (209). No base sequence homology has been found between 5S RNA and 16S or 23S rRNA using the technique of DNA-RNA hybridization (56). Recently Monier's group (130) demonstrated the presence in exponentially growing *E. coli* cells of a precursor of 5S RNA. The precursor was longer by 1-3 nucleotides. A similar precursor was synthesized in the presence of chloramphenicol (247).

**Heterogeneity of Ribosomal RNA**

DNA-RNA hybridization experiments have clearly shown that genes for 16S and 23S rRNA are present in multiple copies (137-140). For instance, Spadari and Ritossa (137) confirmed the existence of 6 genes for 23S rRNA and 6 for 16S rRNA for each *E. coli* chromosome. Because of this redundancy, it is quite possible that the genes for 16S rRNA or those for 23S rRNA are not homogeneous and that there are several chemically different species of 16S rRNA and 23S rRNA.
At least three different loci for 5S RNA cistrons have been localized in *E. coli* (141). The question of the possible heterogeneity of rRNAs is important, since it implies the heterogeneity of each of the ribosomal subunits and is possibly related to some functional differentiation among ribosomes.

On the other hand, the sequence analysis of methylated oligonucleotides of rRNA done by Fellner and Sanger (47) showed that many long oligonucleotides with unique base sequences and chain lengths up to eleven occurred in one, two or four moles per mole of RNA, and never in an amount less than one mole. Thus, both 16S and 23S rRNA from *E. coli* are largely homogenous, at least with respect to the base sequences around methylated nucleotides. Further extensive analysis of fragments of 16S rRNA performed by Fellner et al. strongly indicated homogeneity of 16S rRNA (58, 142). Completion of such base sequence analysis will undoubtedly give a more convincing answer to the question of rRNA heterogeneity. It should be noted that the base sequence of 5S RNA from *E. coli* has proved the homogeneity of this RNA species (42), despite the multiplicity of cistrons for this RNA in the bacterial genome (48).

**Ribosomal Proteins**

It appears that the 30S ribosomal subunit of *E. coli* contains about 21 proteins. This has been confirmed by both Kurland's (33, 210) and Nomura's groups (38). The average
molecular weight is 12,400 per protein.

Traut and his co-workers (34) isolated 36 proteins from the 50S ribosomal subunits of *E. coli* and concluded that the number of 50S proteins could be between 34 and 38. Kurland and his co-workers concluded that the number of 50S proteins is between 25 and 31 with an average molecular weight of 15,200. Kaltschmidt and Wittman found as many as 34 ribosomal proteins on the 50S subunit (123). It is significant to note that there is no protein common to both 50S and 30S particles. This conclusion was obtained by both chemical and immunological studies (33-36, 121-122, 124-126). The results favour the conclusion that there is no extensive structural homology among ribosomal proteins (rProteins).

**Stoichiometry of Ribosomal Proteins**

It has usually been assumed that the ribosome has a defined structure and that the ribosome population is homogeneous. The heterogeneity of rRNA and hence of ribosome populations was suggested by several investigators, but it is only now that Kurland's group has performed careful studies on the stoichiometry of ribosomal proteins that the question has been brought to serious consideration (210).

Thus, when the first experimental studies on this problem by Moore's group (35) showed that most (thirteen) of the ribosomal proteins existed in amounts corresponding to one copy per 30S particle, the conclusion was readily accepted that the ribosomes were homogeneous with respect to their
protein composition. However, later work by Kurland et al. (36) did not agree with this conclusion. They examined 21 ribosomal proteins and found that 12 of them were present in amounts corresponding to about one copy per 30S particle, but that 8 other proteins were present in amounts less than 0.7 copies per ribosome. There was no evidence which suggested that any 30S protein was present in amounts corresponding to more than 1 copy per ribosome. They concluded that there are two kinds of proteins; one which they called "unit protein" is present in all the isolated 30S ribosomal particles and the other which they called "fractional protein" is present in some but not all the isolated 30S particles. Discrepancies between Kurland's and Moore's laboratories were mostly in the molecular weight values assigned to some of the proteins. Although work done by another group also favored the conclusion that all the ribosomal proteins existed in stoichiometric amounts (37), it now appears that the conclusion obtained by Kurland's group is correct, at least with respect to ribosomes obtained as in vitro preparations. Recent investigations of molecular weights of 30S ribosomal proteins by Moore's group has now yielded data consistent with that obtained by Kurland and his collaborators (34). There are several other facts supporting the conclusion of a heterogeneous population of ribosomes. First, there is a striking correlation between proteins classified as unit proteins by Kurland et al. and proteins found by Nomura's group to be required for the
"physical assembly" of ribosomes. Such correlation is consistent with the mechanism of the ordered assembly of 30S particles (38, 211). Second, Kurland and his co-workers were able to show as much as 60% stimulation of activity of 30S particles by incubating them with externally added 30S ribosomal proteins under the conditions optimum for reconstitution. Concomitant with this stimulation, they observed that some externally added proteins were incorporated into the particles and some proteins initially present in the 30S particles were released into the medium (36). Although interpretation of the observed protein exchanges must await exact identification of these exchanged proteins, the observed facts are consistent with the conclusion that the isolated 30S particles are not fully active and that part of the reason for the inactivity is a deficiency in some ribosomal proteins in some of the 30S particles. An alternative explanation is the steady-state hypothesis of Kurland (210) who postulates a functional cycle in which the complement of fractional proteins on a ribosome changes as a given ribosome proceeds through the different operational modes. One example of such a cycle would entail different sets of fractional proteins associated with the ribosomes during chain initiation, propagation, termination and a rest mode. Some of the proteins which are involved in such a cycle might be required for protein synthesis by all ribosomes.

In contrast to the 30S ribosomal proteins, most of the
50S ribosomal proteins appear to be present in amounts corresponding to one copy per 50S particle. Traut et al. (34) found that 31 of the 50S ribosomal proteins existed in stoichiometric amounts and only 2, or possibly 4, of the 50S ribosomal proteins existed in amounts that were much less than one copy per particle. Kurland's group has also failed to detect any significant heterogeneity of 50S ribosomal subunit populations so far. A major conclusion which has emerged from studies on ribosomal proteins is that, because none of the proteins exists as more than one copy per particle, ribosomal subunits have no symmetry. This means that any model of ribosome function involving structural symmetry, for example, the presence of two or more identical sites on a ribosome, can be discarded.

RNA-Protein Interaction and the Internal Organization of the Ribosome

It is possible to assemble 30S ribosomal subunits from free 16S rRNA and a mixture of about 20 different ribosomal protein molecules (38,60). It was found that the assembly reaction required the presence of a specific RNA. In the absence of rRNA, no particles resembling 30S ribosomal subunits were formed. Furthermore, neither 17S cytoplasmic rRNA from yeast nor "16S" RNA prepared from E. coli 23S rRNA could replace the 16S E. coli rRNA in the reconstitution. With these two RNAs no particle sedimenting at 30S was formed. Rat liver 18S rRNA also could not replace 16S E. coli rRNA in the
reconstitution. These experiments clearly show that the rRNA-ribosomal protein interaction is specific and is important for the overall organization of ribosomal particles. Recently Nomura's group has been able to reconstitute 50S ribosomal subunits of Bacillus stearothermophilus (144). They were able to show a partial requirement for 5S RNA in the reconstitution of functional 50S particles. Their results also indicated specificity of binding between the 23S rRNA and ribosomal proteins.

Nomura's group has also shown that all mutational alterations discovered so far which inhibit 30S ribosomal subunit assembly also inhibit 50S ribosomal subunit assembly, whereas many mutations which abolish 50S assembly do not appear to affect 30S assembly (145-146). They proposed that assembly of 50S ribosomal subunits somehow depended on simultaneous assembly of 30S ribosomal subunits in vivo, while the assembly of 30S particles was independent of 50S assembly.

Experiments to date strongly suggest that single-stranded regions are important in the RNA-protein interaction, but do not exclude the possible additional involvement of helical regions in the interaction (234).

The chemical basis of the specificity demonstrated in the RNA-protein interaction is still a matter of speculation. From the foregoing discussion, it is clear that one cannot make a detailed model of ribosome structure. The topological relationship of the many different molecular components is
unknown. Even the very elementary questions as to which proteins are on the surface and which proteins are buried inside, or which part of the rRNA molecule is exposed on the surface of the ribosome, are not answered. However, it is believed that at least a part of the rRNA is exposed and thus the ribosomal structure is drastically different from the common spherical virus structure in which the RNA is completely protected by an outer protein shell. Miskin et al. (234) suggest that a conformational change in a protein, a local rearrangement of a helical portion of RNA, or an alteration in some protein-RNA association are some of the possibilities that can account for the transition between the active and inactive states of the ribosome (62, 63). All detailed structural models must be considered highly speculative at this time.

**Binding of tRNA to Ribosomes**

(a) Non-Specific Binding of Free tRNA

Both tRNA and aa-tRNA were shown in early studies to attach to ribosomes (148, 251-252). The tRNA-ribosome complex is stable only in solutions of high Mg\(^{++}\) ion concentration. It is still unknown what forces are involved in holding the two components together in a stable complex. Presumably, magnesium bridges are formed not only between subunits but also between the tRNA-ribosome complex. The Mg\(^{++}\) ions tend to overcome the mutual repulsion of the phosphate groups of RNA to allow binding. Bound tRNA will not wash off in high
Mg$^{++}$ ion concentration but can easily be displaced by free tRNA from the surrounding medium (148). This exchange is not affected by charging tRNA with amino acids (249-250), by temperature, or by puromycin or chloromphenicol (148). The nonspecific association of tRNA and ribosomes takes place in the cold and does not require the supernatant enzymes, GTP, ATP or an energy source other than the thermal energy of the reacting components. If the terminal pCpCpA sequence of the tRNA is damaged this binding does not occur. If the terminal adenosine is removed by oxidizing deacylated tRNA with periodate followed by treatment with cyclohexylamine (253), the ability of the tRNA to bind to the ribosomes is destroyed (148). If the terminal sequence is reformed (254) by incubating the tRNA with the supernatant fraction and ATP, or ATP and CTP, the ability to bind is partially re-established (252). The requirement for the intact pCpCpA terminus common to all tRNA molecules, indicates that the terminal sequence plays a decisive role in the binding of tRNA to ribosomes. The binding is specific for 50S ribosomal subunits; 30S subunits do not have any affinity for tRNA in the absence of mRNA. Quantitative studies showed that there is only one binding site per 70S or 50S ribosome (148). According to Cannon et al. (148) this does not eliminate the possibility of several sites, but if there are, either they are not all equivalent, or if they are equivalent they have the property that binding tRNA to any one weakens the binding ability of
the others. They suggest two models for the binding and exchange of tRNA. In the first the ribosome has one site on which the tRNA binds in rapid equilibrium with the tRNA in solution. In the second model the tRNA is tightly bound to a site and the complex dissociates very slowly. The exchange would take place by a second tRNA molecule altering the site and displacing the first. One can consider two equivalent sites for the binding: either site alone can bind the tRNA tightly but if both are full, both tRNA molecules are bound loosely. Such a structure would show rapid exchange associated with the loose binding for the two molecules but only slow loss and tight binding with one tRNA. The basic distinction between the two models lies in the composition of the intermediate state during the exchange process: the intermediate state in the second model is a ribosome and two bound tRNA molecules, in the first model it is a free ribosome.

In the absence of protein synthesis or mRNA, and at 0.1 mM Mg\(^{++}\) ion concentration, the ribosomes are dissociated into their subunits and in the ensuing process all the bound tRNA is completely removed (148). After protein synthesis in a cell-free extract of *E. coli*, a small fraction of the tRNA that is bound to the ribosomes in high magnesium (10 mM) becomes resistant to being washed off the ribosomes in low magnesium (0.1 mM). This amounts to about half a molecule of tRNA per ribosome (148). This has been interpreted as
due to the presence of a nascent polypeptide chain on the tRNA which stabilizes the binding of the tRNA to the ribosome.

Takanami (252, 255) has also observed, using rat liver ribosomes, that the ribosomes will bind tRNA on a roughly one-for-one basis. This binding is magnesium dependent, but after in vitro protein synthesis, some of the bound tRNA remains tightly bound to ribosomes in low magnesium. He has shown that this tightly bound tRNA is not covalently linked to the rRNA but rather is attached to the end of the nascent polypeptide chain.

(b) Non-Enzymatic Specific Binding of Aminoacyl-tRNA

In the presence of template RNA, tRNA attaches to ribosomes with specificity (149) and inhibits the binding of aa-tRNA to ribosomes, presumably by competing with aa-tRNA for ribosomal binding sites (255). Poly U stimulates the binding of both deacylated tRNA\textsuperscript{Phe} and Phe-tRNA to ribosomes (250). At high Mg\textsuperscript{++} ion concentration, tRNA\textsuperscript{Phe} binds to ribosomes with approximately the same affinity, and to the same extent as Phe-tRNA. Since both tRNA and aa-tRNA recognize codons, the ratio of tRNA to aa-tRNA may sometimes regulate the rate of protein synthesis. It has been shown, however, that with addition of a soluble enzyme fraction and GTP, the inhibition of Phe-tRNA binding to ribosomes by tRNA is greatly reduced (250, 256). The presence of ribosomal initiation factors (257) and GTP reduces considerably the inhibition, due to tRNA, of AUG-dependent binding of N-fMet-
tRNA. Specific binding could be shown to occur in solution of high Mg$^{++}$ ion concentration; a binding which was enhanced by K$^+$ or NH$_4^+$ ions (149-152). This finding does not demand a special energy source other than the thermal energy of the reacting components. The inability of the mRNA-ribosome complex to distinguish between free tRNA and its amino acid charged form (149, 153) confirms the adaptor hypothesis (154) and demonstrates that the amino acid per se is not involved in the translation step (153-155). With respect to the binding forces that mediate the mRNA-dependent binding of tRNA to ribosomes, this result, in addition, suggests that the 3'-hydroxyl group of the pCpCpA-terminus has no influence on the stability of the complex (153).

A first hint as to the number of specific tRNA-binding sites on the ribosome came from the discovery that poly U-directed binding of phenylalanine tRNA to 30S ribosomal subunits is stimulated approximately twofold by the present of 50S ribosomal subunits at high Mg$^{++}$ ion concentration and in the absence of protein synthesis (156-158). This finding is consistent with the hypothesis that one molecule of aa-tRNA can bind to the 30S particle (acceptor site) and that the second binding site is generated by formation of the 70S ribosome (donor site).

Aside from the anticodon region, the remaining part of the tRNA-molecule may interact nonspecifically with the ribosome. It is tempting to assume that the 30S subunit of the 70S ribosome has this function. In fact, the ability of
isolated 30S particles to bind aa-tRNA in response to mRNA indicates, besides the specific codon-anticodon relationship, a strong nonspecific interaction between aa-tRNA and the 30S ribosomal subunit (156, 159). Furthermore, the response of the specific aa-tRNA binding reaction to a large array of inhibitors of protein synthesis, some of which act specifically on the 30S ribosomal subunit, provides some evidence for the nonspecific association between the ribosome and a part of the aa-tRNA molecule which comprises base sequences outside of the anticodon region.

Yet, it is very likely that both ribosomal subunits of the 70S ribosome take part in the nonspecific binding. The integration of peptidyl-transferase within the structure of the 50S ribosomal subunit implies the occurrence of two tRNA-binding sites on the 50S particle accessible to all tRNA molecules (160). Further support comes from the observation that antibiotics which act specifically on the 50S ribosomal subunit inhibit the binding reaction (158). Moreover, the aminoacyl ester bond of the aa-tRNA bound to a 70S ribosome in response to mRNA is protected from alkaline hydrolysis (161). Because the presence of the 50S particle is absolutely necessary for the protection of the ester, the tRNA appears to be intimately associated with the 50S particle. The same conclusion was drawn from the observation that aa-tRNA bound to 70S ribosomes in the presence of a template is resistant to digestion by pancreatic RNase; again, the protection occurs
as a result of the association of aa-tRNA with the 50S ribosomal subunit (162). For specific binding of tRNA to 30S ribosomal subunits, the adenosine terminus is not important, but the binding to 70S ribosomes involves both the anticodon region and the terminal adenosine of tRNA (163). In summary, the successful formation of a peptide bond requires the co-operative interaction between all components that participate in this reaction (164).

Studies concerning the substrate specificity at the catalytic center of the 50S ribosomal peptidyl transferase should make possible a more detailed characterization of tRNA-binding sites on the ribosome. In general, the function of peptidyltransferase seems to be favored by specificity at the acceptor site toward substrates with a free α-amino group and by the specificity at the donor site toward substrates with an amido group in that position (165).

There are almost no experimental data on the nature of these nonspecific binding forces. It was found that treatment of 70S ribosomes with proteolytic enzymes under mild conditions abolished the binding capacity of ribosomes for specific tRNAs (166). Their capacity to bind mRNA was not affected by this treatment. Although it leaves the question unanswered whether ribosomal proteins participate directly or indirectly in the binding of tRNA to ribosomes, this result nevertheless provides evidence that the specific tRNA-binding reaction requires the structural integrity of the ribosome. Treatment of ribosomes
with p-dinitrofluorobenzene, that causes loss of the mRNA-directed tRNA binding activity, yields some weak indication that amino-, thiol-, phenol-, or imidazol-groups of ribosomal proteins are involved, directly or indirectly, in the interaction of tRNA with the template-ribosome complex (167). Furthermore, the reaction of ribosomes with diethanoldisulfide destroyed the aa-tRNA binding activity, whereas the association of mRNA with ribosomes modified in such a way proceeded normally (168). The loss of binding activity may be due to the fact that free thiol-groups are necessary to maintain the proper configuration of the ribosomal binding site or that they participate directly in the binding reaction. Since the binding of N-acylated aa-tRNA or peptidyl-tRNA is not affected by sulfhydryl-reagents but only the binding of aa-tRNA, it was concluded, firstly, that the binding site for peptidyl-tRNA is distinct from that for aa-tRNA and, secondly, that only the acceptor site is altered by sulfhydryl reagents. The 30S ribosomal subunit is the major site of inactivation (169). Sulfhydryl reagents do not inhibit the formation of polyphenylalanyl-puromycin or of formylmethionyl-puromycin catalysed by 50S ribosomal subunits (160). The occurrence of a pentanucleotide of constant sequence in a single-stranded loop of the clover leaf model of all tRNA-species analyzed so far and the existence of a complementary sequence in 5S RNA suggests a direct interaction between tRNA and 50S ribosomal 5S RNA, mediated by hydrogen bonds (258-259).
(c) Enzyme-Specific Binding of Aminoacyl-tRNA

This will be discussed in the following section titled "The Ribosome and Protein Synthesis (see pages 24-28).

(d) Summary

There are site(s) on the ribosomes which bind tRNA. The actual number of sites is unknown and may depend on the experimental conditions. For instance, Warner and Rich (260) using intact rabbit reticulocytes came to the conclusion that each ribosome active in protein synthesis has two molecules of tRNA attached to it. By contrast, the inactive single ribosomes bound approximately one molecule of tRNA, and this binding was less firm than that seen in the polysomes. In their extensive study of in vitro binding of tRNA to E. coli ribosomes, Cannon et al. (148) found that washed ribosomes bound one molecule of tRNA per ribosome and this was rapidly exchangeable at 4°. Under conditions of all-free protein synthesis, the same amount of tRNA became attached to the ribosomes, and a portion of this was more firmly bound. On the other hand, Takanami (252, 261) had found that tRNA will become attached to rat liver ribosomes only in the presence of a transfer enzyme during incubation. With polysomal reticulocyte ribosomes there is little or no exchange at 4°, while inactive ribosomes have a limited exchange (260). It is possible that some of these differences are due to the various species involved. The in vitro environment differs in many respects from the environment in vivo. In particular,
the rate of protein synthesis is so much lower in *vitro* that it is not clear that the difference between the amount of tRNA bound by active and inactive ribosomes would have been detected in the *in vitro* studies.

According to Wettstein and Noll (262) ribosomes engaged in protein synthesis bind at least two and at most three tRNA molecules and that the tRNA bound to rat liver polyribosomes occurs in three different states which exhibit different binding properties. Both the free aminoacyl-changed and the peptide-linked tRNA are tightly bound and not removable by washing, even at low magnesium, as long as the structural integrity of the active complex is preserved. Moreover, their attachment is irreversible and requires transfer enzymes as well as energy. These three different states in which ribosome-bound tRNA is encountered correspond to at least two, and probably three, distinct sites on the active complex. The first site, decoding site, selects the charged tRNA matching the specified codon. Single ribosomes devoid of mRNA do not have this selective binding site. The second site, condensing site, which is found on the 50S ribosomal subunit can bind tRNA in the absence of mRNA; however, during protein synthesis this site is only accessible from the activated state of the first site. Gilbert has shown that tRNA-linked nascent polyphenylalanine remains attached to the 50S particle even after complete dissociation of the ribosomes into subunits (263). Elson (264-265), on the other hand, observed the release of
4S RNA from the 50S particle during its conversion to a particle with a lower sedimentation coefficient in the presence of high salt. The third site, exit site, is specific for uncharged tRNA. Not all of the three sites are equally occupied during protein synthesis in vitro. During protein synthesis in vivo, the decoding and condensing sites are both fully occupied; however, under in vitro conditions, all of the condensing and exit sites, but somewhat less than half of the decoding sites are filled at any one moment. This would indicate that the rate-limiting step in vitro is the selection of tRNA, and in vivo it is the formation of the peptide bond.

The Ribosome and Protein Synthesis

Although ribosomes may have several other functions in vivo, for example, stimulation of RNA synthesis, or regulation of the biosynthesis of RNA or of ribosomes themselves, their only clearly established functions are those related to the synthesis of proteins (70) and this is discussed below.

In prokaryotes the initiation of protein synthesis requires the formation of an initiation complex consisting of the 30S ribosomal subunit, mRNA and formyl methionyl-tRNA (fMet-tRNA_f) (9-11). In a solution of low Mg^{++} ion concentration initiation factors as well as GTP are required for this step. Initiation factors are proteins which were originally obtained from crude ribosomes by washing with 1M NH_4Cl and were found to be required for the translation of natural mRNA (12-14). At least three initiation factors, F_1, F_2 and F_3
(also called A, B and C respectively) are known (15, 16). The possibility of the presence of a new initiation factor, $F_{AB}$, is now undergoing investigation. In vivo the initiation site on the natural mRNA contains an AUG codon which codes for fMet-tRNA$_f$ (17-19). The codons AUG and GUG serve as initiator codons in vitro. The presence of a tRNA involved solely in the initiation of protein synthesis in bacterial systems was discovered by Marcker and Sanger (71). This tRNA so far appears to initiate synthesis of all bacterial proteins (72-75) and probably all proteins in mitochondria (76, 77), chloroplasts (78) and blue-green algae (79). Preliminary experiments indicate that in some mammalian systems the mRNA codon assignments for peptide chain initiation are identical to bacterial systems (235-239, 245). E. coli has two methionine accepting tRNAs: tRNA$_f^{Met}$ and tRNA$_m^{Met}$. These are both changed by the same methionyl-tRNA synthetase (80) but only methionine attached to tRNA$_f^{Met}$ can be formylated by a transformylase (17) which has been purified from E. coli (81).

Very recently transformylases from wheat germ chloroplasts (229) and from Saccharomyces cerevisae mitochondria (230) have been isolated. According to Ochoa (117-118), the formation of the initiation complex involves two steps: (a) the $F_1$ and $F_3$-dependent binding of natural mRNA to the ribosomes, and (b) the GTP requiring $F_2$-dependent binding of fMet-tRNA$_f$ to the mRNA-ribosome complex. After formation of the initiation complex consisting of the 30S ribosomal subunit,
mRNA, fMet-tRNA and the initiation factors, the 50S ribosomal subunit joins to form the 70S initiation complex (23, 24). Although the details are still unclear, studies indicate that fMet-tRNA_f is initially attached to the A site (on the 30S subunit) and is subsequently translocated to the P site (on the 50S subunit). GTP is cleaved before or during this translocation (25, 82).

The next step is the binding of a second aminoacyl-tRNA to the A site. This binding is directed by the codon next to AUG and requires GTP as well as two soluble protein factors, Ts and Tu (26). The T factors (Ts and Tu) interact first with GTP and then with an aminoacyl-tRNA, with the exception of fMet-tRNA_f and Met-tRNA_f (27). The GTP-aminoacyl-tRNA-T factor complex then reacts with the ribosome leading to binding of the aminoacyl-tRNA at the A site. GTP appears to be hydrolyzed at this step (28).

The next step in protein synthesis is the formation of a peptide bond between fMet-tRNA_f (or peptidyl-tRNA) and the second aminoacyl-tRNA bound to the ribosome. This peptide bond formation does not require any supernatant protein factor, and is catalyzed by peptidyl transferase, an enzyme present on the 50S ribosomal subunit (30). The peptide bond formation occurs by transfer of the acyl group at the P site to the amino group of the aminoacyl-tRNA at the A site. The energy for peptide bond formation is supplied by the relatively high energy ester bond between the tRNA and the peptidyl moiety (83). After formation of the first dipeptide bond, the fMet...
aminoacyl-tRNA stays at the A site, and the discharged tRNA$_f$ stays on the ribosome, probably at the original P site.

The next step, translocation, involves movement of fMet aminoacyl-tRNA (or peptidyl-tRNA) from the A site to the P site. Release of discharged tRNA$_f$ from the P site accompanies this translocation step (31). Translocation requires a soluble protein factor (G factor) and GTP which is hydrolyzed to GDP and Pi (32). Factors similar to T and G and which function in the same manner have been isolated from mammalian systems (238-242) and yeast (243). Simultaneously with the translocation, the ribosome moves along the mRNA, in the 5' to 3'-direction, by the length of one codon, leaving the third codon ready for the binding of a new aminoacyl-tRNA to the A site. These processes are repeated and polypeptide chain elongation continues until the ribosome encounters one of the chain termination codons (UAG, UAA and UGA). Recently the mRNA codon assignments for peptide chain termination have been found to be identical in mammalian systems (97, 233). For termination to take place, the peptidyl-tRNA must be on the P site (37, 38). Chain termination leads to cleavage of the polypeptide from the tRNA and the subsequent release of this tRNA from the ribosome.

Since initiation of protein synthesis takes place on the 30S ribosomal subunit, the ribosomes after protein synthesis must subsequently undergo dissociation. Subramanian et al. (110) isolated a dissociation factor (DF) from the 30S fraction fraction which was found to carry out this function. Very
recently it has been shown (111-112, 231) that this DF factor was actually initiation factor F₃. Therefore F₃ has now been shown to have both RNA binding and ribosome dissociation activities. Some workers believe that chain termination liberates 70S ribosomes (113-114) which dissociate subsequently (113) by interaction with F₃, released from 30S ribosomal subunits when the 50S ribosomal subunit joins the 30S initiation complex. This would imply that F₃ recycles between a ribosome-bound and a free form. Other investigators hold the view that ribosomes dissociate at chain termination (115-116, 120). If so, F₃ might remain ribosome-bound throughout the entire cycle so long as ribosomes, bearing F₃, can associate and remain associated when they carry aminoacyl- or peptidyl-tRNA. At present, the precise manner in which F₃ functions in the ribosome cycle remains an open question.

**Release Factors and the Mechanism of Termination**

When a ribosomal : peptidyl-tRNA complex reaches a terminator codon on a mRNA, some mechanism must bring about hydrolysis of the ester bond between the peptidyl and tRNA moieties. This would then allow the completed protein to be released.

Most of the information about the mechanism of chain termination was obtained in two assay systems. In one of these, RNA from a mutant R17 or f₂ phage with a UAG nonsense codon early in the coat protein gene was used as the messenger. *In vitro*, this messenger directs the synthesis of the free
(not tRNA-linked) amino terminal hexapeptide of the coat protein (75, 84-85). Bretscher (84) prepared a cell-free system which included only those aa-tRNA species needed for forming the hexapeptide. If a codon is untranslatable in consequence of the lack of a required aa-tRNA, this leads to the stoppage of peptide chain propagation but not to chain termination. The fact that, in the presence of the mRNA from a nonsense mutant, chain termination occurred even in the absence of all tRNAs, except the six species added, suggested that if RNA was involved in chain termination, it was not contained in the tRNA fraction (75, 84, 86-87) obtained from the high speed supernatant from *E. coli*. Capecchi (75) was able to prepare a substrate for studying the mechanism of termination in the following way. The formation of a hexa-peptidyl-tRNA as specified by the R17 RNA was blocked at the pentapeptidyl-tRNA stage by omitting from the in vitro system the amino acid coded by codon six. The ribosome-mRNA-pentapeptidyl-tRNA complex was then separated from the supernatant fraction by centrifugation and the aa-tRNA needed to complete the hexapeptidyl-tRNA was added in the presence of GTP. The last amino acid was then added to the coat protein fragment. The resulting hexapeptidyl-tRNA remained attached to the mRNA-ribosome complex. This product made possible the study of the unique requirements of the release step. It was found that the release of free hexapeptide from this complex depended on a protein component from the high speed supernatant of *E. coli* This component was designated release factor (R factor) (43).
Following Capecchi's lead, Nirenberg's group has been very active in studying the factors involved in chain termination (88-98). Caskey et al. (88) used a different termination assay. fMet-tRNA$_f$ was bound to ribosomes in the presence of the trinucleotide AUG to form an AUG-fMet-tRNA$_f$-ribosome complex. Then a terminator trinucleotide and crude R factor were added and the release of free fMet was measured. With this assay they were able to resolve R into 2 components, R$_1$ and R$_2$; R$_1$ responded to UAG and UAA but not UGA whereas R$_2$ was active with UGA and UAA but not UAG (89, 91). Capecchi and Klein (107) used antisera to purified R$_1$ and R$_2$ to test their role in release of completed proteins in a cell-free system directed by R17 RNA. Their results indicated that these factors were required for release of completed proteins and that either factor could promote release of either the coat protein or the replicase; this implied that both cistrons terminated with UAA since this was the only one of the three terminator codons recognized by both R$_1$ and R$_2$. Nichols (108) sequenced the portion of R17 RNA at the end of the coat protein cistron and found two consecutive terminator codons, UAAUAG. This may mean that, at least in some systems, two terminators are required to ensure that release occurred between cistrons. This seems most likely in view of the existence of nonsense suppressors which allow UGA or UAG to be read as specifying an amino acid, instead of serving as terminator signals.

A third component, S, has been found to increase the rate of formation or stability of the terminator codon : ribosome :
factor R complex (90, 92). Peptidyl transferase may also be involved in release since inhibitors of this enzyme also inhibit release. The presence of the terminator complex may allow peptidyl transferase to act as a hydrolase and thus break the ester bond holding the completed protein to the last tRNA (peptidyl-tRNA) (93, 99). It was previously suggested that the non-ribosomal enzyme, N-acylaminoacyl-tRNA hydrolase may catalyze the release reaction (102, 106) but this now seems unlikely in view of the results of Caskey et al. (88) who showed that fMet-tRNA is cleaved during the release reaction while it is known that free fMet-tRNA is a very poor substrate for the hydrolase. Recently Jost and Bock (232) isolated an N-substituted aminoacyl-tRNA hydrolase from yeast supernatant. They suggested that there are two hydrolases, one, a ribosomal hydrolase, may be involved in the release of nascent peptide chains during the normal process of chain termination, and the other, the supernatant hydrolase, could hydrolyze any oligopeptidyl-tRNA present in the cell sap.

Recent studies indicate that R factor and suppressor tRNA compete for the translation of terminator codons (96, 100). Recently Goldstein et al. (97, 233) have isolated an R factor from rabbit reticulocytes which, in the presence of the fMet-tRNA-AUG-ribosome complex and the UAA terminator codon causes the release of fMet. They also found that antibiotics which inhibited peptidyl transferase activity also inhibited release.

Very recently Ishitsuka and Kaji (109) isolated another
factor called tRNA release factor (TR) from the high speed supernatant of E. coli. It was found to facilitate the removal of tRNA from ribosomes and was distinctly different from the G factor in that it did not require GTP for its action. They postulate that this factor may act at the last step in protein synthesis in the following way: A completed polypeptide chain linked to tRNA through the -COOH terminal group of the polypeptide is probably bound to the donor site of the ribosome with the acceptor site having the termination codon. The codon-specific chain-termination factor would then bind to the acceptor site of the ribosome resulting in the splitting of the polypeptide group from the tRNA. The tRNA is left on the donor site of the ribosome and cannot be removed by the G factor which releases tRNA only as a consequence of translocation. The TR factor would then remove the last tRNA from the ribosome. This hypothesis complements that suggested by Vogel et al. (104) who postulated that peptidyl transferase might cleave the substrate, with the R factor acting to change the specificity of the peptidyl transferase reaction, so that nascent peptide was now transferred to water instead of to a molecule of aa-tRNA. In this event, the R factor might interact directly with peptidyl transferase, in which case the factor could be looked on as part of a multimeric release enzyme. Alternatively, the R factor might itself cleave the bond between nascent peptide and tRNA. The factor has been shown to be unable to do this
when free in solution (104) but it might be able to catalyze the reaction when the substrate is bound to a ribosome.

From the foregoing discussion, it can be seen that the mechanism of the actual release reaction—the cleavage of the ester bond between nascent protein and tRNA and the subsequent release of the tRNA—is still unknown. It remains to be seen whether the R factors do recognize the termination signals directly or whether other molecules, which in turn interact with the appropriate species of R factors, are involved in this process.

Ribosomal Structure and Function

The foregoing discussion had illustrated the complexity of ribosome structure and some of the known events of its function in protein biosynthesis. Related to this is the recent finding by Lodish (212) that ribosomes from \textit{E. coli} initiated synthesis \textit{in vitro} of all three of the proteins coded by phage \textit{f}_2, but ribosomes from \textit{B. stearothermophilus} initiated synthesis of only one. He was able to show that the specificity of initiation depended on the source of the 30S ribosomal subunits: the origin of the 50S ribosomal subunits, initiation factors, tRNA or supernatant enzymes had no effect. Thus the 30S particle selects regions of messengers as signals for the initiation of polypeptide synthesis. It appears that the control of translation is dependent on the conformation of mRNA (244). Presumably the tertiary structure of the ribosome plays a crucial role. The work of Steitz (18)
and Hindley (19) suggested that the site on the messenger to which the 30S particle attached was identical to the site at which protein synthesis was initiated. It has also been shown that in f2 RNA there are AUG sequences which can initiate protein synthesis, but are prevented by the RNA structure from doing so (18, 19, 213-215). To make the problem even more complicated, after infection of *E. coli* by T4 phage ribosomes bind appreciably only to the maturation protein initiation site of R17 RNA (216). Apparently this change lies in the initiator factor fraction.

Recently in order to get a clearer picture of the ribosome, studies on the low molecular weight RNA bound to ribosomes have been started by many groups with the hope of being able to correlate the findings with a possible clearer understanding of the structure of the ribosome with respect to its function.

Until recently RNA was considered to belong to one of those categories: transfer, ribosomal or messenger. Development of more sophisticated analytical techniques, such as polyacrylamide gel electrophoresis and improved chromatographic techniques, has permitted the detection of a number of new RNA species, among which are RNAs of relatively low molecular weight (5-8S). Such small RNAs have been found in or associated with ribosomes (170, 175), microsomal membrane (185), smooth endoplasmic reticulum (186), nuclei (181, 182, 187, 188, 191, 194) and nucleoli (188, 190, 193).

Knight and Sugiyama (170) found a new class of tRNA in
E. coli ribosomes and supernatant and in HeLa cell mitochondria but not in HeLa cell cytoplasm. Most of the tRNA is not associated with ribosomes. This minor tRNA was found to have a molecular weight, judged by mobility on polyacrylamide gels, intermediate between the major part of tRNA and 5S RNA. The minor tRNA of E. coli can be changed with amino acids, however, its amino acid specificity is different from that of the major class of tRNA.

A further study by Knight (171) revealed that ethidium bromide treatment of growing HeLa cells eliminated this minor tRNA from the mitochondria and reduced the major species by 50% without affecting the synthesis of 5S RNA. The minor species was found to be unmethylated while the major species had a completely different methylation pattern from the cytoplasmic 4S RNA. No significant differences were observed between the 5S RNA of the cytoplasm and that occurring in the mitochondrial fraction. Recently precursor tRNA (pre-tRNA) has been isolated from HeLa cell cytoplasm (183, 184). This pre-tRNA which is not methylated migrated on gel electrophoresis between tRNA and 5S RNA. It has also been shown that the tRNA from the mitochondria of rat liver and Neurospora have different specificites from their cytoplasmic counterparts (172, 173). The mitochondrial tRNA was found to hybridize more efficiently to the mitochondrial DNA than the corresponding cytoplasmic tRNA suggesting that mitochondrial tRNA is transcribed from mitochondrial DNA. In a recent report, Nass and Buck (174) observed that the four mitochondrial tRNAs
they studied from rat liver differed in base sequences from their cytoplasmic counterparts. These mitochondrial tRNAs could only be acylated by mitochondrial synthetases and not by cytoplasmic enzymes.

Recently 7S RNA has been isolated from HeLa cell ribosomes (175). It was found to be associated with the 28S rRNA and as such may play a role in the conformation of the 28S rRNA. They also present evidence that 7S RNA derives from the same polynucleotide precursor as does its accompanying 28S molecule (175, 217). 7S RNA was also found in Chinese hamster and chicken fibroblast 28S rRNA (175). There appears to be one 7S RNA molecule for every 28S molecule and it appears to be attached to the larger rRNA by non-covalent bonds. The 7S contains approximately one methylated base. Electrophoretic mobility of 4, 5 and 7S RNA reveals about an equal separation between 4 and 5S RNA and 5 and 7S RNA.

Weinberg and Penman (181) have observed six distinct low molecular weight species of RNA in the nucleoplasm and nucleolus of HeLa cells. The nuclear RNA species range in size from 4 to 6S RNA, and appear to be stably associated with the nucleus. Some are associated principally with the nucleoplasmic and others with the nucleolar fraction. These species are methylated and have base compositions unlike any of the other nuclear RNA classes. These species represent only about 0.4% of the total cellular RNA. In the nucleus, 5S RNA is present in great excess relative to 7S RNA (175;
this RNA exhibits both the sedimentation velocity and electrophoretic mobility of a species of approximately 5.5S RNA, the term 7S RNA originally given to this species by Pere et al. is thus a misnomer) which is consistent with the existence of a sizeable pool of 5S ribosomal RNA in the nucleus. The 5.5S and 5S RNA species are present in equimolar quantities in cytoplasmic ribosomes. Similar species of nuclear RNA are found in mouse fibroblast cultures and in the developing chick embryo brain. Preparative gel electrophoresis has been used to isolate four methylated low molecular weight RNA molecules from Chinese hamster cells (187). They have been shown to be distinct from tRNA, 5S and 5.5S rRNA and are found in a fraction enriched with respect to nuclei. In a more recent investigation, Weinberg and Penman have isolated three new nuclear low molecular weight RNA species (182). One species migrates with identical electrophoretic mobility to that of 5S rRNA and is methylated while the other two species, also methylated, migrate slower than 5.5S RNA. These RNAs are found almost solely in the nuclei of interphase cells and are quite loosely associated with the nucleoprotein complexes of the nucleus which is in marked contrast to the other previously described six species of nuclear RNA. All nine nuclear RNA species differ from each other in several of their properties; for example, stability, size, synthesis, quantity, etc., which may reflect differences in their function in vivo.

Busch's group has shown that the nuclei and nucleoli of
normal rat liver (188) and a number of tumor cells (189) consistently contained 4 to 6S RNA. Recently nucleolar 4 to 6S RNA has been isolated from Novikoff hepatoma ascites cells and separated into three main fractions by exclusion chromatography on Sephadex G-100 (190). The elution pattern was found to be similar to the ribosomal 4 to 6S RNA of the Novikoff hepatoma. Peak I (the fraction that emerged in the void volume) contained two major components, both of lower mobility than ribosomal 5S RNA. Only one band migrated in 10% polyacrylamide gels and was similar to the RNA derived from ribosomes. The main component in Peaks II and III migrated with mobilities similar to 5S and 4S RNA respectively. Peacock and Dingman (191) reported the presence of several low molecular weight RNA species (4 to 7S RNA) in rat liver nuclei which were not present in the cytoplasm. Hodett and Busch isolated this nuclear fraction and characterized two U-rich RNA fractions (192, 195, 197). These U-rich fractions, with mobility less than 5S RNA, are apparently unrelated to those found by other workers for HeLa cell ribosomes (175) and cytoplasmic fractions (185). Each of these other fractions seem to be present primarily in the rRNA or ribosomal subfractions. Busch suggests that since this U-rich RNA has a lower rate of labeling than any other nuclear RNA, this RNA would appear to be stable and might serve a structural role but since this RNA is less hydrogen-bonded than tRNA or rRNA it might also exert some role in template activity. Recently two other low molecular weight RNAs have been liberated from the nucleolar 28S
RNA fraction and referred to as 8S and U3 RNA (193). These two RNAs are not associated with ribosomal 28S RNA. The molar ratio of 8S and U3 RNA to nucleolar 28S RNA is only approximately 1:2, suggesting that only some of the nucleolar 28S RNAs are bound to these molecules. They may have some role in the formation of the ultimate cytoplasmic ribosomal particle. It is possible that they may also serve as essential components for the movement of nucleolar products to the nuclear ribonucleoprotein network, or for the addition of special proteins that are components of the ribosomes. An 8S nuclear RNA has also been isolated from KB cells (246). More recently U3 RNA and 4.5S RNA have been isolated from the nucleus of Novikoff hepatoma cells and from rat liver nuclei (194-195). This 4.5S RNA was subsequently separated into three fractions. It should be noted that the 3'-terminal of one fraction is blocked (196). Recently the nucleolar U3 RNA was separated into 4 distinct bands (198). It has been suggested that these RNAs may function in processing of nucleolar ribosomal RNA precursors into ribosomal precursor particles and finally into mature ribosomes. The low turnover of these molecules suggests that some might be stable messenger RNAs for certain proteins, perhaps ribosomal or ribosomal precursor proteins. It is also possible that these RNAs may play a role as initiation factors in ribosomal RNA synthesis.

Various RNAs have been isolated from rabbit reticulocytes besides 4, 5 and 7S RNA (207-208). Two RNAs sedimenting at 8S and 10S have properties expected of mRNA.
Sea urchin 26S rRNA has been shown to contain a hydrogen-bonded 5.8S rRNA (199), which is unmethylated and which is similar to the 5.5S RNA associated with rRNA of HeLa cells (175), chicken fibroblasts (175) and Novikoff ascites tumors (193). Sy and McCarty suggest (199) that the 5.8S rRNA or the 5.5S rRNA is involved in maintaining the correct 3-dimensional configuration of either the 28S or 26S rRNA necessary for its proper interaction with proteins in ribosome maturation.

Yeast ribosomes have been found to contain besides 4S and 5S RNA, a 5.8S RNA molecule which is unmethylated and which is non-covalently attached to the 25S rRNA (200). It is suggested that the 5.8S RNA is derived from a part of the 35S precursor RNA, whereas the 5S RNA is made de novo.

It has been found, just recently, that the mitochondrial ribosomes of *Neurospora crassa* are devoid of 5S RNA (201). A 7S RNA component was also absent.

After infection with adenovirus 2 or 7, human epithelioid cells synthesize a discrete species of low molecular weight RNA (176). This is found predominantly in the soluble fraction of the cytoplasm of these cells. Its function is unknown, and its primary structure is different from transfer RNA and from the two principal low molecular weight RNA components found in the ribosomes of the uninfected KB cells (177). Low molecular weight RNA synthesis in T5-infected *E. coli* occurred predominantly 3 to 4 mins. after complete infection (178). This type of RNA synthesis was characterized by seven discrete bands of molecular weight range 8.0 to 3.1 x 10^4 and a broad
band migrating equivalent to host 4S RNA. The data suggested
that all species of molecular weight between 5.3 and 2.6 x 10⁴
were probably cleavage products of RNA of higher molecular
weight. Only one band, molecular weight, 5.3 x 10⁴ has been
shown to be bound to polysomes. Altman has isolated tRNA
precursors from E. coli infected with bacteriophage φ80 (205).
The precursor migrated on polyacrylamide gels between 4S and
5S RNA. Many species of low molecular weight RNA ranging
from 4S to 10S have been isolated from Rous Sarcoma Virus
(179, 180). Purified RSV contain a homogeneous population of
methylated 4S RNA which is indistinguishable from host tRNA
on the basis of electrophoretic mobility, although differences
in nucleotide composition are detectable. A minor homogeneous
RNA component, with sedimentation velocity and electrophoretic
mobility approximating the 7S RNA molecule, and a few methyl
residues has been detected. Its significance and possible
function are presently obscure.

Recently the nucleotide sequence of 6S RNA from the
supernatant fraction of E. coli has been established (202).
This RNA had been noted previously although no function has
been assigned to it (203-204). Its relationship with RNAs
found in higher organisms is unclear. It may be related to
one or other of several bands of rather similar electrophoretic
mobilities to 6S RNA on acrylamide gel electrophoresis
(believed to be nuclear RNAs) (191, 197). It is, however,
unlikely to be related to the low molecular weight ribosomal
RNA, called 7S RNA (175), for 6S RNA of *E. coli* is not found on ribosomes.

In summary, a new and exciting chapter has recently opened up in the field of RNA chemistry and particularly that of the biochemistry of nucleolar, nuclear and rRNA; for example, the uniquely localized low molecular weight RNA. The eventual elucidation of their functions will help in the understanding of ribosomal structure and function.
OUTLINE OF THE PROBLEM

The events involved in the initiation of protein synthesis are relatively well understood. However, the mechanism of chain termination in protein biosynthesis is still an unsolved problem. The discovery of R factors was a great step forward, but in contrast to the case of initiation where a specific tRNA is definitely involved, the requirement for a specific tRNA in termination has not, as yet, been shown and the present understanding of the problem is that such a tRNA is not required. The chain-terminating experiments outlined in a previous section (Release Factors and the Mechanism of Termination, p. 28) in which highly purified tRNAs were used, nevertheless were not properly controlled since it can be argued that the terminating tRNA could remain bound to the ribosomes. The previous investigators failed to show that their ribosomes or ribosomal subunits were devoid of 4S RNA. Perhaps this hypothetical chain-terminating tRNA is different in some manner from the normal tRNA such that it is not removed from the ribosome during the cleaning procedure. In any event, it may combine in some manner with R factor, or some other protein, such as peptidyl transferase in the termination mechanism, or it may simply be present to stabilize ribosomal conformation for the termination process. These are only some of the questions that must be answered before a final mechanism can be hypothesized.

Recently investigations have led to considerable understanding of the ribosomal structure. Both subunits have been
reconstituted with particular emphasis on the 30S subunits and the order in which the proteins are reassembled to form the functional unit. Less is known about the biological role of the RNA associated with ribosomes. In particular the nature and function of many small molecular weight RNAs associated with ribosomes is unknown.

Thesis Proposal

This thesis was devoted to the investigation and characterization of the low molecular weight RNA bound to *E. coli* ribosomes which had been prepared by standard procedures. Methods were also studied for preparing ribosomes devoid of all 4S components with particular emphasis on the role of tRNA in the chain termination mechanism.

Ribosomes from *E. coli* were chosen for study not only because they are so well characterized and have been used in previous chain termination studies but also because of the ease with which these active ribosomes can be obtained. The experimental outline is: (a) isolation and preparation of active ribosomes (WRib), (b) removal of bound tRNA from WRib and subsequent characterization, (c) characterization of the low molecular weight RNA from whole ribosomes and the subunits, and (d) exchange of labeled ribosomal bound tRNA with unlabeled tRNA and subsequent characterization.
MATERIALS AND METHODS

Chemicals

Common chemicals obtained commercially were of the highest purity or reagent grade. Individual radioactive amino acids including the amino acid mixture (¹⁴C-labelled) and ³H-labelled uracil were obtained from New England Nuclear Corporation; adenosine 5'-triphosphate (disodium) (ATP) and guanosine 5'-triphosphate (trisodium) (GTP), from Calbiochem; polyuridylic acid (poly U), from Miles Chemical Company; puromycin dihydrochloride (PM.2HCl), from Nutritional Biochemicals Corporation; N,N'-Methylenebisacrylamide (Bis), N,N,N',N'-Tetramethylethylenediamine (TEMED), 2-mercaptoethanol, riboflavin and aurintricarboxylic acid (ATA), from Eastman Organic Chemicals; methylene blue from Fisher Scientific Co.; acridine orange (basic orange 14), acrylamide and 3'-dimethylaminopropionitrile (DMAPN), from Matheson, Coleman and Bell; lanthanum acetate from K&K Laboratories, Inc.; cesium chloride (CsCl), A grade for density gradients, from Calbiochem; sucrose, density-gradient grade (ribonuclease-free), from Mann Research Laboratories, Inc.; transfer RNA (tRNA) from E. coli B, from General Biochemicals; and DNase I from Worthington Biochemical Corp.

Preparation of E. coli B

(a) Cells grown to the late log phase were obtained from the Grain Processing Co., Muscatine, Iowa. The growth medium used was as follows: 1% glucose, 1% yeast extract in a
phosphate buffer medium, the buffers being monopotassium and
dipotassium phosphate. The starting pH was 7.0 to 7.1. After
the growth period was completed, the cells were harvested as
follows: (1) the contents of the fermentor were cooled from
37° to 16° and then centrifuged, (2) the cells were recovered
from the centrifuge and washed in a medium made up of 0.5%
KCl and 0.5% NaCl, (3) the cells were recentrifuged and frozen
at -20°.

(b) One hundred ml s of medium (see below) in a 125-ml erlen-
meyer flask was inoculated with E. coli previously grown on the
surface of an agar slant and the flask was aerated by shaking
at 100 rpm on a Metabolite Water Bath Shaker (New Brunswick
Scientific Co., Inc.) set at 37°. When growth had reached mid-
log phase, the contents of the flask, which served as the
inoculum, were added to a carboy containing 15 liters of the
following minimal medium at pH 7.0 (g/L):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_2HPO_4$</td>
<td>7.0</td>
</tr>
<tr>
<td>$KH_2PO_4$</td>
<td>3.0</td>
</tr>
<tr>
<td>Na citrate.2$H_2O$</td>
<td>0.5</td>
</tr>
<tr>
<td>$MgSO_4.7H_2O$</td>
<td>0.2</td>
</tr>
<tr>
<td>$NH_4Cl$</td>
<td>1.0</td>
</tr>
<tr>
<td>glucose</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Throughout the growth period the cells were vigorously
aerated. When the cells had reached the late log phase, they
were quickly cooled by adding ice and then harvested by
centrifugation and stored at -70°.

**Preparation of E. coli B Pyrimidine-_req_uiring Mutant 12632**

(American Type Culture Collection, ATCC 13135)
CHART I*

Preparation of E. coli ribosomes

E. coli
a) ground with glass beads in buffer A (10 mM Mg(OAc)$_2$)
b) centrifuged at 10,000 x g

cellular debris
a) incubated with DNase I
b) centrifuged 2x at 30,000 x g

precipitate
a) centrifuged at 105,000 x g

ribosomal pellet
a) mixed with (S100)
   buffer B (0.1 mM Mg(OAc)$_2$)
b) centrifuged at 105,000 x g

ribosomal pellet S100A
a) mixed with buffer C (1 mM Mg(OAc)$_2$)
b) centrifuged at 105,000 x g

ribosomal pellet
a) mixed with buffer D (5 mM Mg(OAc)$_2$)
b) centrifuged at 105,000 x g

ribosomal pellet
a) mixed with buffer E (10 mM Mg(OAc)$_2$)
b) centrifuged at 105,000 x g
c) repeated a) and b) 3x

ribosomal pellet
mixed with buffer F (10 mM Mg(OAc)$_2$)

ribosomal suspension (WRib)
a) put on DEAE-cellulose column equilibrated with buffer G and wash with same buffer
b) eluted with buffer H

centrifuged at 105,000 x g

ribosomal suspension

ribosomal pellet
mixed with buffer F

ribosomal suspension (RSI)

* Buffer A-F described previously in List of Buffers.
An inoculum was prepared from the *E. coli* mutant grown on the surface of an agar slant as in (b) above. When growth has reached the mid-log phase, the inoculum was added to a 15-liter carboy containing the same medium as (b) above except that 4 μg uracil/ml medium was added. Where labelled cells were required, ^3^H-labelled uracil was added to the carboy prior to log phase in presence of the same amount of unlabelled uracil. The cells were grown and harvested in the same manner as (b) above and finally stored at -20°.

**Preparation of E. coli B Ribosomes (WRib) (see Chart I)**

Ribosomes were prepared from *E. coli* by a combination of many methods (218-220) which were intended to remove all non-ribosomal material. This was achieved by employing a series of buffers with the same high ammonium concentration but with varying magnesium concentrations. All operations were performed at 0°-4°. Frozen or fresh cells were ground with three times (3x) the weight of Superbrite glass beads (3M Company, previously cleaned in 6N HCl and washed with distilled water) by means of a Virtis 45 homogenizer running at top speed in the presence of an equal volume of buffer A (10 mM Tris, 10 mM Mg(OAc)$_2$, 10mM NH$_4$Cl, 10 mM mercaptoethanol, pH 7.8). The cells were homogenized for four 2 minute intervals with five minute cooling periods in between. The homogenizing flask was surrounded by ice throughout these operations.

Following the last homogenization, the homogenate was centrifuged at 10,000 x g for 20 mins in order to remove
cellular debris. The supernatant fraction was collected and 3 µg of pancreatic DNase I was added with gentle mixing to each milliliter of *E. coli* extract and incubated for 10 mins. The extract was then centrifuged at 30,000 x g for 20 mins. The supernatant solution was removed and the centrifugation repeated. The upper four-fifths of the supernatant solution was removed and centrifuged at 105,000 x g for 5 hrs in a Model-L ultracentrifuge. The supernatant was removed and dialyzed versus buffer A overnight (30:1 ratio) and then stored at -70°. This was labelled S-100 and is the source of aminoacyl-tRNA synthetases and factors required for protein synthesis and was used in many subsequent analyses.

The ribosomal pellet was dissolved in buffer B (10 mM Tris, 0.1 mM Mg(OAc)$_2$, 0.5-1.0 M NH$_4$Cl, pH 7.4) and mixed for 2 hrs. Aggregates were removed by centrifugation at 10,000 x g for 5 mins and then the ribosomal mixture was centrifuged at 105,000 x g for 15-24 hrs. The supernatant, S100A, was saved and stored at -70°. This is the source of initiation and transfer factors required for protein synthesis.

The ribosomal pellet was dissolved in buffer C (10 mM Tris, 1 mM Mg(OAC)$_2$, 0.5-1.0 M NH$_4$Cl, pH 7.4) and mixed for 2 hrs. Aggregates were removed by centrifugation at 10,000 x g for 5 mins and then the ribosomal mixture was centrifuged at 105,000 x g for 10-15 hrs. The supernatant was discarded and the ribosomal pellet dissolved in buffer D (10 mM Tris, 5 mM Mg(OAc)$_2$, 0.5-1.0 M NH$_4$Cl, pH 7.4) and mixed for 2 hrs. Aggregates were removed by centrifugation at 10,000 x g for
5 mins. and then the ribosomal mixture was centrifuged at 105,000 x g for 7-10 hours. The supernatant was discarded and the ribosomal pellet dissolved in buffer E (10 mM Tris, 10 mM Mg(OAc)$_2$, 0.5-1.0 M NH$_4$Cl, pH 7.4) and mixed for 2 hrs. Aggregates were removed by centrifugation at 10,000 x g for 5 mins and then the ribosomal mixture was centrifuged at 105,000 x g for 5 hrs. The supernatant was discarded and the mixing of the pellet with buffer E and subsequent centrifugation was repeated three times more. The ribosomal pellet was finally dissolved in buffer F (10 mM Tris, 10 mM NH$_4$Cl, 10 mM Mg(OAc)$_2$, pH 7.6) and mixed for 2 hrs. Aggregates were removed by centrifugation at 10,000 x g for 5 mins. The ribosomal mixture (WRib) was checked for amino acid acceptor activity (the method of which will be outlined in detail below) and finally stored at -70° in small 4-ml vials containing 1 ml aliquots.

Assay for Ribosomal Activity and Amino Acid Incorporation

Ribosomal activity was determined by following the uptake of $^{14}$C-Phe in a poly U-dependent phenylalanine incorporation system. The assay system contained the following components (total volume 250 μl):

- 50 mM Tris, pH 7.6
- 14 mM Mg(OAc)$_2$
- 100 mM NH$_4$Cl
- 6 mM ATP
- 2 mM GTP
- 100 μM unlabelled Phe
- 5 μl $^{14}$C-Phe (1:2 with H$_2$O)
- 50 μg poly U
- 50 μl WRib
- 100 μl S100
- 10 μl S100A
The previously frozen components were thawed and then placed on ice and all additions were made at 0°-4°. The S100 and S100A vials were discarded after each usage. After all additions were completed (the ¹⁴C-Phe was added last¹) the tubes were incubated at 37° for 15 mins. After the incubation the tubes were quickly placed in ice and mixed with 3 ml of ice cold TCA (trichloroacetic acid). The tubes were then heated at >90° for 20 mins. They were then removed and kept at 0° for 15 mins. The protein was then collected on Millipore filters which were subsequently dried under a heating lamp. The dried filters were placed in scintillation vials and 5 ml of scintillation fluid [containing 3g of PPO(2,5-diphenyloxazole) and 0.1 g of dimethyl-POPOP(1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene per liter of toluene] was added and counted in a scintillation counter.

Assay for Amino Acid Acceptor Activity

The assay system was essentially that of Keller (229) and was as follows: 0.2 ml of assay mix (250 mM Tris, 100 mM Mg²⁺, 12.5 mM ATP, pH 7.6)

0.7 ml tRNA
0.1 ml enzyme (freshly prepared)
5 μl ¹⁴C-amino acid mixture (1:2 with H₂O)

All additions² were made at 0°-4°. The tubes were incubated at 37° for 20 mins. After the incubation the tubes were quickly placed in ice and mixed with 2 mls of ice cold TCA to stop the reaction. The contents were then collected on Millipore filters which were placed in scintillation vials and dried with the aid of a heat lamp. Five mls. of toluene

¹,² See Appendix, page 163
scintillation fluid was added and the vials were then counted on a scintillation counter.

**Preparation of E. coli Aminoacyl-tRNA synthetases**

*E. coli* aminoacyl-tRNA synthetases were prepared by a two-step procedure. Step (a) was employed to remove all the tRNA while step (b) was used to remove all low molecular weight material. In this way a relatively clean preparation of synthetases could be isolated.

(a) Treatment on DEAE-cellulose column

A fiberous form of the resin, DE-22 was used. The DE-22 was washed with 1N NaOH and then with 1N HCl by the standard procedures as described in the Whatman information leaflet. DE-22, in the fully protonated form, was equilibrated at the pH meter by stirring with enough NaOH so that the pH on continued stirring remained at about 7. This produces a material with a high capacity to bind tRNA (mean small ion capacity 1.0 meq/g dry resin). Each mg of tRNA requires a column volume of 0.15 ml. The column was used only once and then the resin was discarded.

The following steps were all carried out at 0°-4°. Three ml of S100 (approximately 0.6 mg RNA/ml) was thawed out and 0.75 ml of 1.0 M KCl was added to give 3.75 ml of 0.2 M KCl. This enzyme mixture was then put through a 0.5 ml DE-22 column previously equilibrated with 10 ml buffer G (0.2 M KCl, 10 mM Tris, pH 7.4). The first 0.4 ml of effluent (the void volume) was discarded and then the next 3.7 mls was collected and put
through a Sephadex G-25 column (see below).

(b) Treatment on Sephadex G-25 Column

The Sephadex G-25 was prepared as described in the Pharmacia handbook, "Sephadex-gel filtration in theory and practice." The following steps were carried out at 0°-4°. A 20 ml Sephadex G-25 column was prepared and equilibrated with 35 ml of freshly prepared buffer H (20 mM Tris, 10 mM cysteine, pH 7.4).

Three mls of effluent from (a) above was eluted through the column with buffer H. Collection of effluent was started as soon as the effluent from (a) was pipetted onto the top of the column. Protein started to emerge from the column at the void volume which was about 35% of the column volume. Since the void volume varied with the packing of the column, small aliquots were removed from the column and tested with 1M HClO₄. A distinct turbidity occurred when the concentration of protein in the test solution was 0.2 mg/ml or more. At this point a further 4.2 ml of effluent was collected (or until 1M HClO₄ test is negative) and made 40% with respect to glycerol and stored at -20°. It has been found that certain aminoacyl-tRNA synthetases maintained their activity when stored for one year in glycerol. In the case of seryl-tRNA synthetase the activity increased in the presence of glycerol while with some others the activity decreased in absence of glycerol (279). The enzyme mixture was used as prepared without prior removal of the glycerol. The column was washed
with 30 ml of distilled water to wash out the buffer and was ready for use again.

Preparation of Benzoylated DEAE-cellulose (BD-cellulose)

BD-cellulose (fully benzoylated), prepared by reaction of DEAE-cellulose with benzoyl chloride as described by Gillam et al. (228) was ground and sieved in the wet state through a 50 mesh (0.3 mm opening) screen and freed of fine particles by repeated settling and decantation. During the sieving and the removal of the fines, the BD-cellulose was maintained in solutions containing dilute (0.1-0.5 M) NaCl to prevent excessive generation of fines. The BD-cellulose was packed into columns by adding a slurry of the resin in 2 M NaCl (which had been freed of trapped air by evacuation) to a column half filled with 2 M NaCl. The slurry was allowed to settle until approximately 2-3 cm of BD-cellulose had packed. The column stop-cock was then opened but the liquid level was always kept above the packed surface of the exchanger. The slurry was added until the desired depth of bed was obtained. The packed column was washed with 2 M NaCl until the eluate had acceptably low absorbance (A$_{260}$ nm 0.025). Following these washing steps, the column was equilibrated with the solution used to start the elution and the tRNA (dissolved in the latter solution or a solution of equal or less conductivity) was applied to the column. After a brief rinsing with the solution used to equilibrate the column, positive linear gradients of NaCl were applied in the usual
manner (292, 293).

Treatment of Ribosomes with Puromycin

The following incubation mixtures (in a total volume of 5 ml) were prepared and made 0.5 mM with respect to puromycin (PM):

A. 10 mM Tris 6 mM ATP 14 mM Mg(OAc)$_2$ 100 mM NH$_4$Cl 2 mM GTP 2 yl S100 100 yl S100A 46 mg WRib 200 mM amino acid mix

B. 10 mM Tris 6 mM ATP 14 mM Mg(OAc)$_2$ 100 mM NH$_4$Cl 2 mM GTP 100 yl S100A 20 mg WRib

C. 5 mM Tris 14 mM Mg(OAc)$_2$ 100 mM NH$_4$Cl 2 mM GTP 100 µl S100A 13 mg WRib

Incubation mixture A (148, 272) was brought up to pH 7.6 and preincubated for 5 mins at 37° prior to PM addition and then further incubated for 30 mins at 37°. Following the incubation the mixture was centrifuged at 105,000 x g for 5 hrs. The pellet was then mixed in buffer I containing 10 mM Tris, 0.1 mM Mg(OAc)$_2$, pH 7.6 and dialyzed overnight against the same buffer. After centrifugation at 105,000 x g, the pellet was mixed in 10 mM Mg(OAc)$_2$ buffered at pH 7.8 (buffer A) and dialyzed overnight against the same buffer.

Incubation mixture B (148, 272) was brought up to pH 7.6 and then incubated at 30° for 15 mins while incubation mixture C (148, 272) was brought up to pH 7.6 and incubated at 30° for 15 mins. Following the incubation both mixtures were made one molar with respect to NH$_4$Cl and centrifuged at 105,000 x g for 5 hrs. The pellets were mixed in 10 mM Mg(OAc)$_2$
buffered at pH 7.6 (buffer F) and stored at -20°.

**Treatment of Ribosomes with Periodate (HIO₄)**

The WRib were incubated for two hours at 37° in a mild alkaline buffer (0.5 M Tris, pH 9.0). This process stripped the bound tRNA of amino acids (273, 294). The liberated amino acids were removed by dialysis against distilled water at 4°. The ribosomal pellet was obtained by centrifugation at 105,000 x g and mixed in a buffer containing 100 mM KOAc, pH 5.0 plus 1.1 μmoles NaIO₄/mg RNA. This mixture was incubated at room temperature for 45 minutes in the dark. After the incubation period 1.0 ml of glycerol was added to reduce any unreacted periodate and the mixture was reincubated for an additional 30 mins. The mixture was spun at 105,000 x g and the pellet mixed in 10 mM Mg(OAc)₂, buffered at pH 7.6 (buffer F).

**Analytical Polyacrylamide Gel Electrophoresis**

(a) A modification of the Davis system was used (221). The following stock solutions were prepared and stored in brown bottles in the cold room.

Stock Solutions

<table>
<thead>
<tr>
<th>(A)</th>
<th>(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1N HCl</td>
<td>48 ml</td>
</tr>
<tr>
<td>Tris</td>
<td>36.6 gm</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.23 gm</td>
</tr>
<tr>
<td>8M urea to 100 ml (pH 8.9)</td>
<td>8M urea to 100 ml (pH 6.7)</td>
</tr>
<tr>
<td>Tris</td>
<td>5.98 gm</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.46 gm</td>
</tr>
<tr>
<td>8M urea to 100 ml (pH 6.7)</td>
<td>8M urea to 100 ml (pH 6.7)</td>
</tr>
</tbody>
</table>
Stock Solutions continued

(C)  
acrylamide  28.0 gm  
Bis  0.735 gm  
8M urea to 100 ml  

(D)  
acrylamide  10.0 gm  
Bis  2.5 gm  
8M urea to 100 ml  

(E)  
riboflavin  4 mg  
8M urea to 100 ml  

(F)  
sucrose  40 gm  
8M urea to 100 ml  

The following working solutions were prepared the day of the run and then discarded.

<table>
<thead>
<tr>
<th>Small-pore solution #1</th>
<th>Small-pore solution #2</th>
<th>Large-pore solution</th>
<th>Stock buffer solution for reservoirs*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 part A</td>
<td>ammonium persulfate</td>
<td>2 parts B</td>
<td>Tris 6.0 gm</td>
</tr>
<tr>
<td>2 parts C</td>
<td>0.14 gm</td>
<td>4 parts D</td>
<td>glycine 28.8 gm</td>
</tr>
<tr>
<td>1 part 7M urea</td>
<td>7M urea to 100 ml</td>
<td>2 parts E</td>
<td>water to 1 liter</td>
</tr>
<tr>
<td>pH 8.9</td>
<td></td>
<td>2 parts F</td>
<td>pH 8.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* diluted 1:10 with water

The gels were prepared in cylindrical glass tubes, 0.5 x 10 cms. The clean glass tubes were first placed in an upright position in a tube stand. Stands are conveniently made by cementing hollow rubber stoppers, for example, the B-D Vacutainer stoppers, in a single row, a few cms apart, with the closed end down, to a flat piece of plastic. The open end of the cap should fit snugly around the gel tube to prevent leakage of the ingredients. The large pore solution was prepared and run into the glass tubes. A water layer was then placed on top of the gel solution in such a manner as not to disturb the gel surface. The tube stand was now placed directly under a fluorescent bulb for about 30 mins to allow for photopoly-
merization. Following photopolymerization the water layer was removed and the gel tubes completely filled with small-pore gel solution, prepared by mixing equal volumes of small-pore solutions #1 and #2. The gels were allowed to polymerize while being protected from strong light.

Following polymerization of the gels, the glass tubes were removed from the tube stand and placed in the cold room on an electrophoretic apparatus, as illustrated by Davis (221), containing the appropriate buffer. The electrodes were attached to the apparatus and to a power supply set at a constant current of 5 mA/tube. Prior to turning on the power supply, a drop or two of 0.001% bromophenol blue was added to serve as the marker dye.

After the run the glass tubes were removed from the electrophoretic apparatus and the gels were subsequently removed by rimming the tubes under water with a wire. The gels were stained for 1 hr in a 15% HAc solution containing 2% acridine orange (specific for nucleic acids) and 1% lanthanum acetate (fixative) (222). The gels were destained overnight in 15% HAc and then stored in 7% HAc in the cold room.

(b) A modification of the Dingman and Peacock system was used (223-225). The following working solution for the preparation of a 10% polyacrylamide gel was prepared the day of the run and then discarded:
7.9 ml acrylamide mixture (19.5% acrylamide + 0.5% Bis)
4.65 ml H$_2$O
0.95 ml DMAPN (6.4%)
1.5 ml buffer (undiluted) (Tris 108 gm, EDTA diNa 9.3 gm, Boric Acid 55 gm: to 1 liter with H$_2$O, pH 8.3)
10 mg ammonium persulfate

The gels were prepared in cylindrical glass tubes, 0.5 x 10 cms. The glass tubes were first placed in an upright position in a tube stand. The gel mixture was prepared, mixed and run into the glass tubes for a distance of 7 cms. A water layer was then placed on top of the gel solution in such a manner as not to disturb the gel surface and the gels were allowed to polymerize. After polymerization, the water layer was removed, and the glass tubes were filled with diluted buffer (1:10 with H$_2$O). The tubes were then removed from the tube stand and placed in the cold room on an electrophoretic apparatus, as described in (a), containing the same diluted buffer. The tubes were then preelectrophoresed for 30 mins at 5mA/tube.

After preelectrophoresis, the buffer was removed from the glass tubes and up to 200 µl of sample in 10% sucrose, in the presence of a drop or two of bromophenol blue, was layered onto the gel surface. The rest of the glass tube was filled with diluted buffer so as not to disturb the sample layer and then placed in the cold room on the electrophoretic apparatus. The electrodes were attached and electrophoresis was carried out at 10 volts per cm length of glass tube.

After the run the gels were obtained as described in (a)
and then stained for 1 hr in 0.2% methylene blue dissolved in an acetate buffer (0.4 M NaOAc, 0.4 M HAc, pH 4.7). The gels were destained overnight in distilled water and then stored in distilled water in the cold room.

This was found to be the best method for the separation of low molecular weight RNA and was subsequently used for the identification of these RNAs in the Results section.

(c) A modification of the method of Moriyama et al. (195) was used. The only difference between this procedure and that used in (b) was the type of gel preparation.

Preparation of the gel

The following working solution for the preparation of a 10% polyacrylamide gel was prepared the day of the run and then discarded.

0.5 ml of 10% ammonium persulfate and 10 μl TEMED was added to 25 ml of 10% gel solution (9.75% acrylamide + 0.25% Bis in 40 mM Tris, 20 mM NaOAc, 2 mM EDTA, pH 7.4, 25°) previously degassed for a few seconds.

Preparative Polyacrylamide Gel Electrophoresis

A Canalco preparative disc electrophoresis apparatus with a PD-2/320 upper column was used (194). The following stock solutions were prepared and stored in brown bottles in the cold room.

(A)                      (C)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1N HCl</td>
<td>48 ml</td>
<td>acrylamide</td>
<td>28.0 g</td>
</tr>
<tr>
<td>Tris</td>
<td>36.6 g</td>
<td>Bis</td>
<td>0.735 g</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.23 g</td>
<td>8M urea to 100 ml</td>
<td>8M urea to 100 ml</td>
</tr>
<tr>
<td>8M urea (pH 8.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The following working solutions were prepared the day of the run and then discarded.

<table>
<thead>
<tr>
<th>Small-pore solution #1</th>
<th>Small-pore solution #2</th>
<th>Elution buffers</th>
<th>Stock buffer solutions for electrode compartments*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 part A</td>
<td>ammonium persulfate</td>
<td>(1) 1 part A</td>
<td>(1) Tris 6.0 g glycine 28.8 g</td>
</tr>
<tr>
<td>2 parts C</td>
<td>0.14 g</td>
<td>7 parts H₂O</td>
<td>H₂O to 1 liter, pH 8.3</td>
</tr>
<tr>
<td>1 part 7M urea</td>
<td>7M urea to 100 ml</td>
<td>(2) 40 mM Tris</td>
<td></td>
</tr>
<tr>
<td>pH 8.9</td>
<td></td>
<td>20 mM NaOAc</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mM EDTA,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 7.2</td>
<td></td>
</tr>
</tbody>
</table>

*diluted 1:10 with H₂O

In order to prepare the gel, the bottom end of the upper column was capped with a square of Saran Wrap. The column was clamped in a vertical position with the bottom end resting on a flat surface. The capped upper column was first filled to the mark with small-pore gel solution, prepared by mixing equal volumes of small pore solutions #1 and #2 and then a water-layer was placed on top of the gel solution in such a manner as not to disturb the gel surface. The column was then placed between two fluorescent bulbs.

Following polymerization the water layer was removed along with the Saran Wrap and the upper column was filled into the assembly. The sample in 10% sucrose was carefully layered
on top of the gel surface. The upper electrode buffer was then slowly added, so as not to disturb the gel surface, until the upper column reservoir was filled. The lower electrode reservoir was also filled with the appropriate buffer and then both electrodes were connected to a power supply set at a constant current of 7mA. The appropriate elution buffer was pumped in and out at a flow rate of 60 ml per hr, and collected by means of a fraction collector. The system was kept at 4° by means of two separate cooling systems. A marker dye was not used in order to avoid the possible effects of its contribution to the A260 readings of the collected effluent. The run was terminated when the A260 readings of the effluent had become negligible.

Preparation of 30S and 50S subunits from Ribosomes

The preparation of ribosomal subunits was essentially that of Cannon et al. (226). The ribosomal pellet was mixed with buffer K (5 mM Tris, pH 7.3 + 0.1 mM Mg(OAc)2) and dialyzed against the same buffer for 28 hrs. The ribosomal mixture was then put on a 10-30% discontinuous sucrose gradient which contained equal aliquots of 10, 15, 20, 25 and 30% sucrose prepared in the same buffer and centrifuged at 25,000 rpm for 12 hrs at 4° in a Model L ultracentrifuge using a SW-39 rotor. After centrifugation, fractions were collected through the use of the Beckman fraction recovery system. In this procedure each tube was placed in a tube holder and a recovery cap was screwed on top of the holder to
maintain an air-free system. The tube was pierced from the bottom by a needle, dense sucrose was pumped in slowly through the needle so as not to disturb the gradient and fractions were collected from a rubber tube leading from the recovery cap.

**Preparation of CsCl Gradient**

The method was essentially that of Meselson et al. (227) and Traub and Nomura (60). Each of the ribosomal subunits was centrifuged through a CsCl gradient. The ribosomal subunits were dissolved in a buffer made up of 20 mM Tris, 40 mM MgCl$_2$, pH 7.6. One ml of each subunit suspension was mixed with 4.3 ml of 61% (w/v) CsCl dissolved in the same buffer. The resulting mixture had an index of refraction $n_2^\circ = 1.3950$. Approximately 5.0 ml of this mixture was placed in a Lusteroid centrifuge tube in a Spinco SW-39 rotor. To insure the stability of the subunits, it was found necessary to wash the Lusteroid tubes. This was done for 1 hr in boiling 1 mM EDTA and then in boiling water. Centrifugation was carried out at 36,000 rpm for 36 hrs at 4°. After slow deceleration, each Lusteroid tube was withdrawn and placed in a tube holder (Beckman). The tubes were pierced from the bottom with a needle and fractions were collected by gravity.

**Isolation of Total RNA from Ribosomes**

Ribosomes were mixed with buffer L (10 mM Tris, pH 7.6, 10 mM Mg$^{++}$ + 0.5% sodium dodecyl sulfate (SDS)) and an equal
volume of water-saturated phenol for 20 mins at room
temperature. The aqueous layer was collected after centri-
fugation at 5,000 x g for 10 mins. The phenol layer was mixed
with half a volume of buffer for 20 mins. The aqueous layer
was recovered after centrifugation and pooled with the
initial aqueous layer. It was then mixed with an equal volume
of phenol for 20 mins at room temperature. The aqueous layer
was recovered after centrifugation and the phenol removed by
ether extraction. Residual ether was removed by bubbling N₂
through the solution. Sodium acetate (1.5 M, pH 5.2) was
added to give a 2% solution and the RNA precipitated by
addition of 3 volumes of cold ethanol. The total RNA was
collected by centrifugation and dissolved in either 10 mM
Mg(OAc)₂ buffered at pH 7.6 (buffer F) or water.

Preparation of High and Low Molecular Weight RNA from Ribosomes

The total RNA from ribosomes was prepared as described
above. The RNA was dissolved in 0.1 M Tris, pH 7.5 and then
this mixture was made 2 M with respect to NaCl and kept at
4° for 2 days. The precipitate containing the high molecular
weight RNA was separated from the supernatant containing low
molecular weight RNA by low speed centrifugation. In order
to ensure a better separation of high and low molecular
weight material, the RNA precipitate was dissolved in the
above buffer and reprecipitated when the buffer was made 2 M
with respect to NaCl. The precipitate, containing high
molecular weight RNA, was dissolved in 50 mM NaCl and stored
at -20°. The supernatant was made 10 mM with respect to Mg(OAc)₂ and then NaOAc (1.5 M, pH 5.2) was added to give a 2% solution. The RNA was subsequently precipitated with 3 volumes of cold ETOH. The low molecular weight RNA was collected by centrifugation and dissolved in 50 ml NaCl. It was subsequently further characterized on Sephadex G-100 and by preparative gel electrophoresis.
CHART II*

Procedures for preparing E. coli ribosomes

E. coli
a) ground with glass beads in buffer A (10 mM Mg(OAc)₂)
b) centrifuged at 10,000 x g

cellular debris
a) incubated with DNase I
b) centrifuged 2x at 30,000 x g

precipitate
a) mixed with buffer G
b) centrifuged at 105,000 x g

c) steps a) and b) repeated 2x

ribosomal pellet
a) mixed with buffer H
b) centrifuged at 105,000 x g

ribosomal suspension (RSII)

ribosomal suspension
a) mixed with buffer B (0.1 mM Mg(OAc)₂)
b) centrifuged at 105,000 x g

ribosomal pellet
a) mixed with (S100)
b) centrifuged at 105,000 x g

ribosomal pellet
a) mixed with buffer C (1 mM Mg(OAc)₂)
b) centrifuged at 105,000 x g

ribosomal pellet
a) mixed with buffer D (5 mM Mg(OAc)₂)
b) centrifuged at 105,000 x g
c) repeated a) and b) 3x

ribosomal pellet
a) mixed with buffer E (10 mM Mg(OAc)₂)
b) centrifuged at 105,000 x g
c) repeated a) and b) 3x

ribosomal pellet
a) mixed with buffer F (10 mM Mg(OAc)₂)

ribosomal suspension
a) Put on DEAE-cellulose column equilibrated with buffer M and wash with same buffer
b) eluted with buffer M

ribosomal suspension
a) mixed with buffer F
b) centrifuged at 105,000 x g

ribosomal pellet
a) mixed with buffer F
b) centrifuged at 105,000 x g

ribosomal suspension

* Buffer A-H, M-N described previously in List of Buffers.
TABLE I

The activity of ribosomes prepared by different methods

<table>
<thead>
<tr>
<th>Ribosome preparation*</th>
<th>Concentration A$_{260}$ units</th>
<th>Activity**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WRib</td>
<td>50</td>
<td>29</td>
</tr>
<tr>
<td>RSI</td>
<td>52</td>
<td>74</td>
</tr>
<tr>
<td>WRib</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>RSI</td>
<td>18</td>
<td>33</td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>³H-WRib***</td>
<td>11.6</td>
<td>23</td>
</tr>
<tr>
<td>RSII</td>
<td>10.9</td>
<td>20</td>
</tr>
<tr>
<td>RSIII</td>
<td>9.6</td>
<td>24</td>
</tr>
<tr>
<td>WRib</td>
<td>10.0</td>
<td>2</td>
</tr>
</tbody>
</table>

* The description of the preparations are given in Chart II.

** Activity determined by following the incorporation of $^{14}$C-Phe in a poly U-directed synthesis of polyphenylalanine described in Materials and Methods. The activity of the blank containing no ribosomes was set at one (200 cpm).

*** This preparation is identical to WRib except the cells originated from a pyrimidine-requiring mutant of *E. coli* B (ATCC 13135) grown in the presence of $^3$H-uracil.
RESULTS

The Activity of Ribosome Preparations

The ribosomes prepared as outlined in Materials and Methods were found to have an amino acid acceptor activity 50-100 fold over the blank level which was set at one. The \( A_{260}/A_{280} \) ratio of these ribosomes was found to be 2:1 and was comparable and in many cases greater to other reported preparations (282). These ribosomes (WRib) were subsequently used in all future experiments. Ribosomes were prepared by three other procedures as outlined in Chart II and compared with WRib with respect to activity (Table I) and bound low molecular weight RNA (Figure 1).

The RSI (Table I) which had been put through a DE-22 column was much more active than WRib. This activity was still less than that of the original fresh preparation prepared two months earlier which showed 82-fold amino acid acceptor activity over the blank level. In experiment I, Table I, the activity of the ribosomes increased as the concentration of the ribosomes (measured in \( A_{260} \) units) increased.

In experiment II, Table I, freshly prepared labelled WRib from a pyrimidine-requiring \( E. \ coli \) mutant had only a 23-fold increase in activity over the blank. Perhaps this was due to the type of cell used, the stage of growth at which the cells were harvested or the manner in which the ribosomes were prepared (see Materials and Methods). At the particular concentration used, the \(^3H-\)WRib, RSII and RSIII had virtually
FIGURE 1

Photograph of polyacrylamide gel (10% gel, pH 8.3, and 0.5 x 7 cm) electrophoresis patterns of low molecular weight RNA obtained from different preparations of \textit{E. coli} ribosomes. From left to right *: WRib, RSI, RSII, RSIII and the control (commercial \textit{E. coli} tRNA).

* The abbreviations are those designated in Chart II.

The 4S and 5S RNA regions indicated have been predetermined by numerous investigators and verified by the use of appropriate standards (223-225).
the same activity. The WRib which showed only slight activity was the same as that used in the first experiment, Table I, except that it had been thawed and frozen one more time. Perhaps this was one of the reasons for its drastically lowered activity.

Figure 1 shows the polyacrylamide gel electrophoresis patterns of the low molecular weight RNA obtained from the various ribosome preparations outlined in Chart II. There appears to be much less 4S and 4.5S RNA* bound to ribosomes which have been washed through a DEAE-cellulose column with 1 M NH₄Cl. This may be correlated with the increased ribosomal activity observed under these conditions (Table I). The region designated X may be 6S RNA.

Attempts to Remove Bound tRNA from Ribosomes

The WRib were treated under various conditions in an effort to remove all the bound tRNA.

(a) Puromycin (PM)

The WRib were treated with PM (referred to as PM-WRib) as described in Materials and Methods. After the incubation period, the PM was removed with 1 M NH₄Cl. The capacity of these PM-WRib to support protein synthesis was found to be at least as high as the untreated ones and in some cases the activity was found to be considerably higher. Figure 2 shows the

---

* This species of RNA (4.5S) has been found to have a mobility greater than 5S RNA but less than 4S RNA (194). Transfer RNA has been found to have the same mobility as 4S RNA and is considered to be synonymous with 4S RNA. 5S RNA was identified by its mobility relative to 4S RNA in 10% polyacrylamide gels (223-225).
Photograph of polyacrylamide gel (10%, pH 8.3, and 0.5 x 7 cms) electrophoresis patterns of low molecular weight RNA obtained from puromycin-treated *E. coli* ribosomes (PM-WRib). From left to right: PM-WRib (preparation B in Materials and Methods), PM-WRib (preparation C), PM-Mg treated WRib (preparation A), PM-Mg treated WRib (preparation A but with twice the PM concentration) and the control (commercial *E. coli* tRNA).
separation of low molecular weight RNA from the various PM-WRib on polyacrylamide gels. These PM-WRib were found to contain bound tRNA.

In some experiments after the incubation period with PM, these PM-WRib were subsequently suspended in 0.1 mM Mg(OAc)₂ buffered at pH 7.6 (buffer I) and dialyzed against the same buffer (referred to as PM-Mg treated WRib). These PM-Mg treated WRib were found to have very low ability to support protein synthesis and the results in Figure 2 showed that there was only a trace amount of tRNA bound to these PM-Mg treated WRib. This experiment was repeated using a higher PM concentration, 1 mM and then these PM-WRib were subsequently suspended and dialyzed against buffer I. A negligible amount of bound tRNA was detected in these PM-Mg treated WRib (Figure 2). These ribosomes were also found to have very low amino acid acceptor activity.*

(b) Periodate

WRib were treated with HIO₄ in 100 mM KOAc, pH 5.0 after prior mild alkaline hydrolysis to strip amino acids from bound tRNA as described in Materials and Methods. Unfortunately HIO₄ completely disrupted the ribosomal structure such that the characteristic ribosomal pellet could not be obtained even after extended periods of ultracentrifugation. Therefore this procedure had to be abandoned (273).

* Prior to the activity studies the PM was readily removed from the WRib by incubation in 1 M NH₄Cl which breaks the amide linkage between the PM and the WRib.
FIGURE 3

Photograph of polyacrylamide gel (10%, pH 8.3 and 0.5 x 7 cm) electrophoresis patterns of low molecular weight RNA obtained from Mg-treated E. coli ribosomes. From left to right: Mg-treated WRib (0.1 mM Mg^{++}), Mg-treated WRib (0.1 mM Mg^{++} followed by 10 mM Mg^{++}) and the control (commercial E. coli tRNA).

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FIGURE 4

The sedimentation pattern of *E. coli* ribosomes on a 10-30% discontinuous sucrose gradient. The ribosomes (200A_{260} units) were first suspended in buffer K, then dialyzed against the same buffer and subsequently centrifuged through a sucrose gradient at 25,000 rpm for 12 hrs at 4°. One ml fractions were collected. A complete description is given in Materials and Methods.

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(c) **Dialysis against 0.1 mM Magnesium**

WRib were mixed in a buffer containing 5 mM Tris, pH 7.4 plus 0.1 mM Mg(OAc)$_2$ and dialyzed against the same buffer for 2 days with frequent changes in the buffer. The ribosomal pellet was collected by centrifugation, mixed with 10 mM Mg(OAc)$_2$ buffered at pH 7.6 (buffer F) and an aliquot was stored at -20°. The rest of the suspension was dialyzed against the same buffer overnight and then subsequently frozen at -20°. Electrophoresis in a 10% polyacrylamide gel of the low molecular weight RNA from these Mg$^{++}$-treated WRib is shown in Figure 3. The WRib dialyzed only against low Mg$^{++}$ showed a strong 5S RNA band but very faint 4.5S and 4S RNA bands relative to the 5S RNA. The WRib dialyzed against low and then high Mg$^{++}$ showed a dense 5S RNA band and a strong 4.5S RNA band but negligible 4S RNA band relative to the 5S RNA. It appears that dialysis against low Mg$^{++}$ removes virtually all the tRNA normally bound to WRib.

**Studies on the 30S And 50S Ribosomal Subunits**

*E. coli* ribosomal subunits were prepared as described in Materials and Methods. Figure 4 shows the separation of the subunits on a 10-30% discontinuous sucrose gradient. Fractions were collected from the top as described in the text. The 4S RNA was identified as tRNA by the fact that it had amino acid acceptor activity. Figure 5 shows the results of electrophoresis of the RNA from the subunits in a 10% polyacrylamide gel. The gel containing only the RNA from the 30S ribosomal
FIGURE 5

Photograph of polyacrylamide gel (10%, pH 8.3 and 0.5 x 7 cm) electrophoresis patterns of low molecular weight RNA obtained from *E. coli* ribosomal subunits. From left to right: 30S RNA*, CsCl-treated 30S RNA**, 50S RNA*, 50S RNA (5% gel)*, 50S RNA*, CsCl-treated 50S RNA**, tRNA from sucrose gradient and the control (commercial *E. coli* tRNA).

* Represents the total RNA from the 30S and 50S ribosomal subunits respectively.

** The 30S ribosomal subunit was centrifuged through a CsCl gradient as described in Materials and Methods and then the total RNA was isolated from these subunits as previously described. The 50S ribosomal subunit was treated similarly.

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FIGURE 6

Sedimentation pattern of \textit{E. coli} ribosomal subunits in a CsCl solution. The subunits were prepared as described in Figure 4 and then centrifuged at 36,000 rpm for 36 hrs at 4\degree \text{C} in 61\% (w/v) CsCl as described in Materials and Methods.

A. Represents the sedimentation of 30S ribosomal subunits (69\text{A}_{260} \text{ units}) through CsCl. One ml fractions were collected and 5 \text{\mu l} aliquots were removed for the absorbance readings.

B. Represents the sedimentation of 50S ribosomal subunits (483\text{A}_{260} \text{ units}) through CsCl. One ml fractions were collected and 5 \text{\mu l} aliquots were removed for the absorbance readings.

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Elution pattern of 100 mg of commercial *E. coli* tRNA from Sephadex G-100. The RNA was chromatographed on a 3 x 200 cm column and eluted with 50 mM NaCl at a flow rate of 6 ml/hr. Five ml fractions were collected and one ml aliquots were removed for absorbance readings.
subunit shows only one dense region at the top of the gel indicating that it contains only high molecular weight RNA. The gel containing only the RNA from the 50S ribosomal subunit also has a dense region at the top of the gel but in addition a double 5S RNA band with what appears to be a trace of 4.5S and 4S RNA. The gel containing the tRNA fraction from the sucrose gradient also shows a distinct band in the 5S region.

The subunits were each put through a CsCl gradient as described in Materials and Methods. Figure 6 shows the sedimentation density pattern of each of the CsCl-treated subunits. Each shows a single peak. The CsCl-treated subunits were then electrophoresed in a 10% polyacrylamide gel. The results are shown in Figure 5. The gel containing only the RNA from CsCl-treated 30S ribosomal subunits shows a number of bands from the top of the gel to the 5S RNA region where there appears to be a trace band. The corresponding gel containing the RNA from CsCl-treated 50S ribosomal subunits shows a strong 5S RNA band and definite 4.5S and 4S RNA bands.

**Characterization of the Ribosomes**

Figure 7 shows the optical density pattern of commercial *E. coli* tRNA (General Biochemicals) fractionated on a long Sephadex G-100 column (200 x 3 cm). The first peak is high molecular weight RNA, the second peak 5S RNA and the third peak, the major peak, contained the 4S RNA material. Figure 8 shows a 10% polyacrylamide gel pattern of peaks II and III
FIGURE 8

Photograph of polyacrylamide gel (10%, pH 8.3 and 0.5 x 7 cm) electrophoresis patterns of different fractions of commercial \textit{E. coli} tRNA chromatographed previously on a Sephadex G-100 column (see Figure 7). From left to right: peak II, peak III and the control (commercial \textit{E. coli} tRNA).

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Elution pattern of total ribosomal RNA (8300A₂₆₀ units) from *E. coli* on Sephadex G-100. The RNA was fractionated on a 3 x 200 cm column and eluted with 50 mM NaCl at a flow rate of 6 ml/hr. Tubes 1-25 contain 20 ml and all subsequent tubes contain 5 ml fractions. One ml aliquots were removed for absorbance readings. Solid line: A₂₆₀; dotted line: ¹⁴C- amino acid acceptor activity.
TABLE II

The Distribution of RNA in *E. coli* Ribosomes

<table>
<thead>
<tr>
<th>Peak</th>
<th>Fraction Number*</th>
<th>Total A$_{260}$ Units</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20 - 43</td>
<td>7897</td>
<td>97.2</td>
</tr>
<tr>
<td>II</td>
<td>45 - 64</td>
<td>128</td>
<td>1.5</td>
</tr>
<tr>
<td>III</td>
<td>65 - 120</td>
<td>103</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* The fractions are those shown in Figure 9.
from Figure 7. The gels were run using an aliquot from the combined fractions within each peak region. The 4S RNA fraction (peak III) is well separated from the 5S RNA fraction (peak II) although it does contain a trace of 4.5S RNA. The 5S RNA fraction also contains the major part of the 4.5S RNA and a trace of 4S RNA material. The reason trace amounts of other types of RNA were found in both peaks was because peak tubes were not used in the electrophoresis runs. This will be clearly demonstrated further on in the thesis.

Figure 9 shows the fractionation of the total RNA obtained from *E. coli* WRib run on the same Sephadex G-100 column. The major peak is high molecular weight ribosomal RNA, the second peak contains 5S rRNA and the third peak, 4S RNA as confirmed by acceptor studies. The last peak contained residual phenol left over from the procedure used to obtain the total ribosomal RNA which was described in Materials and Methods. Table II shows the amount of RNA in each peak region. The $A_{260}$ ratio of 4S : 5S RNA is 0.87 which indicates that there are approximately 1.3 molecules of 4S RNA bound to the ribosomes per molecule of 5S RNA*. Figure 10 shows the separation of peaks II and III from Figure 9 by electrophoresis in 10% polyacrylamide gels. The gels were run using an aliquot

---

* If one assumes that one molecule of 5S RNA is bound per ribosome and that in this population of ribosomes one molecule of 5S RNA represents $128A_{260}$ units, then the number of molecules of 4S RNA bound can be easily calculated given the molecular weight of 5S and 4S RNA as $4 \times 10^3$ and $2.5 \times 10^3$ respectively.
FIGURE 10

Photograph of polyacrylamide gel (10%, pH 8.3 and 0.5 x 7 cm) electrophoresis patterns of peaks II and III as designated in Table II. From left to right: peak II, peak III and the control (commercial E. coli tRNA).

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TABLE III

Specific amino acid acceptor activity of tRNA bound to E. coli ribosomes

<table>
<thead>
<tr>
<th>14C-amino acid</th>
<th>Ribosomal bound tRNA p moles/A260 unit</th>
<th>Total tRNA* p moles/A260 unit</th>
<th>Numbers of known specific tRNAs in E. coli**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>13.4</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Arg</td>
<td>60.0</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Asn</td>
<td>46.4</td>
<td>(19.0)</td>
<td>3</td>
</tr>
<tr>
<td>Asp</td>
<td>43.4</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Gly</td>
<td>16.1</td>
<td>(15.2)</td>
<td>3</td>
</tr>
<tr>
<td>Glu</td>
<td>37.8</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>His</td>
<td>45.7</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Ile</td>
<td>14.8</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Leu</td>
<td>109.4</td>
<td>106.9</td>
<td>5</td>
</tr>
<tr>
<td>Lys</td>
<td>108.5</td>
<td>(59.0)</td>
<td>2</td>
</tr>
<tr>
<td>Met</td>
<td>209.0</td>
<td>73.6</td>
<td>2</td>
</tr>
<tr>
<td>Phe</td>
<td>65.5</td>
<td>42.7</td>
<td>3</td>
</tr>
<tr>
<td>Pro</td>
<td>33.0</td>
<td>(68.9)</td>
<td>4</td>
</tr>
<tr>
<td>Ser</td>
<td>26.3</td>
<td>72.3 (43.4)</td>
<td>4</td>
</tr>
<tr>
<td>Thr</td>
<td>65.1</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Trp</td>
<td>383.0</td>
<td>27.3</td>
<td>5</td>
</tr>
<tr>
<td>Tyr</td>
<td>12.7</td>
<td>43.6 (29.3)</td>
<td>2</td>
</tr>
<tr>
<td>Val</td>
<td>163.6</td>
<td>(76.4)</td>
<td>2</td>
</tr>
</tbody>
</table>

* Results shown in brackets were done in this laboratory by R. Chase. The other results in this column are those of Bartz et al. (291). Total RNA refers to cytoplasmic and ribosomal bound tRNA.

** References 274, 275.

3 See Appendix, page 163.
Elution pattern of the low molecular weight RNA (1368A$_{260}$ units) bound to *E. coli* ribosomes from Sephadex G-100. The RNA was fractionated on a 3 x 200 cm column and eluted with 50 mM NaCl at a flow rate of 6 ml/hr. Five ml fractions were collected. Solid line: A$_{260}$; dotted line: $^{14}$C-amino acid acceptor activity.

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from the combined fractions shown in Table II. The results were identical to those found in similar peaks in Figure 8. The dotted lines in Figure 9 indicate the region of amino acid acceptor activity. The peak tubes were pooled and acceptor studies were done on all the amino acids except glutamine and cysteine. The results are shown in Table III. E. coli ribosomes have bound tRNA which has acceptor activity for all the amino acids. The amount of changing varies from one tRNA species to another.

Another experiment was carried out in order to get more definitive data on the distribution of the major low molecular RNA species bound to ribosomes. The total ribosomal RNA was isolated from WRib using the phenol technique. The recovery of rRNA by this procedure was 66.3% on the basis of the total A₂₆₀ units recovered from the starting material. The rRNA was dissolved in 100 mM Tris, pH 7.5 and the solution was made 2 M with respect to NaCl and kept at 4°C for a day. The supernatant, which contained the low molecular weight RNA, made up 9% of the total ribosomal RNA. This RNA was precipitated with cold ETOH, dissolved in 0.05 M NaCl, loaded into a Sephadex G-100 column (3 x 200 cm) and eluted with the same buffer. The results are shown in Figure 11. The major peak contained high molecular weight RNA, the second peak contained the 5S RNA and the minor peak, the 4S RNA as determined by acceptor studies. The dotted lines indicate the region of amino acid acceptor activity. The recovery of RNA from the
**TABLE IV**

Distribution of low molecular weight RNA in *E. coli* ribosomes isolated by Sephadex G-100 chromatography*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total $A_{260}$ units</th>
<th>% of Total</th>
<th>Actual % bound to ribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak I</td>
<td>656</td>
<td>50.7</td>
<td>95.5</td>
</tr>
<tr>
<td>Peak II</td>
<td>404</td>
<td>31.2</td>
<td>2.8</td>
</tr>
<tr>
<td>Peak III</td>
<td>230</td>
<td>18.1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* The data are from Figure 11.
FIGURE 12

Photograph of polyacrylamide gel (10%, pH 8.3 and 0.5 x 7 cm) electrophoresis patterns of the peak fraction of each peak region of Figure 11. From left to right: fraction 86, fraction 116, fraction 132, fractions 116 and 132 and the control (commercial E. coli tRNA).

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FIGURE 12a

Chromoscan tracing of a mixture of fractions 116 and 132 (peaks II and III) from Figure 11. Equal concentrations of both fractions were mixed.

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column was 95%. Table IV shows the distribution of RNA in these peak regions. The $A_{260}$ ratio of 4S : 5S RNA was approximately 0.58 : 1 which indicates that there is approximately 0.90 molecule of 4S bound to the ribosomes per molecule of 5S RNA*. The peak fraction in each peak region of Figure 11 was run on a 10% polyacrylamide gel. The results are shown in Figure 12. Peak I (Fraction 86) contained one major band which remained at the top of the gel and had presumably a molecular weight in excess of 6S. Peak II (fraction 116) contained 3 major bands - a double 5S RNA band and the 4.5S RNA band while peak III (fraction 132) contained the bands corresponding to 4S RNA. Equal concentrations of fractions 116 and 132 (peaks II and III) were combined and electro-phoresed in a 10% polyacrylamide gel (Figure 12). This gel was subsequently scanned in a Joyce-Loebl Chromoscan. The results are shown in Figure 12a. The 4S RNA appears as a probably double band. The material preceding 4S RNA is degraded RNA. Without taking the degraded RNA into account, the 4.5S RNA represents 2.7% of the total RNA. Even though this 4.5S RNA is spread throughout the 4 to 5S RNA region, it still represents much less than one molecule per molecule of 5S RNA.

Other Techniques used to Fractionate Low Molecular Weight RNA

(a) Preparative Gel Electrophoresis

Commercial E. coli tRNA (5 mg) was fractionated in a 10%

* If one assumes that one molecule of 5S RNA is bound per ribo-
some and that in this population of ribosomes one molecule of
5S RNA represents 404A_{260} units, then the number of molecules
of 4S RNA bound can be easily calculated given the molecular
weights of 5S and 4S RNA as $4 \times 10^3$ and $2.5 \times 10^3$ respectively.
Electrophoretic pattern in a 10% preparative polyacrylamide gel of 5 mg of commercial *E. coli* tRNA. The fractionation procedure is given in Materials and Methods. Flow rate was 60 ml/hr and 5 ml fractions were collected. One ml fractions were removed for absorbance readings.
FIGURE 14

Electrophoretic pattern of 100 mg of commercial *E. coli* tRNA in a 10% preparative polyacrylamide gel. The fractionation procedure is given in Materials and Methods.

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FIGURE 15

Chromatography of 100 mg of commercial *E. coli* tRNA on DEAE-Sephadex A-50. The RNA was eluted with a 0.45-0.60 M NaCl gradient buffered in 20 mM Tris, pH 7.6. The flow rate was 20 ml/hr and 3.2 ml fractions were collected.

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FIGURE 16

Photograph of polyacrylamide gel (10%, pH 8.3 and 0.5 x 7 cm) electrophoresis patterns of the peak fraction of each peak region of Figure 15. From left to right: fraction 90 (peak I), fraction 113 (peak II), fraction 173 (peak IV) and the control (commercial E. coli tRNA).

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polyacrylamide gel using a Canalco preparative gel electrophoresis apparatus as described in Materials and Methods. The results are shown in Figure 13. Aliquots from the peak fractions were then electrophoresed in a 10% polyacrylamide gel. The major peak contained 4S RNA, the second peak, 4S RNA and a trace of 5S RNA, the third peak, 4.5S RNA and the last peak, 5S RNA. Figure 14 shows the fractionation on a much larger scale where 100 mg of tRNA were used. Aliquots from the peak fractions were then electrophoresed in a 10% polyacrylamide gel. Peak I contained 4S RNA with a trace of 5S RNA, peak II contained 5S RNA, 4S RNA with trace amounts of 4.5S RNA and 5.8S RNA while peak III contained trace amounts of 4S and 5S RNA.

(b) DEAE-Sephadex Chromatography.

Commercial E. coli tRNA (100 mg) was loaded onto a DEAE-Sephadex A-50 column (0.9 x 120 cm) previously equilibrated with 0.45 M NaCl buffered in 20 mM Tris, pH 7.6. The tRNA was eluted with a 0.45-0.60 M NaCl gradient (283). The gradient was checked radiometrically by use of a conductivity meter. The results are shown in Figure 15. The optical density pattern is almost identical to that in Figure 13. The peak fractions were electrophoresed in a 10% polyacrylamide gel and the results are shown in Figure 16. Peak I contains virtually all the 4S RNA and some 4.5S RNA, peak II contains 5S RNA and some 4S RNA while peak IV contains only 5S RNA.

Since the preparative gel electrophoresis gave a much better separation of 4S RNA than the DEAE-Sephadex A-50, it
FIGURE 17

Electrophoretic pattern of the low molecular weight RNA (1940 $A_{260}$ units) from *E. coli* ribosomes in a 10% preparative poly-acrylamide gel. The fractionation procedure is given in Materials and Methods. Flow rate 60 ml/hr and 3 ml fractions were collected. Solid line: $A_{260}$; dotted line: $^{14}$C-amino acid acceptor activity.

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FIGURE 18

Photograph of polyacrylamide gel (10%, pH 8.3 and 0.5 x 7 cm) electrophoretic patterns of the peak fraction of each peak region of Figure 17. From left to right: fraction 26 (peak I), fraction 35 (peak II), fraction 70 (peak III) and the control (commercial E. coli tRNA).

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FIGURE 19

Spectrum of fraction 26 (peak I) of Figure 17. An aliquot was first chromatographed on Whatman No. 1 paper and a spectrum was run on the eluted spots (A and B) as described in the Results.
was used to carry out the fractionation of ribosomal RNA.

**Fractionation of Ribosomal RNA By Preparative Gel Electrophoresis**

The total low molecular weight ribosomal RNA was obtained as described in Materials and Methods and separated in a 10% preparative gel. The results are shown in Figure 17. The dotted lines indicate regions of amino acid acceptor activity. The main absorbance peak did not correspond to the main peak of acceptance activity. Aliquots from the fractions of peak absorbance were electrophoresed in a 10% polyacrylamide gel. The results are shown in Figure 18. The first peak contains 4S RNA, and a trace amount of 5S RNA. The second peak contains 4S RNA and some 5S RNA, and the last peak contains 5S RNA only.

Efforts were made to characterize the main peak since it was a region of comparatively low amino acid acceptor activity (a) An aliquot from the main peak was spotted onto Whatman No. 1 paper and the paper chromatogram was run for 16 hours at room temperature in a solvent system consisting of isobutyric acid : concentrated NH₄OH:H₂O (66:1:33). Two spots were obtained, one at the origin and a fluorescent spot which migrated beyond any of the standards (ADP, ATP GDP, GTP). The spots were cut out and eluted off the paper with H₂O using Heppel's technique (284). A spectrum was run on the eluted spots. The results are shown in Figure 19. Tracing A, the fluorescent spot does not show the typical RNA trace while tracing B, the spot at the origin, shows a more "typical" RNA
FIGURE 20

The effect of pH on the spectrum of fraction 26 (peak I) of Figure 17. A is at pH 11.0; B at pH 7.0; C at pH 1.0.

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trace. (b) A spectrum of the peak fraction at various pH values was run and the results are shown in Figure 20. There appears to be very little change in the spectrum with changes in pH at least as far as the λ max and λ min are concerned.

(c) A small amount of the peak fraction (7.2A₂₆₀ units) was put on a small DEAE-cellulose column (0.5 x 6 cm) and eluted with an (NH₄)₂CO₃ gradient. The principal fraction (2.8A₂₆₀ units) was eluted in the void volume. It had an λ max of 254 and an λ min of 244. The pH of the solution was 9.6. A minor fraction (0.5A₂₆₀ units) was eluted with approximately 1.9 M (NH₄)₂CO₃ but did not show a typical RNA spectrum. It was similar to that of tracing B, Figure 19. There was still approximately 50% of the sample unaccounted for. No further sample was eluted with 2 M (NH₄)₂CO₃ in 7 M urea, 0.1 N KOH or 1.0 N KOH. (d) Approximately 28A₂₆₀ units of the peak fraction was put on a DEAE-Sephadex A-50 column (0.9 x 32 cm) and eluted with a NaCl gradient (0.45 -1.0 M) in 20 mM Tris buffer containing 7M urea at pH 7.4. A single peak was eluted in the void volume which showed an absorbance tracing similar to the principal fraction of (c) above. Recovery was approximately 74%.

The principal fractions in (c) and (d) were electrophoresed in a 10% polyacrylamide gel but no definitive results could be obtained. At the present moment the characterization of the main peak remains to be established. Besides containing some tRNA, the principal peak must contain RNA of lower
FIGURE 21

Growth and pH curves of an *E. coli* pyrimidine-requiring mutant (ATCC 13135). The description of the growth medium is given in Materials and Methods. Solid line: cell growth, dotted line: pH.

Symbols:

- - - - - growth at 4 μg/ml uracil
- - - - - growth at 300 μg/ml uracil
- - - - - pH at 4 μg/ml uracil
- - - - - pH at 300 μg/ml uracil

The arrow indicates addition of one ml uracil (300 μg/ml).

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molecular weight since presumably this would be eluted first from the preparative gel. This low molecular weight RNA may just be degraded RNA and the presence of such a high concentration of this material may be due to the nature of the preparative gel procedure. This may be one of the pitfalls of the procedure and this technique was abandoned in favor of the demonstrated superiority of Sephadex G-100 as a means of fractionating low molecular weight RNA.

Exchange Experiments

An E. coli pyrimidine-requiring mutant (ATCC 13135) was grown in a minimal-salts medium in the presence of 4 µg uracil/ml as described in Materials and Methods. Figure 21 shows the growth curve of the mutant at two different uracil concentrations. When the cells were grown in the lower concentration of uracil, cell growth proceeded normally and then levelled off. Addition of the higher concentration of uracil resulted in an immediate marked increase in cell growth.

When the cells were grown in the higher concentration of uracil, cell growth also proceeded normally and then levelled off. The addition of the same concentration of uracil had no further effect on cell growth.

The pH of the medium decreased during cell growth but remained constant during the stationary periods regardless of the concentration of uracil in the medium.

Once the growth pattern of this mutant strain was esta-
FIGURE 22

Displacement of labelled tRNA bound to E. coli ribosomes. The E. coli mutant described in Figure 21 was used. The preparation of the ribosomes is described in Materials and Methods.

Symbols:
- Tube 1  ••• stripped tRNA + ATA
- Tube 2  Δ---Δ aa-tRNA + PM
- Tube 3  ■—■ stripped tRNA + ATA + PM
- Tube 4  ○···○ aa-tRNA + ATA + PM

Conditions of displacement:

At 0 wash each tube contained in a total volume of 10 ml, 2 mg of the particular unlabelled tRNA and 150A_{260} units WRib buffered in 10 mM Tris, 10 mM Mg(OAc)$_2$, pH 7.6. Tubes 1, 3 and 4 were made 70 μM with respect to ATA and tubes 2, 3, and 4 lmM with respect to PM. Tubes 1 and 2 were incubated for 30 mins at 0° and tubes 3 and 4 for 30 mins at 30°*.

After the second wash each tube was incubated at 30° in the presence of 10 mg of unlabelled tRNA.

After the fourth wash each tube was incubated at 24° for 20 mins in a solution containing 0.5 mM GTP, 3 mM ATP, 50 mM NH$_4$Cl, 750μl S100, 400μl S100A and 10 mg of unlabelled tRNA.

After the fifth wash each tube was incubated at 24° for 20 mins in a buffer containing 50 mM Tris, 5 mM Mg(OAc)$_2$, pH 7.6 and 10 mg of unlabelled tRNA.
FIGURE 22 - continued

After the sixth wash each tube was incubated at 37° for 30 mins in a solution containing 50 mM KCl and 10 mg of unlabelled tRNA.

After the seventh wash each tube was incubated at 0° with 0.1 mM Mg(OAc)$_2$ buffered at pH 7.3 (buffer K) and 10 mg of unlabelled tRNA.

* After each incubation period, the WRib obtained by centrifugation were resuspended in 10 ml of solution containing the particular components for displacement.

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blished the cells were grown in the same minimal-salts medium but this time 8 μg uracil/ml of medium was added. One milli-curie of uracil-6-\textsuperscript{3}H (9.16 μg) was also added to label the cells. The cells were grown to the late log phase and then harvested. Ribosomes were prepared in the usual manner and were found to be very active in a protein-synthesizing system (Table I). A preliminary experiment was carried out to study the conditions necessary to displace the labelled tRNA bound to these ribosomes. The results are shown in Figure 22. Displacement appears to be virtually complete after six washes regardless of the conditions used.

It was decided to carry out an exchange experiment in the same manner as was done by Cannon et al. (148). In this experiment, a buffer system is employed under certain conditions which enables exchange between unlabelled tRNA and labelled ribosomal bound tRNA to readily take place. The \textsuperscript{3}H-WRib were mixed in a buffer containing 10 mM Tris, 10 mM Mg\textsuperscript{++} at pH 7.4. Unlabelled \textit{E. coli} B tRNA (General Biochemicals) was added (15x the amount of labelled tRNA calculated to be present on the WRib) and the mixture was incubated at 0-4° for 30 mins with occasional stirring. After the incubation period the mixture was centrifuged at 105,000 x g for 5 hrs. The pellet was mixed with fresh buffer, an aliquot was removed to determine the amount of exchange that had taken place and then unlabelled tRNA was added and the incubation repeated. The washings were repeated until
TABLE V

Exchange of labelled tRNA bound to

*E. coli* ribosomes with unlabelled tRNA

<table>
<thead>
<tr>
<th>No. of washings</th>
<th>Ribosomal pellet** (cpm)</th>
<th>Non-pelleted ribosomes* (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33884</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>25809</td>
<td>57187</td>
</tr>
<tr>
<td>2</td>
<td>23548</td>
<td>4663</td>
</tr>
<tr>
<td>3</td>
<td>18333</td>
<td>31026</td>
</tr>
<tr>
<td>4</td>
<td>15080</td>
<td>16968</td>
</tr>
<tr>
<td>5</td>
<td>14974</td>
<td>3579</td>
</tr>
<tr>
<td>6</td>
<td>13728</td>
<td>1047</td>
</tr>
</tbody>
</table>

* The non-pelleted ribosomes were put through a Millipore filter (1.2 μ). Ribosomes with bound tRNA remained on the filter (290). The filter was dried and counted in a toluene scintillation mix.

** The ribosomal pellet was mixed with 10 ml of buffer (see Text) and a 5 μl aliquot was counted in a dioxane scintillation mix.
FIGURE 23

Exchange of labelled tRNA bound to E. coli ribosomes with unlabelled tRNA. Ribosomes were prepared as mentioned in Figure 22 from the E. coli mutant described in Figure 21. Details of the exchange conditions are given in the Results.
exchange was complete (Table V and Figure 23). According to the table a great number of counts were found in the supernatant (non-pelleted ribosomes). The extremely high levels of counts in some of the supernatants was probably due to the presence of some ribosomes resulting from incomplete centrifugation. Although exchange appeared to be virtually complete after four washes, two more exchanges were carried out until the loss of counts to the supernatant was minimal. This minimal loss was achieved by centrifuging the ribosomal mixture for extended periods of time to ensure that all of the ribosomes had been pelleted. These ribosomes were found to be inactive in a protein-synthesizing system.

Following the exchange experiment, the total ribosomal RNA was prepared from these $^3$H-WRib as described in Materials and Methods. This RNA was dissolved in 10 mM Mg(OAc)$_2$ buffered at pH 7.6 (buffer F) which was then made two molar with respect to NaCl. The high molecular weight RNA precipitated immediately and was collected by centrifugation while the low molecular weight RNA remained in the supernatant. The high molecular weight RNA was redissolved in buffer F and reprecipitated in 2 M NaCl. The supernatant was combined with the previous one. Sodium acetate (1.5 M, pH 5.2) was added to give a 2% solution and the low molecular weight RNA was subsequently precipitated by the addition of 3 volumes of cold ethanol. This low molecular weight RNA was put on a Sephadex G-100 column (200 x 3 cm) and eluted with 50 mM NaCl.
Elution pattern from Sephadex G-100 of the low molecular weight RNA (2000A$_{260}$ units) bound to labelled *E. coli* ribosomes after exchange with unlabelled tRNA. The ribosomes and the exchange conditions are described in Figure 23. The RNA was fractionated on a 3 x 200 cm column and eluted with 50 mM NaCl at a flow rate of 6 ml/hr. Five ml fractions were collected.
FIGURE 25

Photograph of polyacrylamide gel (10%, pH 8.3 and 0.5 x 7 cm) electrophoresis patterns of various fractions from Figure 24. From left to right: fractions 85, 106, 116, 121, 125, 130, 135, 142 and the control (commercial E. coli tRNA).

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TABLE VI

Distribution of labelled $^3$H-RNA in *E. coli* ribosomes after exchange with unlabelled tRNA

<table>
<thead>
<tr>
<th>Fraction</th>
<th>cpm/A$_{260}$ unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>high molecular weight</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>19465</td>
</tr>
<tr>
<td>5S RNA</td>
<td>2707</td>
</tr>
<tr>
<td>4S RNA</td>
<td>55</td>
</tr>
</tbody>
</table>
The results are shown in Figure 24. The first peak contained high molecular weight RNA while the rest contain the major part of the RNA as indicated. Aliquots of the peak fractions and a number of fractions between the second and third peaks were electrophoresed in 10% polyacrylamide gels. The results are shown in Figure 25. The first peak (fraction 85) also contains a small amount of 5.8S and 5S RNA. The second peak (fraction 116) contains only a trace of 4S RNA while the third peak (fraction 142) contains virtually all the 4S RNA and some 4.5S RNA. The A$_{260}$ ratio of 4S : 5S RNA had increased to almost 3.5 in ribosome exchanged with unlabelled tRNA. This is comparable to about 5.7 molecules of 4S RNA to one molecule of 5S RNA*. In untreated ribosomes the ratio of 4S : 5S RNA on a total RNA basis varied from 0.58 (Table IV) to 0.90 (Table II). This amounted to 1-2 molecules of tRNA bound per molecule of 5S RNA; a value which compared very favourably with the literature (286-289).

Aliquots of the 3 peaks fractions in Figure 24 were taken and the amount of labelled $^3$H-RNA was determined. The results are shown in Table VI. It appears that almost 100% exchange had taken place between unlabelled tRNA and the labelled tRNA originally bound to the ribosomes.

* If one assumes that one molecule of 5S RNA is bound per ribosome and that in this population of ribosomes one molecule of 5S RNA represents the total amount isolated in A$_{260}$ units, then the number of molecules of 4S RNA bound can be easily calculated given the molecular weights of 5S and 4S RNA as $4 \times 10^3$ and $2.5 \times 10^3$ respectively.
FIGURE 26

Chromatography on BD-cellulose of 4S RNA (140A$_{260}$ units) bound to E. coli ribosomes after exchange with unlabelled tRNA. Fractions 135-170 (Figure 24) were fractionated on a column (100 x 0.9 cm) with the indicated (dashed line) gradient of NaCl containing 10 mM MgCl$_2$ in a total volume of one liter. The flow rate was 42 ml/hr and 5 ml fractions were collected. At the indicated point, elution was continued with a 0-30% ethanol gradient in 1 M NaCl containing 10 mM MgCl$_2$ in a total volume of 400 ml. The flow rate was 42 ml/hr and 5 ml fractions were collected.

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FIGURE 27

Chromatography on BD-cellulose of 4S RNA (140A$_{260}$ units) normally bound to E. coli ribosomes. Fractions 126-140 (Figure 11) were fractionated on a column (112 x 0.9 cm) with the indicated (dashed line) gradient of NaCl containing 10 mM MgCl$_2$ in a total volume of one liter. The flow rate was 42 ml/hr and 5 ml fractions were collected. At the indicated point, elution was continued with a 0-30% ethanol gradient in 1 M NaCl containing 10 mM MgCl$_2$ in a total volume of 400 ml. The flow rate was 42 ml/hr and 5 ml fractions were collected.

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The acrylamide gel analysis (Figure 25) of various fractions from the Sephadex G-100 column (Figure 24) indicated that fractions 135-170 contained virtually all the 4S RNA and some 4.5S RNA but were devoid of 5S RNA. These fractions were put on a BD-cellulose column (100 x 0.9 cm) previously equilibrated with 0.35 M NaCl in the presence of 10 mM Mg++. The RNA was eluted with a (0.35-1.0 M) NaCl gradient in a total volume of one liter. This was followed by a 0-30% ethanol gradient in 1 M NaCl and 10 mM MgCl₂. Total volume of this gradient was 400 ml. The results are shown in Figure 26. Four peak regions were isolated in the NaCl gradient although the second peak region is itself actually made up of 3 peaks. The ethanol gradient contains one definite peak and a shoulder which may actually be a second component. Figure 27 shows the pattern of low molecular weight RNA normally bound to *E. coli* ribosomes. It is somewhat different from the pattern shown in Figure 26. In place of the first peak (Figure 26) there is a shoulder. There is a sharp, well-defined second peak and a broader third peak which shows a small peak on its trailing edge. The peaks in the ethanol gradient were much better separated than in the previous experiment (Figure 26). Figure 28 shows the optical density pattern obtained when commercial *E. coli* tRNA was fractionated on BD-cellulose. The salt gradient eluted two definite peaks, the second having a slight shoulder which may indicate the possibility of a third component. The ethanol fraction contained only one component.
FIGURE 28

Chromatography on BD-cellulose of commercial \textit{E. coli} tRNA. The RNA (3650A$_{260}$ units) was fractionated on a column (106 x 1.5 cm) with the indicated (dashed line) gradient of NaCl containing 10 mM MgCl$_2$ in a total volume of three liters. The flow rate was 90 ml/hr and 20 ml fractions were collected. At the indicated point, elution was continued with 1.10 NaCl containing 10 mM MgCl$_2$ in 10\% (v/v) ethanol.

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but this may have been due to the fact that a gradient was not employed.

Aliquots were taken from every second fraction (Figure 26) to see if the labelled tRNA that remained bound to *E. coli* ribosomes after the exchange with unlabelled tRNA was spread evenly throughout the elution pattern, or whether it was concentrated in a certain region. It was found to be spread uniformly throughout the fractions eluted with the NaCl gradient. The radioactivity amounted to 25-50 cpm per $A_{260}$ unit. In the ethanol gradient the radioactivity reached a maximum level of 250 cpm per $A_{260}$ unit. An aliquot of the peak fraction was electrophoresed in a 10% polyacrylamide gel and was found to contain some 4.5S RNA. This may account for the high counts in this particular fraction. Another possibility is that according to Table III, tRNA$^\text{Trp}$ is bound to WRib in the highest amount and therefore it may not be as readily exchanged as the other tRNAs. Since tRNA$^\text{Trp}$ is found solely in the ethanol fraction (295) it therefore could account for the high counts. The former explanation seems to be more plausible since there is no reason to believe that one particular tRNA is less vulnerable to exchange than another.
DISCUSSION

The experiments described in the previous sections were carried out in an attempt to clarify the biological role of the low molecular weight RNAs associated with E. coli ribosomes. In particular, these experiments were designed to characterize the RNA bound to E. coli ribosomes which had undergone extensive washing procedures. Some of these RNAs were clearly identified but in most cases their function could not be established. Nevertheless, a method was found for preparing ribosomes devoid of all 4S components but no evidence was obtained for a specific 4S fraction which might function during protein synthesis in the chain termination mechanism. This result helps to confirm recent reports on the non-existence of a chain-terminating tRNA. The mechanism of chain termination in protein biosynthesis is still an unsolved problem. Present evidence suggests that there may be three nonsense or terminating codons, UAA, UAG and UGA. According to the Wobble Hypothesis (307), however, UGA may also code for Cys and Trp. No suppressor has yet been found which suppresses only ochre mutants (UAA), although suppressors exist which suppress both ochre and amber mutants (UA^G_A). Since UAA has been found to be the terminator codon for all the phage proteins thus far identified, it is possible that at least one specific terminating tRNA could be involved. However, the groups investigating this problem could not demonstrate such a tRNA species in the termination step.

Most of the information about the mechanism of chain
termination was obtained by the use of two assay systems. In one of these, Capecchi (43, 75) used as mRNA, the RNA from a mutant R17 phage, in which the seventh codon in the coat protein gene was a nonsense codon (UAG). The formation of the hexapeptidyl-tRNA was carried out as described previously (p. 29). The resulting hexapeptidyl-tRNA remained attached to the mRNA-ribosome complex. The release of free hexapeptide from this complex depended on a protein component, designated release factor (R factor) from the high speed supernatant (S100) of E. coli. Bretscher (84), using the same assay system, incubated the S100 supernatant under mild alkaline conditions and subsequently treated it with periodate to destroy the amino acid acceptor abilities of the endogenous tRNAs. He then added only those aa-tRNA species needed to form the hexapeptide plus the periodate-treated supernatant and got chain-termination.

Nirenberg's group (88), using a AUG·UAA·ribosome complex as the termination assay, was able to get chain termination to take place upon addition of the crude R factor. They subsequently found that the R factor was actually two different enzymes with different codon specificities (89, 91). Another protein, called S factor, which served to catalyze the termination reaction, was also isolated (90, 92).

Recently Ishitsuka and Kaji (109) isolated a TR factor (tRNA release factor) which they suggested worked hand-in-hand with the R factor—the R factor hydrolyzed the ester link between the peptide and the tRNA while TR displaced the
tRNA from its site on the ribosome.

Each of the groups working in this field stipulated in their discussions that their results were not conclusive evidence for the nonexistence of a chain-terminating tRNA. In contrast to the case of initiation where a specific tRNA is definitely involved, the requirement for a specific tRNA in termination has not, as yet, been shown but the present understanding of the problem is that such a tRNA is not required. The chain-terminating experiments outlined above in which highly purified tRNAs were used (84, 88), nevertheless were not properly controlled since it can be argued that the terminating tRNA could have remained bound to the ribosomes. These previous investigators failed to show that their ribosomes or ribosomal subunits were devoid of 4S RNA. This tRNA could occupy a site on the ribosome distinct from the normal tRNA sites. Thus, this specific tRNA or tRNA-like component could be an integral part of the ribosome.

Previous investigators studied the proteins involved in the termination mechanism and from their results concluded that the possible involvement of a specific RNA component was remote. This report is the first known direct study of the RNA bound to ribosomes with respect to its possible involvement in chain-termination.

The following questions which have yet to be posed concerning chain-termination could be asked:

(1) What is the nature of the low molecular weight RNA associated with purified ribosomes?
(2) Is there a specific low molecular weight RNA other than 5S RNA which cannot be equilibrated with tRNA?

(3) If such an RNA exists is it involved in chain termination?

In order to begin to answer the questions it was necessary to obtain a clean ribosome preparation which was active in protein synthesis. Ribosomes from *E. coli* were chosen for the study for three reasons: (a) they are well characterized, (b) the ease with which active ribosomes could be obtained, and (c) all previous chain-termination studies had been carried out with these ribosomes. The latter reason (c) was of particular importance since it would be easier to correlate the results obtained in these studies with those reported in the literature. At the beginning of the project Nirenberg's group had reported the best ribosome preparation (WRib) and their procedure was followed.

The ribosomes used in all experiments to be discussed were readily prepared from *E. coli* B cells grown to the mid-log or late log phase (see Materials and Methods). The preparation involved seven incubations with 0.5-1.0 M NH₄Cl at various concentrations of magnesium buffered at pH 7.4. These steps removed all enzymes (and RNA) not tightly bound to ribosomes. A number of investigators had also shown that prolonged washing with 0.5 M NH₄Cl deactivated or removed ribosomal RNase I (296-298). Others have found variable results in that some of the RNase activity had been removed by NH₄Cl washing but the ribosomes still retained a significant level of RNase (298). Ribosomal activity was determined
by following the uptake of $^{14}$C-Phe in a polyU-dependent phenylalanine incorporation system. The activity of the preparations varied between 50-100 fold over the control or background level (see Results, p. 68). The ribosomes were 67% RNA as contrasted with the usual value of 60-63% (6) which was additional confirmation of the high purity obtained.

The choice of the purification procedure will, of course, depend on the use to which the ribosomes are to be put, the nature of the impurities which are to be removed, etc. Often a balance must be found between the purification and over-handling of the ribosomes since handling may destroy their biological activity. In general, there is no absolute standard of purity. Each preparation must be judged by an operational criterion.

Keeping the latter statement in mind, a development occurred at a stage when the project was already considerably advanced. Iwasaki et al. (219) reported a new and very quick method of obtaining very active ribosome preparations. The method essentially involved washings in 1 M NH₄Cl followed by elution through a DEAE-cellulose column (RSI, Chart II). The activity of these fresh ribosome preparations was approximately 150-fold over the control or background level. Because of the advanced stage of the present project, this latter method of preparing ribosomes was not adopted since it would have been necessary to repeat all the experiments already completed. For this reason and those already mentioned, the ribosome preparation in use was deemed suitable for the experiments in which
it would be employed.

The activity of the ribosome preparations was quite variable as shown in Table I. Perhaps the difference in activity levels was caused by variations in handling during the work-up of the ribosomal preparation. It is well known that ribosomes which are quite stable at low temperature and ionic strength become unstable when either the temperature or ionic strength is raised or the concentration of Mg$^{++}$-ions is reduced. Under the former conditions, the ribosomes were found by numerous investigators to have latent ribosomal RNase I activity while the latter conditions tended to activate the enzyme (298). Following such treatments which disrupt the structure of the ribosome, the enzyme, if present, is able to attack both the ribosomal RNA and added free RNA under conditions of temperature, ionic strength and Mg$^{++}$-ion concentration where intact ribosomes showed no RNase activity (298).

The ribosomal preparations used in Table I (RSI, II and III) were two month old WRib which had been stored at -70°. The original fresh preparation had an activity 82-fold greater than the blank which contained no ribosomes (Table I). Most investigators have indicated that their ribosomal preparations remained active for up to three weeks (219) while others have obtained active preparations with six month old preparations (218). Although Nirenberg (218) was able to freeze and thaw his WRib preparations several times without undue loss of activity, the WRib preparation used in experiment II, Table I was almost inactive after freezing and thawing twice. This
may have been due to the fact that the preparation was already two months old. The reason for our inability to maintain active preparations over extended periods of time may also have been the result of the method used to freeze the preparations. They were quickly frozen in dry ice prior to storage at -70°. On the other hand Nirenberg froze his extracts in liquid N₂ prior to storage in liquid N₂ refrigerators. Even though the preparations were kept at -70° the possible presence of some nuclease activity cannot be discounted. For instance, Szer (299), using an RNase I⁻ strain of E. coli, found almost complete disappearance of 23S RNA with the concomitant formation of 16S RNA in the larger ribosomal subunit at 0°. The final products suggested the involvement of an RNase IV found in the RNase I⁻ strain. RNase II, which is also present in this strain, loses 90% of its activity after 24 hrs when kept cold or frozen (300). However, when our "old" WRib preparations were passed through a DE-22 column and eluted with 1 M NH₄Cl, the original activity of the WRib, at the particular concentration used, was reestablished (RSI, Table I, Chart II). A similar phenomenon was recently reported by Scheps et al. (268) who observed that the activity of a ribosomal extract could be restored by a temperature dependent preincubation in the presence of 0.56 M NH₄Cl followed by incubation in 1 M NH₄Cl. They suggest that this is due to a decrease in the amount of 70S ribosomes which were found to have increased in the formerly inactive preparations. They also found that the defect involved both subunits and was not due to an inactive
S100 which contains the synthetase activity. It should also be borne in mind that as mentioned earlier, washing ribosomes with 0.5 M NH₄Cl tends to remove some of the ribosomal bound RNase I (296-298). But Ochoa's group (219) found that after two prolonged washings with 0.5 M NH₄Cl the ribosomes still contained a high level of RNase activity. However, when the ribosomes were chromatographed on a DEAE-cellulose column the RNase activity was reduced by about 99%. These ribosomes were active in protein synthesis. This may account for the very active WRib preparations which were obtained when the WRib were prepared by washing through a DE-22 column (RSI, Chart II, Table I). The reason for not using this preparation in all experiments was already discussed. These observations show that RNase can be removed from E. coli ribosomes without causing their inactivation; the enzyme normally appears, however, to be bound very firmly to the ribosomes. Unfortunately, none of the fresh ribosomal preparations used in the present studies was checked for the presence of RNase activity, and although they were extensively treated with 1 M NH₄Cl they were not subsequently eluted through a DEAE-cellulose column. It may be reasonable to assume that since these RNases are normally so tightly bound to the ribosomes, at least some RNase activity remained associated with them (WRib).

It is also known that as a bacterial culture enters the late logarithmic and stationary phases its ability to yield an extract active in synthesizing peptides decreases. This has been observed for several bacterial species (218, 266, 267)
and is seen as well with natural mRNA and in the polyU-directed synthesis of polyphenylalanine. To compare the activity of cell-free protein-synthesizing extracts from different lots of cells, the cells should be harvested in the same physiological state, a condition which is often difficult to obtain (experiment II, Table I). The cells used in all the experiments except the exchange studies were obtained commercially in two batches. One batch was harvested at the mid-log phase while the other was harvested at the late log phase. The exchange experiments were carried out with cells grown in the presence of labelled uracil and harvested in the late log to stationary phase. Although WRib prepared from each batch of cells were very active in protein synthesis, the activity per A₂₆₀ unit varied from batch to batch and this may have been due, in part, to the stage at which the cells had been harvested.

Figure 1 shows the separation of low molecular weight RNA from the different ribosome preparations (Chart II, Table I) after electrophoresis in a 10% polyacrylamide gel. There appears to be a marked difference in the amount of tRNA bound to ribosomes which have been put through a DEAE-cellulose column and subsequently eluted with 1 M NH₄Cl. It appears quite likely that the column has not only removed ribosomal aggregates but also loosely bound tRNA which may block sites on the ribosomes, and/or alter the conformation of the ribosome in such a way as to prevent the normal tRNA exchange from taking place.

Once an active ribosome preparation was obtained experi-
ments were undertaken to prepare WRib devoid of all 4S components while at the same time maintaining the activity of the ribosome. Various methods were used to remove bound tRNA from ribosomes. The results shown in Figure 2 indicated that puromycin (PM) treatment alone did not remove all the bound tRNA. This was expected since PM occupies only one site on the ribosome, the peptidyl site. Aminoacyl-tRNA bound to the acceptor site does not react with PM (269, 285). Kuriki and Kaji (285) have shown that while PM reduced the amount of bound peptidyl-tRNA, it did not alter the amount of ribosome-bound tRNA. In the absence of soluble enzymes and GTP, the isolated complex of ribosomes, tRNA and peptidyl-tRNA bound additional tRNA suggesting that the ribosome contained two sites for aminoacyl-tRNA and one site for peptidyl-tRNA. These data are consistent with the hypothesis that during polypeptide synthesis the site for peptidyl-tRNA and one site for aminoacyl-tRNA are constantly occupied but the other site for aminoacyl-tRNA is occupied transiently. Only in the absence of peptide bond formation are both sites for aminoacyl-tRNA constantly occupied. At any one time, 50% of the total bound tRNA should be displaced by PM and this would account for the decreased amount of bound tRNA observed in Figure 2. These PM-treated WRib were also found to be more active than the original preparation; a result that is in agreement with that observed by Scheps et al. (268). They suggested that the inability of the ribosome to respond to polyU was due to their being blocked by unfinished polypeptide chains. PM overcame this by displacing the peptidyl-tRNA,
causing the release of growing peptide chains.

Dialysis of PM-treated WRib against low Mg\(^{++}\) removed all the bound tRNA (Figure 2). This was to be expected since the removal of the nascent polypeptide chain by PM would remove the stabilizing effect it exerted on the binding of the tRNA to the ribosome. Under these conditions the ribosomes would be converted to their subunits and it has been found by some investigators that ribosomal subunits are devoid of 4S RNA material (265, 270, 271). Other investigators have suggested that ribosomal subunits are only devoid of 4S RNA material when treated first with PM but in the absence of PM the 50S ribosomal subunit contains peptidyl-tRNA (115, 272). Unfortunately the dialysis against low Mg\(^{++}\) left the WRib virtually inactive. This may have been due to the fact that the decreased Mg\(^{++}\) concentration destabilized the nucleoprotein structure resulting in the activation of the latent ribosomal RNase I (298).

Dialysis of the WRib against low Mg\(^{++}\) ion concentration (0.1 mM) removed virtually all the bound tRNA (Figure 3). Cannon et al. (148) have found that in the absence of protein synthesis or mRNA, and at 0.1 mM Mg\(^{++}\) ion concentration, the ribosomes were dissociated into their subunits and in the ensuing process all the bound tRNA was shown to be completely washed off. After protein synthesis in a cell-free extract of E. coli, a small fraction of the tRNA that was bound to the ribosomes in high Mg\(^{++}\) ion concentration became resistant to being washed off in low Mg\(^{++}\) ion concentrations. According to
Cannon et al. this amounted to about half a molecule of tRNA per ribosome. This had been interpreted as due to the presence of a nascent polypeptide chain on the tRNA which stabilized the binding of the tRNA to the ribosome. As was found in the previous experiments with PM, dialysis against low magnesium not only removed all 4S RNA but also left the ribosomes virtually inactive. Once again, the reason for the inactive preparations may have been due to nuclease action.

Ribosomal subunits were prepared from these dialyzed ribosomes by centrifugation through a sucrose gradient. The results (Figure 5) showed that there was virtually no 4S RNA material bound to the 50S ribosomal subunit and absolutely none bound to the 30S ribosomal subunit. Although the subsequent CsCl treatment of these subunits should have removed only the split proteins leaving behind the 23S and 40S ribosomal subunits (60), it appears that some degradation must have occurred to account for the additional bands observed in Figure 5; for example, a 5S RNA band in the 30S particle and a 4S RNA band in the 50S particle. It has been shown that in the absence of mRNA, 30S particles do not have affinity for tRNA and that binding is specific for the 50S ribosomal subunits. In the presence of messenger there is also specific binding to the 30S particle. Gilbert has shown that tRNA-linked nascent polyphenylalanine remains attached to the 50S particle even after complete dissociation of the ribosomes into subunits (263). Elsen (264, 265), on the other hand, observed the release of 4S RNA material from the 50S particle in the presence
of high salt.

Attempts were made to obtain an active ribosomal preparation using the individual subunits instead of whole WRib. The subunits, instead of WRib, were used in the assay system for determining polyphenylalanine synthesis as described in Materials and Methods. Twice as much of the 50S ribosomal subunit was added to the system as 30S ribosomal subunit because of the differences in molecular weight (93). The ribosomal activity was found to be just above the blank which contained no ribosomes. The following reasons may account for the failure to achieve ribosomal activity: (a) the subunits were prepared from 2-month old WRib and this together with variations in handling during the work-up and preparation of subunits may have inactivated the preparation, (b) the preparation of ribosomal subunits involved dialysis against low magnesium which in turn causes the destabilization of the nucleoprotein structure resulting in the activation of the latent ribosomal RNase I activity. This RNase has been found to be located exclusively on the 30S subunit (298). However, it has been estimated that no more than one ribosome in about ten would carry a molecule of RNase (298). Szer (299) found that if freshly isolated 70S ribosomes from an RNase I- strain of *E. coli* are fractionated into subunits and kept at 0°, the degradation goes further and both 23S and 16S RNAs are halved. The final products suggested the involvement of an RNase IV found in this RNase I- strain. The effect of RNases on whole WRib as compared to their subunits will be discussed later,
(c) the conditions used to observe ribosomal subunit activity were not optimal. For instance, the ratio of Mg\(^{++}/\)ATP will determine the degree of attachment of the amino acids to the tRNAs (278). The ratio which is optimal in a system containing WRib may not be optimal in the same system containing the subunits instead of WRib. According to Pestka and Nirenberg (159) the activity of different 30S ribosomal subunit preparations varied and 70S ribosomes formed by reassociation of purified 30S and 50S ribosomal subunits were only about half as active in binding aa-tRNA as non-dissociated 70S ribosomes.

It should be noted here that after the above experiments had been completed Tompkins et al. (93) prepared subunits from ribosomes prepared in an identical manner as in the present work and with these subunits, they were able to obtain an active ribosomal preparation. This preparation was also active in chain termination using the Caskey termination assay (88). Since our subunits contained negligible amounts of tRNA and since this termination assay contained only initiator tRNA (fMet-tRNA) it may be assumed, indirectly, that the chain-termination mechanism does not require a specific terminating tRNA. This point will be clarified further on in the thesis.

At this stage in the development of the thesis the following points had been established: (a) a method for preparing active ribosomes, (b) treatment of WRib with PM removed some of the bound tRNA leaving the preparation more active than the original, and (c) dialysis against low magnesium dissociated the ribosomes into subunits devoid of 4S RNA and inactive in
protein synthesis.

Nirenberg was able to get chain-termination using whole ribosomes and since active ribosomes could be readily prepared it was felt that the proposed studies of the low molecular weight RNA, particularly the tRNA bound to ribosomes, could be carried out with these preparations.

As mentioned earlier the WRib used in all the experiments may have contained some nucleases as evidenced by the fact that ribosome activity could be restored by elution through a DEAE-cellulose column which is known to remove RNases. The same effect could have been due to the removal of ribosomal aggregates or tRNA which blocked sites on the ribosome and in so doing prevented the normal tRNA exchange from taking place. Previous evidence suggested that nuclease activity on WRib remained latent provided the variations in handling during the preparation of WRib were minimized and therefore this was the only precaution taken to eliminate nuclease activity. Since experiments were carried out only with active preparations, control experiments to determine the nuclease activity of these preparations were considered unnecessary. Most of the pertinent data recorded in the literature also tended to suggest that tRNA bound to whole ribosomes was RNase resistant. For instance, Cannon et al. (148) found that tRNA bound to 70S ribosomes was resistant to pancreatic RNase. Pestka (162) found that in the presence of ribosomes and polyU, Phe-tRNA was substantially protected from hydrolysis. Although binding of this tRNA to 30S subunits in response to polyU was substan-
tial, the tRNA was not protected from pancreatic RNase digestion. The presence of both subunits was required for substantial protection of the tRNA from RNase digestion. The data also indicated that the Phe-tRNA which was bound to the ribosomes and resistant to RNase remained intact, that is, the aminoacyl end of the aminoacyl-tRNA was protected by the ribosome (149, 255). Neu and Heppel (301) found that ribosomal RNase acted without appreciable destruction of endogenous ribosomal RNA. Gilbert (263) found that in the presence of polyU, treatment of ribosomes with pancreatic RNase in the cold had no effect. Delihas (302) found that with his ribosome preparation only 3% of the total absorbance at 260 nm was released in the presence of pancreatic RNase and there was no effect on ribosomal activity. Ehresmann and Ebel (303) found that T1 RNase caused less degradation in whole 70S ribosomes than when the treatment was done on 30S and 50S ribosomal subunits. At least 35-40% of the 16S RNA was accessible to nuclease action. Gupta et al. (304) found that RNase T1 degraded all parts of the f2 RNA except that protected by the attached ribosomes. Rich's group (305) obtained similar results with pancreatic RNase.

Equipped with this information an extensive characterization of the low molecular weight RNA bound to E. coli ribosomes was then carried out. The results from Table III clearly show that the tRNA bound to WRib has acceptor activity for all the amino acids. A similar phenomenon was observed recently for rabbit reticulocyte ribosomes (276). The results of Culp et al.
(276) differed considerably from those shown in Table III. They also gave results for reticulocyte tRNA which were different again from that bound to their ribosomes. Smith and McNamara (277) have also done acceptor studies on rabbit reticulocyte tRNA and their results differ from those of Culp et al. (276). This may be due to different isolation techniques. The results of Stearn and Horowitz (280), using the total RNA from *Neurospora crassa*, compare quite favourably with many of the specific activities shown in Table III.

It should be stressed that the amino acid acceptor studies were not carried out under optimum conditions. For instance, the ratio of Mg\(^{++}\)/ATP will determine the degree of attachment of the amino acids to the tRNAs (278). Some synthetases require the presence of a sulfhydryl group while others do not (275, 279). Since each tRNA species has probably different optimal conditions for maximal amino acid charging, the present experiments do not, therefore, lead to quantitative results. Under the conditions used, the maximum amino acid incorporation for each tRNA was obtained. The main point that should be established here is that the ribosomes contain species of tRNA which have acceptor activity for all the amino acids and that the amount of acceptance for each amino acid varies. This may be due to the relative amounts of the specific tRNA bound to the ribosomes, or due to conditions under which the assays were carried out, or both. Bartz et al. (291) found that there are fluctuations in the amounts of specific tRNAs in *E. coli* during cell growth. For example, tRNA\(^{Leu}\) reached its maximum
level in the stationary phase of cell growth whereas tRNA\textsuperscript{Cys} reached its maximum level during the early log phase and then declined after that. It is also possible that different ribosomal preparations will have different amounts of specific tRNAs bound or it could be that certain specific tRNAs are more strongly bound to the ribosomes than others. From the previous discussion, it seems very unlikely that the variation in the relative amounts of specific tRNAs bound to ribosomes is due to the removal of a portion of some specific tRNAs by the action of exonucleases. The problem of nuclease contamination; for example, finger nucleases, in various types of systems has occupied the attention of many investigators. As far as this laboratory is concerned, separations of tRNA, for instance on BD-cellulose columns at room temperature for up to four days have been successfully carried out without degradation of the tRNA and with full retention of the capacity for amino acid acceptance. One should also bear in mind that it is presently impossible to calculate, from the available data, the maximum number of tRNA molecules which could be bound to active ribosomes because one would not know what percentage of the ribosomes was actually functional. This is another area in which the studies on tRNA bound to ribosomes will have to be pursued in the future.

The distribution of low molecular weight RNA from \textit{E. coli} ribosomes (Table IV) parallels exactly the results obtained by Busch's group (190) for their fractionation of both low molecular weight nucleolar and ribosomal RNA of Novikoff hepatoma.
ascites cells.

According to Tables II and IV the $A_{260}$ ratio of 4S : 5S RNA varied between 0.58 and 0.90 which amounted to 0.9 to 1.3 molecules of tRNA bound to the ribosomes per molecule of 5S RNA. The percentage of total RNA bound to ribosomes in the form of tRNA varied between 1.3 and 1.7% while the percentage of the total RNA bound to ribosomes in the form of 5S RNA varied between 1.5 and 2.8%. The latter values in both of these cases compare quite favourably to those obtained by other investigators (55, 286). Similarly, these values compare quite favourably to the expected amounts of rRNA, 5S RNA and tRNA using molecular weights as the only criterion. It should be borne in mind that some 4.5S RNA was present in the 5S RNA fractions (Figures 10, 12 and 12a) while only a slight amount was present in the 4S RNA fractions (Figures 10 and 25). For instance, 2.7% of the total low molecular weight RNA in the 4-5S RNA region was 4.5S RNA and this was all found in the 5S RNA fraction (Figures 12 and 12a). Thus the amount of tRNA bound to the ribosomes calculated on the basis of the amount of 5S RNA should be slightly higher. The 4.5S RNA also represents much less than one molecule per molecule of 5S RNA. The 4.5S RNA in _E. coli_ has been shown by the Cambridge group to be a unique component and not an artefact and work is well underway in sequencing this particular species (308). As yet, the function for this component has not been clarified. The mobility of this species with respect to 4S and 5S RNA has been verified by Dixon's group (309).
An attempt was made to characterize further the low molecular weight RNA bound to *E. coli* ribosomes by electrophoresis in a 10% preparative gel (Figure 17). The main absorbance peak did not correspond to the main peak of acceptance activity. Efforts were made to characterize this main absorbance peak. Originally it was thought that GTP would be found in this peak since it is a major requirement in protein synthesis but paper chromatography revealed only two components—one at the origin indicative of a large molecular weight species and one moving beyond the GTP standard. The component at the origin gave the typical RNA tracing (Figure 19). Further attempts to characterize this peak by chromatography on DEAE-cellulose or DEAE-Sephadex A-50 columns resulted in only a single peak being eluted in the void volume in each case.

At the present moment the characterization of the main absorbance peak remains to be established. Besides containing some tRNA, this principal peak must contain RNA of lower molecular weight since presumably this would be eluted first from the preparative gel. It is quite possible that this RNA is actually tRNA that has been partially degraded. In order to confirm this it would be necessary to separate this RNA from the intact tRNA but unfortunately this cannot be done readily. Alkaline hydrolysis studies of this RNA species should confirm whether the sample is tRNA but unfortunately insufficient material was available to carry out the analyses. Because of the experimental difficulties and the limited amount of information that could have been obtained the characterization of
this peak was left for future consideration.

At this stage in the thesis the following additional points had been established in addition to those previously mentioned (see p. 132-133): (a) the ribosomes contain species of tRNA which have acceptor activity for all the amino acids and that the amount of acceptance for each amino acid varied, (b) 1-2 molecules of tRNA are bound to the ribosomes per molecule of 5S RNA, (c) the presence of 4.5S RNA bound to E. coli WRib was confirmed and this species represented much less than one molecule per molecule of 5S RNA.

According to my hypothesis on chain-termination, which was thoroughly discussed previously, if a terminating tRNA exists it must be tightly bound to the ribosomes and probably it would occupy a site on the ribosome distinct from the normal tRNA sites. Such a species of RNA would not be expected to exchange with the normal tRNA. Therefore, exchange experiments were carried out with two purposes in mind: (1) to see if some tRNA are more tightly bound to ribosomes than others, and (2) to confirm the presence or absence of a terminating tRNA.

It had been shown by numerous investigators that exchange between free tRNA and tRNA bound to ribosomes readily takes place (148, 249, 250, 306). This exchange was not affected by charging tRNA with amino acids or by temperature. Bound tRNA will not wash off in high Mg++ ion concentration but could easily be displaced by free tRNA from the surrounding medium. The nonspecific association of tRNA and ribosomes took place in
the cold and did not require the supernatant enzymes, GTP, ATP or an energy source other than the thermal energy of the reacting components.

An exchange experiment was carried out in the same manner as by Cannon et al. (148). Ribosomes were prepared from a pyrimidine-requiring *E. coli* mutant grown in the presence of $^3$H-uracil and thoroughly washed in the usual manner. Exchange was considered to be complete when the number of counts in the ribosomal pellet had remained virtually constant within experimental error (Table V, Figure 23). When labelled ribosomal-bound tRNA was exchanged with unlabelled tRNA virtually all of the labelled tRNA was removed (Table VI). The $A_{260}$ ratio of 4S : 5S RNA on a total ribosomal RNA basis had increased to almost 3.5 in ribosomes exchanged with unlabelled tRNA (Figure 24). This represented about 5.7 molecules of tRNA bound per molecule of 5S RNA as compared with 1-2 molecules of tRNA bound in a system that has not undergone exchange. These results may be explained by considering a multiplicity of tRNA binding sites previously postulated by Warner and Rich (260) as well as by Wettstein and Noll (262) for mammalian ribosomes (see Introduction p. 22-24). Quantitative studies by Cannon et al. (148) showed that there was only one binding site per 70S or 50S ribosome in the absence of mRNA. In the presence of messenger the amount of binding doubled. Other investigators (285) have suggested the presence of three sites, two for aminoacyl-tRNA and one for peptidyl-tRNA. The second aminoacyl-tRNA site was occupied only in the absence of peptide bond formation.
According to Cannon et al. (148) this does not eliminate the possibility of several sites, but if there are, either they are not all equivalent, or if they are equivalent they have the property that binding tRNA to any one weakens the binding ability of the others. This might account for the loss of labelled ribosomal bound tRNA. Two models are suggested for the binding and exchange of tRNA. In the first, the ribosome has one site on which the tRNA binds in rapid equilibrium with the tRNA in solution. The second model can be characterized as the ribosome having an exchange site. The tRNA bound to the site is tightly bound and the complex would dissociate very slowly. The exchange would take place by a second tRNA molecule altering the site and displacing the first. One can consider two equivalent sites for the binding; either site alone can bind the tRNA tightly but if both are full both tRNA molecules are bound loosely. Such a structure would have rapid exchange associated with a loose binding for the two molecules but slow loss and tight binding for the first tRNA bound. A complete discussion of the number of sites available on the ribosome for binding tRNA has already been given (pages 14-24). The data presented here do not exclude the possibility that other kinds of tRNA binding sites with different requirements could be demonstrated under different conditions.

Unfortunately after the last exchange (Figure 23) the ribosomes were found to be inactive in protein synthesis although after the initial exchange these ribosomes were as active as the original preparation. Many reasons could explain
this loss in activity. For example, because of the increased number of tRNAs bound to the ribosomes as compared to 5S RNA, all the available sites on the ribosome may be blocked and/or the conformation of the ribosome may be altered in such a way as to prevent the normal exchange from taking place and therefore preventing protein synthesis. It has also been found that 5S RNA could be released from the 50S ribosomal subunit by high levels of tRNA (287). Sarkar and Comb (288) could give no explanation for this phenomenon. Their only suggestion was that in cells where most of the ribosomes were involved in protein synthesis, 5S RNA may have occupied one of two sites and at one of these sites it could be displaced by tRNA. Their previous studies on 80S ribosomes demonstrated that two molecules of 5S RNA were bound per large subunit but only one molecule per subunit when tRNA was included in the binding mixture (288). They also found that with the loss of 5S RNA there was a loss in biological activity. They suggest that the loss in biological activity may have been due to slight changes in the conformation of the ribosome and may have no relationship to the loss of 5S RNA. The fact that native 5S RNA present on the 50S ribosomal subunits, when exchanged with isolated 5S RNA, yielded particles completely inactive in polypeptide synthesis, suggested to them that the conformation of the 5S RNA on the ribosome was unique and essential for biological activity and quite different from isolated 5S RNA. Other possible reasons for the loss in biological activity could be due to a conformational change in a protein, a local rearrange-
ment of a helical portion of the RNA or an alteration in some protein-RNA association. Inactivation by a ribosomal RNase is remote due to the fact that the exchange took place at a high magnesium concentration and at 0°; conditions under which the ribosomes are quite stable.

Although after the exchange experiment the ribosomes were found to be inactive and ideally one would have hoped for the maintenance of activity, the ultimate purpose of the experiment was to study the binding ability of specific tRNAs to these ribosomes and to check for the presence of a terminating tRNA—both of which could still be studied (see p. 139).

Therefore after the exchange, the ribosomal-bound tRNA was fractionated on a BD-cellulose column to see if the labelled tRNA that remained bound to E. coli ribosomes was spread evenly throughout the elution profile, or whether it was concentrated in a certain region. The labelled tRNA was spread uniformly throughout the region eluted with the NaCl gradient but there was a peak of radioactivity in the ethanol gradient (Figure 26). Counts in the peak region were 5-10 fold higher than that recorded in the salt gradient. This peak ethanol fraction was also found to contain 4.5S RNA. Although the counts in this ethanol fraction were much higher than those recorded in the salt gradient most of it could be accounted for in the following way: (1) the presence of 4.5S RNA which would be labelled. This 4.5S RNA, which would tend to remain strongly bound to the BD-cellulose column, would be eluted in high ethanol concentrations which would overcome the hydrophobic
interactions between the RNA and the resin, (2) since the ethanol fraction has been found to contain all Ser, Tyr and Trp acceptor activities, about 17% of the Leu acceptor activity and 9% of the Phe acceptor activity (295), this region alone would be expected to give higher counts due to their combined presence in this fraction (see Table III), (3) since tRNA\textsubscript{Trp} was found to be present in the largest amount on ribosomes, presumably it alone could account for some of the counts (Table III); however, there is no reason to believe that one particular tRNA is less vulnerable to exchange than another.

The reasons additional experiments to confirm these results could not be carried out were: (a) acceptor studies with tRNA\textsubscript{Trp} gives a very high background (up to 300 cpm) and since one is dealing with such low counts (250 cpm), it would be impossible to get a definitive result, (b) double-labelling experiments could not be carried out because the background levels would be higher than the counts that were being investigated here, (c) the assay used to determine acceptance activity of specific tRNAs would involve the precipitation of all the tRNAs in the ethanol fraction.

It may be possible to separate the 5 acceptor activities found in the ethanol fraction on a Kelmer reversed-phase column but, once again, since one is dealing with such low counts any small error, for example, in background counts, would lead to an overall large experimental error. These experiments would have been very complex and time consuming and it was felt that any results obtained would not have added
significantly to the conclusions already established.

Taking all these factors into consideration, there would be very few counts left over which could be allocated to the presence of a specific chain-terminating tRNA. This species of tRNA would not only be labelled but would also be expected to be present as a sharp peak of radioactivity. Approximately 50,000 counts attributed mainly to tRNA was distributed over the BD-cellulose column (Figure 24, Table VI). If a mere 1% of this tRNA could be considered due to the presence of a specific tRNA terminator, then there would be enough radioactivity present such that it would show up as a distinct sharp peak. Calculating on the basis of 5S RNA, the counts remaining would indicate less than one molecule of 4S material for every 20 5S RNA molecules. Thus the presence of a specific tightly bound terminating tRNA is not possible.

In summary, tRNA exchange resulted in increased binding of tRNA to the ribosomes; from 1-2 molecules to approximately 6 molecules per molecule of 5S RNA. Exchange, under the conditions used, was almost 100% and also resulted in the loss of ribosomal activity. The tRNA which did not exchange was spread uniformly throughout the elution pattern suggesting that all tRNAs are probably bound to the ribosome to the same degree, that is, one tRNA is not bound more strongly than another. The absence of a sharp peak of radioactivity is further proof of the absence of a specific chain-terminating tRNA, since as previously discussed, such a species of tRNA would be expected to be not only tightly bound to the ribosomes but also non-exchangeable with tRNA.
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APPENDIX

1 50 μl of $^{14}$C-Phe (42 μg and 0.1 mCi/ml) was diluted with 100 μl distilled water. 5 μl of this mixture was used for each assay. Two controls were used throughout. One control contained all the assay components except polyU while the other control contained all the assay components except WRib. The level of radioactivity observed in both controls was the same—approximately 200 cpm. This level of radioactivity served as the background and was set at one.

2 50 μl of $^{14}$C-amino acid mixture (1 mCi/ml) was diluted with 100 μl distilled water. 5 μl of this diluted mixture was used for each assay. Enzyme prepared as described on pages 52-53 was added. All tubes contained the same amount of tRNA. Two controls were used throughout. One control contained all the assay components except tRNA (tRNA control) while the other control contained all the assay components except enzyme. Since the tRNA control gave the highest background level of radioactivity, this background was subtracted from each of the sample tubes.

3 The assay system contained all the components previously described on page 51 in Materials and Methods. All the tubes contained the same amount of tRNA. Enzyme prepared as described on pages 52-53 was used: 5 μl of the specific $^{14}$C-amino acid was added to the particular tube. The concentration and specific activity of the $^{14}$C-amino acids
used are given below:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>ug/ml</th>
<th>mCi/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>76.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Arg</td>
<td>37.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Asn</td>
<td>282.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Asp</td>
<td>86.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Gly</td>
<td>56.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Glu</td>
<td>71.0</td>
<td>0.10</td>
</tr>
<tr>
<td>His</td>
<td>1.2</td>
<td>0.05</td>
</tr>
<tr>
<td>Ile</td>
<td>55.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Leu</td>
<td>52.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Lys</td>
<td>30.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Met</td>
<td>1100.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Phe</td>
<td>42.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Pro</td>
<td>31.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Ser</td>
<td>84.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Thr</td>
<td>37.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Trp</td>
<td>900.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Tyr</td>
<td>48.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Val</td>
<td>63.0</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Two controls were used for each specific amino acid tested. One control contained all the assay components except tRNA (tRNA control) while the other control contained all the assay components except enzyme. Since the tRNA control gave the highest background level of radioactivity, this background was subtracted from the sample tube containing tRNA. The experimental conditions are similar to those used by Keller (229) and Muench and Berg (279).