Protein-RNA interactions in the structure and function of the Escherichia coli ribosome

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Protein-RNA interactions in the structure and function of the *Escherichia coli* ribosome

by

Harry Rice Burrell

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ABBREVIATIONS AND SYMBOLS

A  adenosine
5'-AMP  adenosine-5'-phosphate
ATP  adenosine-5'-triphosphate
ATPase  adenosine-5'-triphosphatase

A 260 unit  the amount of material which in a volume of 1.0 ml will give an absorbance of 1.0 at 260 nm when measured in a cuvette of 1.0 cm path length

C  cytidine

Ci  curie--unit of radioactivity equal to $3.7 \times 10^{10}$ disintegrations per second

cm  centimeter

CPM  counts per minute

DEAE  diethylaminoethyl

DNA  deoxyribonucleic acid

EDTA  ethylenediamine tetraacetate, sodium salt

g  gram

G  guanosine

GTPase  guanosine-5'-triphosphatase

l  liter

M  concentration in moles per liter

mA  milliampere

mg  milligram

ml  milliliter

mRNA  messenger ribonucleic acid

MW  molecular weight in daltons
nm  nanometer
p  when used in conjunction with the symbols for the ribo-
nucleotides, refers to the 3', 5'-phosphodiester linkage
in the nucleic acid or oligonucleotide
pmole  picomole
poly U  polyuridylic acid
psi  pounds per square inch
ψ  pseudouridine
rpm  revolutions per minute
RNA  ribonucleic acid
RNase  ribonuclease
S  Svedberg unit, a unit of sedimentation velocity equal
to 10^{-13} second
T  thymidine
tRNA  transfer ribonucleic acid
U  uridine
μg  micogram
5'-UMP  uridine-5'-phosphate
INTRODUCTION

The biosynthesis of protein molecules is a fundamental process characteristic of all living systems examined so far. It is perhaps the most awe-inspiring facet of molecular biology that the three components of this process, DNA, RNA, and proteins, which individually are incapable of self-replication, can interact in such a manner as to provide for the genetic transmission and expression of information; a prerequisite and even a definition of life. In this respect the selective interactions which take place between specific proteins and nucleic acids can be defined as the controlling elements in genetic expression.

The components involved in the cooperative process of protein biosynthesis, or translation, have become more numerous and the synthetic pathway increasingly more complex in the explosion of research which has taken place since the discovery of the structure of DNA by Watson and Crick (1953) implicating a method of information storage inherent in the stereospecific interactions between the nucleotide pairs of the double helix. DNA, messenger RNA (mRNA), transfer RNA (tRNA) and the cognate aminoacyl synthetases, amino acids, ribosomes, initiation factors, elongation factors, termination factors, GTP, ATP, and various inorganic cations and anions have all been established as functional entities in protein biosynthesis (for a review, see Lucas-Lenard and Lipmann, 1971). However an understanding of the translational process and its control has been limited by a lack of knowledge of the protein-protein and
protein-nucleic acid kinetic interactions which evidently are intrinsic to the function of the synthetic apparatus. The seriousness of this restriction becomes quite apparent when one considers the ribosome, an organelle common to both procaryotic and eucaryotic cells and one which plays a central role in protein synthesis. Previous research has shown that in both procaryotes and eucaryotes, the ribosome consists of an extremely complex array of protein and RNA molecules arranged in a specific three-dimensional structure, and that the procaryotic ribosomal RNA molecules and proteins are quite different from those found in the eucaryotic ribosome (Nomura, 1970). At least in *Escherichia coli*, all of the ribosomal proteins and at least 60%-70% of the ribosomal RNA appear to be accessible to some extent to relatively large molecules present in the outside environment, as suggested by studies on the intact ribosome using limited enzymatic digestion (pancreatic ribonuclease, trypsin), chemical modification (for example, glutaraldehyde binding to available proteins, acridine orange binding to exposed RNA sites), and immunological reactions with antibodies directed against single ribosomal proteins (for a review of these results see Garrett and Wittmann, 1973). These data coupled with results using bifunctional cross-linking reagents (e.g., imidoesters) to isolate and identify pairs of ribosomal proteins proximal *in situ* (see Garrett and Wittmann, 1973) have permitted some preliminary spatial models of ribosome structure (Visentin *et al.*, 1973; Bollen *et al.*, 1974).

Although a good deal of progress is rapidly being made in
revealing the location and configuration of the proteins and RNA within the procaryotic ribosome, and the possible roles of these macromolecules in various ribosomal functions (Cox and Hadjiolov, 1972; Garrett and Wittmann, 1973), little is known about the interactions of these components in the maintenance of ribosomal structure and activity. The discovery by Traub and Nomura (1968) and Nomura and Erdmann (1970) that within certain parameters of ionic strength and temperature a functional bacterial ribosome can be self-assembled from the individual protein and RNA constituents, has illustrated quite strongly the importance of macromolecular interactions in this isolated system and therefore presumably also in the in vivo assembly process. The in vitro ribosomal reconstitution system has subsequently proven to be a valuable tool in the analysis of the function of individual RNA and protein components and has emphasized the high degree of specificity involved in the recognition of binding sites between complimentary ribosomal protein and RNA molecules.

Using the reconstitution method and other approaches, a large amount of data has accumulated from many laboratories during the past few years on ribosome assembly and function. From these results it has become increasingly apparent that a successful delineation of the process of protein biosynthesis will require a clear understanding of the selective nature evidently inherent in these ribosomal protein-RNA interactions. Therefore a study has been undertaken to identify the ribosomal proteins which specifically
recognize and bind to various RNA molecules integral to the structure of the ribosome and to the translational event. The procaryote *Escherichia coli* was chosen for experimental analysis due to the extensive amount of genetic and biochemical information available on this organism. In order to gain a proper perspective on the problem, a brief summary of the current knowledge of bacterial ribosome structure and of the various steps in protein synthesis which involve the participation of ribosomes will be presented. Attention will be focused on established ribosomal protein interrelationships with ribosomal RNA and other RNA molecules and on the possible roles of these ribonucleoprotein complexes in protein synthesis.

In *E. coli* the monosome (MW $2.8 \times 10^5$) sediments at 70S and is a complex of a large, 50S subunit (MW $1.8 \times 10^6$) and a smaller, 30S subunit of MW $1.0 \times 10^6$ (Spirin and Gavrilova, 1969). The 50S subunit is composed of at least 34 distinct proteins (Kaltschmidt and Wittmann, 1970b) and two RNA molecules sedimenting at 23S and 5S, having molecular weights of $1.1 \times 10^6$ and $4.0 \times 10^4$, respectively (Spirin and Gavrilova, 1969). The 30S subunit contains one 16S RNA molecule of MW $0.55 \times 10^6$ (Spirin and Gavrilova, 1969) and at least 21 different proteins (Kaltschmidt and Wittmann, 1970b). The actual number of proteins in each subunit which can be defined as ribosomal proteins has not been firmly established, as evidently during various phases of protein synthesis the composition of the active ribosome is quite fluid with various proteins, such as initiation and elongation factors, associating and dissociating from the polysome. In
this respect the criterion for a ribosomal protein which was used in these studies was that it corresponded to a specific location on the two-dimensional electrophoretogram developed by Kaltschmidt and Wittmann (1970a,b) which has been shown to yield results on the total number of proteins present in the ribosome that are in accordance with those obtained by independent methods of analysis such as isolation of the individual ribosomal proteins by ion-exchange chromatography (Kurland et al., 1969). The electrophoretic method defines 21 proteins in the E. coli 30S subunit and 14 proteins in the 50S subunit. In the nomenclature which has been adopted for the identification of these proteins (Kaltschmidt and Wittmann, 1970b), the 30S proteins are prefixed with the letter S (denoting small subunit) and are numbered from 1 to 21 depending upon their distance of migration from the origin on the two-dimensional gel slab. The 50S ribosomal subunit proteins are prefixed with the letter L (for large subunit) and are numbered in the same fashion as the 30S subunit proteins. The two-dimensional pattern and nomenclature of the E. coli 50S and 30S ribosomal subunit proteins are shown in the Appendix (Fig. 24).

Evidence has accumulated that the ribosomal protein population identified in the above manner is heterogeneous for a given ribosome preparation. Stoichiometric measurements of the relative molar amounts of each of the 55 E. coli ribosomal proteins have shown that although many of the proteins are present in equimolar amounts, i.e., one mole of protein per mole of ribosomes, a significant number
are present in either multiple copies (especially in the 50S sub-unit, e.g. L7/L12 and L18) or fractional amounts (especially in the 30S subunit, e.g. S12 and S13) (Weber, 1972; Deusser, 1972; Bickle et al., 1973; Thammana et al., 1973). This heterogeneity in protein composition may reflect the functional state of the ribosome. Kurland and his co-workers have shown that incubation of *E. coli* 30S ribosomal subunits with externally added 30S ribosomal proteins under conditions optimal for the *in vitro* reconstitution of 30S subunits results in as much as a 60% stimulation in protein synthesis activity (Kurland et al., 1969). Furthermore it was found that some of the externally added proteins were incorporated into the 30S particles with a concomitant release of ribosomal proteins initially present in the subunit (Kurland et al., 1969). These data again emphasize the fluid nature of ribosome constitution.

Much of the initial work on the interactions between individual ribosomal components which are important in the structures of the subunits has been accomplished through use of the reconstitution techniques developed by Nomura and his colleagues (Traub and Nomura, 1968; Nomura and Erdmann, 1970). In this approach each of the ribosomal proteins is tested individually for its ability to bind to ribosomal RNA under conditions used for the reconstitution of the entire ribosomal subunit. The results obtained have made it clear that precise ribosomal protein-nucleic acid interactions occur during the assembly process. Five *E. coli* 30S ribosomal proteins, S4, S7, S8, S15 and S20, have been found to form stable complexes with
E. coli 16S RNA (Mizushima and Nomura, 1970; Schaup et al., 1970; Schaup et al., 1971; Garrett et al., 1971; Muto et al., 1974). There are also conflicting data for a weaker binding of S13 and S16/S17 to 16S RNA (Garrett et al., 1971; Muto et al., 1974). The binding process is specific for the RNA component; yeast cytoplasmic 17S ribosomal RNA, "16S" RNA prepared from ribonuclease treated E. coli 23S RNA, and rat liver 18S ribosomal RNA cannot substitute for E. coli 16S RNA in the reaction (Nomura, 1970). The recognition process has also been shown to be site-specific; treatment of E. coli 16S RNA with pancreatic ribonuclease or ribonuclease T1 results in a series of fragments which will complex with only one or two 30S ribosomal proteins (Zimmerman et al., 1972; Schaup and Kurland, 1972; Schulte et al., 1974). Similar studies have established site-specific binding regions for at least six 50S ribosomal proteins, L2, L6, L16, L20, L23, and L24, on E. coli 23S RNA (Stöffler et al., 1971a,b; Schaup and Kurland, 1972; Branlant et al., 1973; Schulte et al., 1974; Garrett et al., 1974b). Proteins L1, L3, L4, L13, L17, and L19 may also interact directly and independently with 23S RNA, although this evidence is not as conclusive (Garrett et al., 1974b). The E. coli 50S ribosomal subunit proteins L18 and L25 have been determined to bind to E. coli 5S RNA (Horne and Erdmann, 1972; Gray et al., 1973; Yu and Wittmann, 1973); smaller amounts of L5 (Horne and Erdmann, 1972; Yu and Wittmann, 1973) and traces of L20 and L30 (Horne and Erdmann, 1972) were also reported to be present in these reconstituted 5S RNA-protein complexes. As in the case with
IbS RNA, the individual proteins can distinguish the correct binding sites on the RNA. The 50S ribosomal proteins which were found to bind to 23S RNA did so exclusively, even in the presence of 16S RNA (Stöffler et al., 1971b). Although the E. coli 50S ribosomal proteins were found to bind equally well to Bacillus stearothermophilus 5S RNA as to E. coli 5S RNA, no interaction was found with a number of different eucaryotic 5S RNA molecules (Bellemare et al., 1973).

In addition to the primary binding reaction between the individual protein and its specific nucleotide sequence, second-order and higher ribosomal protein-RNA and protein-protein interactions evidently occur since the complex formed between the 50S proteins and 5S RNA itself binds to a discrete site on the 23S RNA molecule; this binding reaction requires an additional 50S ribosomal protein, L6, which recognizes both reaction components (Gray et al., 1972). Supporting evidence for the necessity of certain 50S ribosomal proteins in the binding of 5S RNA to 23S RNA comes from studies on the release of 5S RNA together with a number of ribosomal proteins from E. coli 50S subunits treated with high concentrations of salts such as LiCl or NH₄Cl; the so-called "unfolding" phenomenon (Marcot-Queiroz and Monier, 1967; Gormly et al., 1971). Upon lowering the salt concentration and adjusting the solution parameters to conditions optimal for in vitro reconstitution, quantitative reattachment of 5S RNA to the 5S RNA-deficient ribosomal particles occurs but only in the presence of the released protein
fraction (Gormly et al., 1971; Yu and Wittmann, 1973). The released protein fraction has subsequently been shown to contain the 50S ribosomal proteins which bind 5S RNA to 23S RNA in the isolated reconstitution system, i.e. L6, L18, and L25 (Gray et al., 1972; Garrett et al., 1974a).

There are strong indications that the ribosomal RNA secondary and tertiary structures, as well as the specific nucleotide sequence, are important in defining the protein binding site. One of the 30S ribosomal proteins, S4, which has been found to bind to 16S RNA does not bind to one continuous region on the RNA but to a series of distinct, separate sites (Schaup and Kurland, 1972). These sites may be widely separated in the RNA primary structure but actually could be quite close spatially due to the folding of the polynucleotide chain. In this respect the binding of certain ribosomal proteins may help stabilize a particular ribosomal RNA conformation required for the activity of the particle. Furthermore, solution studies by Schulte et al. (1974) have shown that a structural change in both 16S RNA and 23S RNA, associated with a critical level of magnesium ion concentration, is required for ribosomal protein binding. More direct confirmation of the requirement for a unique three-dimensional ribosomal RNA conformation comes from results by Aubert et al. (1968) using heat or urea denatured E. coli 5S RNA. The authors found that while native 5S RNA could be reattached to 5S RNA-deficient, unfolded particles produced by 2 M LiCl treatment of 50S subunits, denatured 5S RNA could not be reincorporated. Renaturation of the denatured form by heating to 60°C in a high magnesium ion
concentration environment restored its ability to be reattached.

From the above evidence it is possible to state that ribosomal protein-ribosomal RNA interactions play an important role in the maintenance of the ribosome in a biologically active state. However, recent studies have indicated that ribosomal proteins may also interact with nonribosomal RNA molecules and nucleotides during the three phases of protein biosynthesis: initiation, elongation, and termination. Initiation of protein synthesis in procaryotic organisms involves the binding of messenger RNA (mRNA), a specific aminoacyl-transfer RNA, formylmethionyl-tRNA F met (fmet-tRNA ᵃ₉), the nucleotide GTP, and three protein initiation factors, IF-1, IF-2, and IF-3, to the 30S subunit (Lucas-Lenard and Lipmann, 1971). After initiation complex formation, the 50S subunit adds to form the 70S monosome, which may be part of a polysomal complex, and the next mRNA codon-designated aminoacyl-tRNA, together with GTP and a protein elongation factor, EF-Tu, are bound (Lucas-Lenard and Lipmann, 1971). In the proposed two-site model of the ribosome (Watson, 1964), the fmet-tRNA ᵃ₉ is bound to the donor site ("D" site) and the next aminoacyl-tRNA binds to the acceptor site ("A" site). Formylmethionine is then transferred from its tRNA and coupled via a peptide bond to the aminoacyl-tRNA in the "A" site in a reaction catalyzed by the 50S subunit-linked enzyme peptidyl transferase (Maden et al., 1968). The resulting peptidyl-tRNA is then translocated from the "A" site to the "D" site, with a concomitant release of the now deacylated tRNA ᵃ₉ from the "D" site and the exposure of a new mRNA codon, in
the "A" site, by a GTP-dependent mechanism involving a soluble protein factor termed G-factor (Haenni and Lucas-Lenard, 1968). A new aminoacyl-tRNA then binds to the "A" site as directed by the new mRNA codon and the cycle of bonding, translocation, and binding is repeated until a "terminator" mRNA codon is reached, at which point a protein termination factor binds to the ribosome and causes release of the completed protein (Lucas-Lenard and Lipmann, 1971). All of these reactions occur in a region of the ribosome termed the peptidyl transferase center.

As can be seen, the complexity of events during the synthetic sequence suggests a priori that the ribosomal proteins play an active role in this process. Support for this supposition comes from thermal stability studies which indicate that the interaction of the mRNA codon and its complementary tRNA anticodon is not in itself sufficient to provide the necessary stabilization of the tRNA-ribosome-mRNA complex (McLaughlin et al., 1968). Secondly, mutations which alter the structures of two 30S ribosomal proteins, S4 and S12, have been identified with changes in the error frequency of translation (Birge and Kurland, 1969; Zimmerman et al., 1971). Held et al. (1974) have shown that ribosomal 16S RNA and a 30S ribosomal protein, S12, are uniquely involved in the initiation of translation of natural messenger RNA (phage R17 coat protein mRNA) by reconstituting hybrid 30S subunits between ribosomal proteins and RNA from B. stearothermophilus which, under certain conditions, cannot initiate at the coat protein cistron, and E. coli, which can. In addition, it has been
reported recently that a 5S RNA-ribosomal protein complex reconstituted in vitro from both *E. coli* and *B. stearothermophilus* 5S RNA and 5OS proteins, containing those proteins, L5, L18, and L25, previously identified as binding to 5S RNA, has GTPase and ATPase activities and specifically binds the oligonucleotide TpVpCpGp, a sequence found in almost all tRNAs functional in protein biosynthesis except certain eucaryotic initiator tRNAs (Erdmann et al., 1973). Neither the proteins nor 5S RNA alone were active in these assays. Therefore it appears likely that although the Wobble Hypothesis of Crick (1966) may explain the nucleotide triplet code for each amino acid, the selective binding of a designated aminoacyl-tRNA to the ribosome is considerably more complicated than the simple formation of hydrogen bonds between complementary bases in the codon-anticodon pairing scheme. Indeed a considerable amount of evidence has implicated the *in situ* interaction of ribosomal proteins with transfer RNA as well as with messenger RNA. A summary outlining some of the more important evidence for each of the *E. coli* ribosomal proteins is presented. Stoichiometric measurements (Weber, 1972; Deusser, 1972) are included since such calculations may reflect a functional heterogeneity which could be of importance in these considerations. The stoichiometric data appear in parentheses and are expressed as moles protein per mole of ribosomal subunits.
S1. (0.3). This protein may be involved in the binding of messenger RNA to the 30S subunit. The addition of S1 to 30S ribosomal subunits stimulates the binding of the synthetic mRNA, polyuridylic acid (poly U) to the subunits (Garrett and Wittmann, 1973). Also the binding of poly U to the 30S ribosomal subunit protects S1 from trypsin digestion (Rummel and Noller, 1973). Protein S1 may also be important for the function of both the "A" and "D" sites on the ribosome, as both the AUG- and initiation factor-dependent binding of fmet-tRNA\textsuperscript{met} and the elongation factor-dependent binding of phenylalanyl-tRNA to the ribosome are inhibited by a specific anti-S1 immunoglobulin G fragment (Lelong et al., 1974).

S2. (0.4). A mixture of S2, S3, and S14 added to E. coli 30S subunit particles under reconstitution conditions was found to stimulate the elongation factor-dependent binding of aminoacyl-tRNA, whereas mRNA binding remained unaffected (Randall-Hazelbauer and Kurland, 1972). Protein S2 may be also involved in the initiation of protein synthesis as anti-S2 antibody inhibits the binding of fmet-tRNA\textsuperscript{met} \textsubscript{F} (Lelong et al., 1974).

S3. (0.7). A histidine-specific photo-oxidation in the presence of the dye Rose Bengal leads to a loss of tRNA binding ability in the 30S ribosomal subunit which can be attributed to a specific modification of S3 (Noller et al., 1971). The binding
of phenylalanyl-tRNA to the 30S subunit in the presence of poly U results in the protection of S3 from trypsin attack (Rummel and Noller, 1973). A specific anti-S3 antibody fragment when added to 30S subunits blocks both the initiation factor-dependent binding of fmet-tRNA^{met}_F and the elongation factor-dependent binding of phenylalanyl-tRNA, indicating an importance of this protein to the function of both the "A" and "D" sites (Lelong et al., 1974). E. coli 30S ribosomal subunits reconstituted using precursor 16S RNA, which is non-methylated and contains excess oligonucleotides at the 3'- and 5'-ends of the molecule, are inactive in poly U-directed polyphenylalanine synthesis due to the inability of the particles to bind phenylalanyl-tRNA and to associate with the 50S subunits (Wireman and Sypherd, 1974). The inactivity of these precursor 16S RNA containing particles toward the binding of phenylalanyl-tRNA was correlated with their deficiency in the protein S3 (Wireman and Sypherd, 1974).

S4. (1.0). This protein binds to 16S RNA (Muto et al., 1974). It is evidently not essential for tRNA binding to either the "A" or "D" sites as anti-S4 antibody does not inhibit either of these functions (Lelong et al., 1974).

S5. (1.0-1.2). This protein may be part of the "D" site as Lelong et al. (1974) have reported that anti-S5 antibody inhibits the initiation factor-dependent binding of fmet-tRNA^{met}_F to the 30S subunit.
S6. (0.25-0.75). The stoichiometric variation of this protein is directly related to the growth rate of the cells; ribosomes from cells grown in nutrient-rich media were found to have a two- to three-fold greater amount of S6 than ribosomes isolated from cells grown in a nutrient-deficient medium (Garrett and Wittmann, 1973). Ribosomal 30S subunit particles reconstituted without S6 show a drastic reduction in the ability to bind \( \text{fmet-tRNA}^{\text{met}}_F \) as directed by the codon AUG and by initiation factor IF-2 (Nomura et al., 1969). The binding of phenylalanyl-tRNA to the 30S subunit in the presence of poly U protects S6 from trypsin attack (Rummel and Noller, 1973). Anti-S6 antibody fragments block the initiation factor-dependent binding of \( \text{fmet-tRNA}^{\text{met}}_F \) to the 30S ribosomal subunit (Lelong et al., 1974).

S7. (1.0). This protein binds to 16S RNA (Muto et al., 1974). Anti-S7 antibody does not affect the binding of aminoacyl-tRNA to either the "A" or "D" sites (Lelong et al., 1974).

S8. (1.0). This protein binds directly to 16S RNA (Muto et al., 1974) and may also be involved in the "A" site as a specific anti-S8 antibody blocks the elongation factor-dependent binding of phenylalanyl-tRNA (Lelong et al., 1974).

S9. (1.0). There are indications that S9 may be part of the GTP-dependent G-factor (translocase) binding site on the 30S subunit (Garrett and Wittmann, 1973). Also, specific anti-S9
antibodies prevent the elongation factor-dependent binding of phenylalanyl-tRNA to the "A" site (Lelong et al., 1974).

S10. (0.6). S10 may be important to the function of both the "A" site and the "D" site as anti-S10 antibody fragments inhibit the binding of \( \text{fmet-}tRNA^\text{met}_F \) and phenylalanyl-tRNA to the ribosome (Lelong et al., 1974).

S11. (0.4). This protein may bind to 23S RNA (Garrett and Wittmann, 1973). The omission of S11 during 30S subunit reconstitution leads to a drastic increase in the misreading of messenger RNA by the reconstituted particle (Nomura et al., 1969). Anti-S11 antibody was found to block the elongation factor-dependent binding of phenylalanyl-tRNA to the ribosome thus indicating the possible involvement of this protein with "A" site binding (Lelong et al., 1974).

S12. (0.2). This protein may bind to 23S RNA (Garrett and Wittmann, 1973). S12 is required in conjunction with 16S RNA for the translation of natural mRNA (Held et al., 1974). Anti-S12 antibody inhibits the initiation factor-dependent binding of \( \text{fmet-}tRNA^\text{met}_F \) to the 30S subunit indicating that this protein may be important for "D" site binding (Lelong et al., 1974).

S13. (0.7). This protein may be involved in "D" site function as anti-S13 antibody fragments block the initiation factor-dependent binding of \( \text{fmet-}tRNA \) to the 30S ribosomal subunit (Lelong et al., 1974).
S14. (0.4). The binding of phenylalanyl-tRNA to the 30S subunit in the presence of poly U protects S14 from trypsin attack (Rummel and Noller, 1973). S14 may be important for the function of both the "A" and "D" sites as anti-S14 antibody inhibits both the initiation factor-dependent binding of fmet-tRNA$^{\text{met}}_F$ and the elongation factor-dependent binding of phenylalanyl-tRNA to the ribosome (Lelong et al., 1974).

S15. (1.0). This protein binds directly to 16S RNA (Muto et al., 1974). Anti-S15 antibody does not affect the binding of aminoacyl-tRNA to either the "A" or the "D" sites (Lelong et al., 1974).

S16. (1.0). Anti-S16 antibody does not affect the binding of aminoacyl-tRNA to either the "A" or the "D" sites (Lelong et al., 1974).

S17. (Stoichiometry not known). This protein may bind to 16S RNA (Muto et al., 1974).

S18. (0.3-0.4). The modification of one cysteine residue in S18 by treatment of the 70S ribosome with N-ethyl maleimide results in a large reduction of activity in the poly U-directed polyphenylalanine synthesis assay (Moore, 1971). In agreement with this result, Elson et al. (1973), using radioactively labeled N-ethyl maleimide, found that S18 is essential for the enzymatic (initiation factor-dependent) binding of fmet-tRNA$^{\text{met}}_F$ to the 30S subunit and for the association of the 30S subunit
with the 50S particle to form the 70S ribosome. The binding
of phenylalanyl-tRNA to the 30S subunit in the presence of
poly U protects S18 from trypsin digestion (Rummel and Noller,
1973). In addition, S18 appears to be required for "A" site
binding as anti-S18 antibody fragments inhibit the binding of
phenylalanyl-tRNA to the ribosome (Lelong et al., 1974).
Pellegrini et al. (1974) have reported that S18, as well as
L2 and L27, are labeled during the binding of the affinity
probe bromoacetyl phenylalanyl-tRNA to the ribosome.

S19. (0.5). The binding of phenylalanyl-tRNA to the 30S subunit in
the presence of poly U protects S19 from trypsin attack (Rummel
and Noller, 1973). Anti-S19 antibody inhibits the binding of
aminoacyl-tRNA to both the "A" site and the "D" site (Lelong
et al., 1974).

S20. (0.8). This protein binds to 16S RNA (Muto et al., 1974).
anti-S20 antibody fragments block the binding of aminoacyl-tRNA
to both the "A" and "D" sites on the ribosome (Lelong et al.,
1974).

S21. (0.3). Protein S21 may be involved in both the "A" site and the
"D" site on the ribosome as anti-S21 antibody blocks both the
initiation factor-dependent binding of fmet-tRNA and the
elongation factor-dependent binding of phenylalanyl-tRNA
(Lelong et al., 1974).
50S Subunit Proteins

L1. (1.10). This protein may bind to 23S RNA (Garrett et al., 1974b).

L2. (0.9). This protein binds to 23S RNA (Garrett et al., 1974b) and may be important for "D" site function as it is labeled during the binding of the affinity label, peptidyl-tRNA analogs, bromoacetyl phenylalanyl-tRNA (Pellegrini et al., 1974), and p-nitrophenyl-carbamyl-phenylalanyl-tRNA (Czernilofsky et al., 1974) to the ribosome.

L3. (1.4). This protein may bind to 23S RNA (Garrett et al., 1974b).

B. stearothermophilus 50S ribosomal subunits reconstituted in the absence of L3 (E. coli) are not active in aminoacyl-tRNA binding or in polyphenylalanine synthesis as directed by poly U (Fahnstock et al., 1973).

L4. (0.9). This protein may bind to 23S RNA (Garrett et al., 1974b).

Protein L4 may be important for tRNA binding to the ribosome as anti-L4 antibodies inhibit the binding of phenylalanyl-tRNA (Highland et al., 1974). This protein is released from the ribosome during "unfolding" (Garrett and Wittmann, 1973).

L5. (1.4-1.8). As mentioned previously, there are reports that L5 binds weakly to 5S RNA (Horne and Erdmann, 1972; Yu and Wittmann, 1973).

L6. (1.15). Protein L6 binds to 23S RNA (Garrett et al., 1974b) and is involved in complexing 5S RNA to 23S RNA (Garrett and Wittmann, 1973).
L7/L12. (1.0-3.0). The amino acid sequences of these two proteins differ only by the acetylation of the N-terminal serine on L7 (Terhorst et al., 1972). The stoichiometry varies, as with S6, according to cell growth conditions (Garrett and Wittmann, 1973). These proteins are essential for the G-factor catalyzed translocation reaction and for the elongation factor-dependent binding of aminoacyl-tRNA to the "A" site as shown by reaction of 70S ribosomes with antibodies directed against these proteins (Highland et al., 1974).

L8+L9. (1.40).

L10. (0.95).

L11. (1.15). Protein L11 may be at or near the "D" site since it is labeled when E. coli 70S ribosomes react with the peptidyl-tRNA affinity analog, 2-nitro-4-azidophenoxy-4'-phenylacetyl-phenylalanyl-tRNA in the presence of poly U (Hsuing et al., 1974).

L13. (1.40). This protein may bind weakly to 23S RNA (Garrett et al., 1974b).

L14. (0.8). Protein L14 may be at or near the "D" site as reaction of 70S ribosomes with p-nitrophenyl-carbamyl-phenylalanyl-tRNA labels L14 (Czernilofsky et al., 1974).

L15. (Stoichiometry not known). Reaction of 70S ribosomes with p-nitrophenyl-carbamyl-phenylalanyl-tRNA labels L15 (Czernilofsky et al., 1974).
L16. (0.8). This protein binds to 23S RNA (Garrett et al., 1974b). L16 may also be involved with "D" site binding as it is labeled when the affinity label probe p-nitrophenyl-carbamyl-phenylalanyl-tRNA is bound to the ribosome (Czernilofsky et al., 1974).

L17. (1.2). This protein may bind weakly to 23S RNA (Garrett et al., 1974b).

L18. (2.18). Protein L18 binds directly to 5S RNA (Horne and Erdmann, 1972; Yu and Wittmann, 1973; Gray et al., 1973). Protein L18 may also be at or near the "D" site since it is labeled, along with L11, when the peptidyl-tRNA analog, 2-nitro-4-azidophenoxy-4'-phenylacetyl-phenylalanyl-tRNA, is bound to the ribosome (Hsuing et al., 1974).

L19. (1.0). Protein L19 may bind to 23S RNA (results are variable) and may also bind to 16S RNA (Garrett and Wittmann, 1973).

L20. (Stoichiometry not known). This protein binds to 23S RNA (Garrett et al., 1974b) and may bind weakly to 5S RNA (Horne and Erdmann, 1972).

L21. (0.85).

L22. (1.65-2.0).

L23. (1.20). Protein L23 binds to 23S RNA (Garrett et al., 1974b).

L24. (1.60). This protein binds near the 5'-terminus of 23S RNA (Garrett and Wittmann, 1973).
L25. (1.40). Protein L25 binds directly to 5S RNA (Horne and Erdmann, 1972; Yu and Wittmann, 1973; Gray et al., 1973). This protein is released from the ribosome during "unfolding" (Garrett and Wittmann, 1973).

L26. (0.2). The binding of bromoacetyl-phenylalanyl-tRNA to the 70S ribosome labels L26 (as well as L2 and L27), indicating that this protein may be important for the binding of aminoacyl- or peptidyl-tRNA to the "D" site (Pellegrini et al., 1974).

L27. (0.75). This protein may be at or near the "D" site as it is labeled when either bromoacetyl-phenylalanyl-tRNA (Pellegrini et al., 1974) or p-nitrophenyl-carbamyl-phenylalanyl-tRNA (Czernilofsky et al., 1974) is bound to the 70S ribosome.

L28. (0.45).

L29. (1.10).

L30. (0.90). This protein may bind weakly to 5S RNA (Horne and Erdmann, 1972).

L31. (Stoichiometry not known).

L32. (0.60).

L33. (0.50).

L34. (Stoichiometry not known).

Since essentially nothing is known on the molecular basis for the in situ selective interactions of the ribosomal proteins with each other and with nucleic acids, numerous difficulties arise.
in the interpretation of some of these results and in the subsequent attempt to assign a function to each of these proteins. For example, the studies using antibodies or antibody fragments directed against a specific ribosomal protein are subject to several serious limitations. Inhibition of a particular ribosomal function by a given antibody may not necessarily mean that the protein is at the active site, as the inhibitory effect could reflect an allosteric phenomenon caused by cooperative ribosomal protein-protein or protein-nucleic acid interactions. Secondly, the failure of a particular antibody to inhibit a ribosomal function could reside in the fact that it does not react with a determinant that is essential for the in situ activity of the proteins. Similarly the investigations using derivatives of aminoacyl-tRNA carrying reactive side groups attached to the aminoacyl moiety (i.e., bromoacetyl-phenylalanyl-tRNA, p-nitrophenyl-carbamyl-phenylalanyl-tRNA, 2-nitro-4-azidophenoxy-4'-phenylacetyl-phenylalanyl-tRNA) may indicate which proteins may be at or near the tRNA binding sites on the ribosome, but they do not permit one to unambiguously distinguish which ribosomal proteins, if any, are interacting directly with tRNA. Also in several cases using different affinity label probes on the tRNA species the labeling patterns are quite different. For example, while Pellegrini et al. (1974), Czernilofsky et al. (1974), and Hsuing et al. (1974) all found some ribosomal proteins, albeit sometimes different ones, reacting with the affinity label attached to aminoacyl-tRNA, Bis-pink and Matthei (1973), using a nonspecific reactive probe, ethyl...
2-diazaomalonyl-phenylalanyl-tRNA which is capable of reacting with either proteins or RNA, found that the label was exclusively associated with 23S RNA in the 70S E. coli ribosome. However Schwartz and Ofengand (1974) found that only 16S RNA was labeled when E. coli valyl-tRNA containing another nonspecific reactive group, phenacyl-p-azide substituted to a 4-thiouridine residue in the tRNA, was bound to the 70S ribosome. In contrast, the nonspecific affinity label used by Hsuing et al. (1974), 2-nitro-4-azidophenoxy-4'-phenylacetyl-phenylalanyl-tRNA, reacted only with the 50S ribosomal proteins L11 and L18; no ribosomal RNA was labeled. These conflicting results emphasize the problems involved in the in situ study of ribosomal protein interactions.

An alternative approach would be to develop a simple in vitro system which retains the specificity involved in the recognition and binding mechanisms between ribosomal proteins and various RNA molecules, thereby permitting the isolation and study of these interacting species. Affinity chromatography is especially amenable in this regard as it provides a rapid and efficient means of selective isolation based upon an exploitation of the unique biological properties of proteins or nucleic acids to bind ligands specifically and reversibly (for a review see Cuatrecasas and Anfinsen, 1971a). The procedure involves passing a solution containing one component of the interacting species over a bed containing the other component covalently linked to an insoluble polymer or gel; e.g., cross-linked dextran (Sephadex), beaded agarose derivatives (cyanogen bromide-activated Sepharose), cellulose, synthetic polyacrylamide
gels, or controlled-pore glass particles. Macromolecules showing affinity for the insolubilized ligand will bind to the matrix noncovalently, while those not recognizing the ligand will pass through unretarded. The biospecifically absorbed molecules can then be eluted from the solid support by changing the solvent parameters to effect dissociation. With these advantages in mind, an affinity chromatography procedure has been developed using \textit{E. coli} ribosomal RNA or tRNA covalently coupled to agarose in order to study ribosomal protein-RNA interactions. In previous studies other workers have reported the synthesis of water-insoluble RNA derivatives using a variety of techniques. In most procedures the nucleic acid is covalently coupled to the solid support, but in some cases high molecular weight polynucleotides, such as the larger ribosomal RNAs, can be physically immobilized in agarose (Petrovic \textit{et al.}, 1973). Several methods have been employed to immobilize native or chemically modified RNA molecules. Cellulose has been used as a matrix by Gilham (1968) to covalently attach \textit{E. coli} tRNA, previously activated by reaction with a substituted carbodiimide, through the 5'-terminal phosphate residue of the RNA. Similarly, tRNA has been linked to cyanogen bromide-treated agarose either directly (Bonavida \textit{et al.}, 1970) or through a spacer group or "arm." Bartkowiac and Pawelkiewicz (1972) attached \textit{E. coli} isoleucyl-tRNA to bromoacetylamidobutyl-agarose through the free amino group on the esterfied isoleucine. Others have chemically modified the tRNA by periodate cleavage of the 3'-terminal ribose group yielding a reactive dialdehyde configuration. The modified tRNA can then be
covalently coupled to an activated amino group attached to the matrix. In this manner periodate-oxidized *E. coli* tRNA has been coupled to hydrazide-agarose (Remy *et al.*, 1972; Grosjean *et al.*, 1973), polyacrylhydrazide-agarose (Nelidova and Kiselev, 1968), ε-aminocaproyl-agarose (Robberson and Davidson, 1972), and, concomitant in time with the procedure developed here as outlined in the Experimental section, to adipic acid dihydrazide-agarose (Joyce and Knowles, 1974). Transfer RNA immobilized by these different procedures was found to retain its ability to react specifically with other biological macromolecules as indicated by the use of insolubilized tRNA as an immunoabsorbent for the isolation of precipitating antibodies directed against tRNA (Bonavida *et al.*, 1970), to purify the cognate aminoacyl-tRNA synthetases (Nelidova and Kiselev, 1968; Bartkowiac and Pawelkiewicz, 1972; Remy *et al.*, 1972; Joyce and Knowles, 1974), and to examine complementary anticodon interactions between various tRNAs (Grosjean *et al.*, 1973). Robberson and Davidson (1972) have attempted to use *E. coli* 16S RNA coupled to ε-aminocaproyl-agarose through the periodate-oxidized 3′-terminus, to isolate the ribosomal DNA sequences. The coupling of the RNA to the solid support can occur at a discrete site, such as the 3′-terminus in the reaction of periodate-oxidized RNA with a hydrazide substituted matrix, or at a number of sites, as when the unmodified RNA is reacted directly with cyanogen bromide-treated agarose (Berridge and Aronson, 1973). Evidently the ability of an unmodified, native RNA or polynucleotide to couple directly to cyanogen bromide-activated agarose is dependent upon the degree of
secondary and tertiary structure present in the RNA. Poonian et al. (1971) found that double-stranded DNA or RNA attached only very poorly, if at all, to cyanogen bromide-activated agarose, but single-stranded nucleic acids or double-stranded polynucleotides with long single-stranded regions at either terminus were covalently bound, presumably through free aromatic amino groups on the bases.

In the studies to be described here, the affinity absorbent used was synthesized by covalently linking ribosomal RNA or tRNA to cyanogen bromide-activated agarose, via the 3'-terminal nucleotide residue, through an adipic acid dihydrazide spacer group. Agarose derivatives are especially suitable as a solid support since they have a very loose structure which allows molecules with molecular weights in the millions to diffuse readily through the matrix, can easily undergo substitution reactions with cyanogen halides, are very stable, and have a high capacity for substitution (Cuatrecasas and Anfinsen, 1971b). A hydrocarbon "arm" or spacer group was interposed between the RNA ligand and the agarose backbone since steric factors produced by the closeness of the ligand to the agarose bead surface have been shown to strongly inhibit bioaffinity interactions (Cuatrecasas and Anfinsen, 1971b). Adipic acid dihydrazide readily couples to cyanogen bromide-activated agarose and to the free aldehyde groups produced by periodate oxidation of the ribose moiety in nucleotides (Lamed et al., 1973) and has the advantage of being relatively hydrophilic, thereby greatly reducing the possibility of nonbiospecific protein absorption.
(O'Carra et al., 1974). The method of attachment of the RNA to the dihydrazide gel, through the oxidized 3'-terminal nucleotide residue, was chosen since extensive double-stranded regions are present both in E. coli tRNA (Cramer and Gauss, 1972) and ribosomal RNA (Monier, 1972) thus indicating a low probability of attachment directly to cyanogen bromide-activated agarose (Poonian et al., 1971). Previous reports have shown that periodate modification of the 3'-terminal ribose moiety evidently does not affect the biological activity of either 5S RNA or tRNA in many of the reactions in protein synthesis. Fahnstock and Nomura (1972) found that periodate oxidized 5S RNA incorporated into B. stearothermophilus 50S subunits does not affect the polypeptide synthesis activity of the reconstituted particles. Similarly it has been shown that 3'-terminal oxidized E. coli tRNA after reduction with borohydride can be amino-acylated (Cramer et al., 1968; Ofengand and Chen, 1972) and can be bound nonenzymatically to both the ribosomal "A" and "D" sites, although it is inactive in the enzymatic binding reaction directed by elongation factor EF-Tu, in the GTP hydrolysis reaction, and in the peptidyl transferase step (Chinali et al., 1974). These findings indicate that cleavage of the C2'-C3' bond of the 3'-terminal ribose group does not destroy the specificity of the ribosomal protein-RNA interactions in the function of 5S RNA or in the binding of tRNA to the ribosome.
EXPERIMENTAL PROCEDURE

Bacterial Cells

*Escherichia coli* strain B (3/4 log phase) grown on enriched medium was purchased from Grain Processing Corp., Muscatine, Iowa, as a frozen paste. Radioactively labeled ribosomal proteins and RNA were prepared from cells of *E. coli B* grown in a glucose-salts medium (Demerec and Cahn, 1953) containing either 50 μCi/1 of [2-14C] uracil (45 mCi/mmmole), or 250 μCi/1 of [14C] L-amino acid mixture. Cultures were harvested in early exponential growth, as measured by the turbidity at 650 nm in a Beckman Spectrometer 20, washed once in TMA2 buffer (0.01 M Tris-HCl buffer, pH 7.4, containing 0.01 M MgCl₂, 0.06 M NH₄Cl, 0.006 M 2-mercaptoethanol), and stored at -75° until used.

Preparation of Ribosomes and Ribosomal Subunits

Cells were suspended in TMA2 buffer containing 2 μg/ml deoxyribonuclease (ribonuclease-free) and broken in a pre-chilled French Pressure cell at 12,000 p.s.i. In the experiments with radioactively labeled cells, they were broken by grinding with alumina in the cold for 30 minutes. The resulting paste was suspended in TMA2 buffer containing 2 μg/ml deoxyribonuclease. All subsequent operations were performed at 0°-4°. Ribosomes were prepared by differential centrifugation as described by Tissières *et al.* (1959) and were washed twice with TMA2 buffer, then once with
TMA2 buffer containing 0.5 M NH$_4$Cl, and finally dialyzed against TMA2 buffer. The final preparation was frozen and stored at -75°.

Ribosomes were dissociated into subunits by dialysis against 0.01 M potassium phosphate buffer (pH 7.4) containing 0.0001 M MgCl$_2$, 0.01 M 2-mercaptoethanol, and the subunits were isolated by zonal centrifugation for 5.5 hours at 40,000 rpm in the B IV rotor of the Spinco Model L-4 ultracentrifuge in a 10%-25% linear sucrose gradient in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.06 M NH$_4$Cl, 0.0001 M MgCl$_2$, and 0.006 M 2-mercaptoethanol. The separated ribosomal subunits were recovered after centrifugation by using 50% sucrose to pump the gradient out of the rotor and into a fraction collector. Fractions of 40 ml were collected at 0°-4° and their absorbance at 260 nm measured. After pooling the appropriate fractions, the purified subunits were isolated by centrifugation at 105,000 × g for 12 hours in the 35 rotor of the Spinco Model L-4 ultracentrifuge. The 50S subunits were resuspended in TMA2 buffer and stored at -75°. 30S subunits were also stored frozen at -75° in the same buffer except a lower magnesium ion concentration (0.0001 M) was used to prevent aggregation. The subunit preparations generally contained less than 5% cross-contamination as judged by analytical sucrose gradient sedimentation patterns. In addition, the 50S and 30S subunits individually were not active in the in vitro synthesis of poly [14C] phenylalanine directed by polyuridylic acid (Nirenberg and Matthaei, 1961), however an equimolar mixture of the two subunit preparations was fully active.
in this assay when compared to the activity of control, undissociated 70S ribosomes.

The concentration of ribosomes was calculated from the absorbance at 260 nm using a value of $A_{260}^{0.15} = 16$.

Preparation of Ribosomal Proteins

Ribosomal proteins were prepared from purified ribosomal subunits by the urea-LiCl procedure of Spitnik-Elson (1965). An equal volume of 8 M urea - 6 M LiCl was added to the ribosome solution (3-5 mg/ml) and the mixture was incubated at 0° for 48-72 hours. Precipitated RNA was removed by centrifugation. For affinity chromatography studies, the 30S subunit proteins were directly dialyzed against binding buffer (0.005 M potassium phosphate, pH 7.4; 0.3 M KCl; 0.02 M MgCl$_2$; 0.006 M 2-mercaptoethanol; Traub and Nomura, 1968) to remove urea and LiCl. 50S ribosomal proteins were further purified after the urea-LiCl treatment by chromatography on DEAE-cellulose to remove endogenous, solubilized 5S RNA (Nomura and Erdmann, 1970). The ribosomal proteins, in a 0.01 M Tris-HCl buffer (pH 7.4) containing 4 M urea, 0.3 M KCl, and 0.001 M MgCl$_2$, were passed over a DEAE-cellulose column, equilibrated with the same buffer, at 4°. Ribosomal proteins were not retarded on the column. Bound RNA could be eluted from the column by increasing the KCl concentration to 1.5 M in the same buffer. This purification step removed essentially all of the 5S RNA from the protein mixture as determined by addition of a small amount of $[^{14}C]$ 5S RNA to the 50S
proteins before DEAE-cellulose chromatography and subsequent measurement of the radioactivity contained in the fractions eluted with 0.3 M and 1.5 M KCl. No 50S ribosomal proteins were lost by this procedure and all 50S proteins, described by Kaltschmidt and Wittmann (1970b), were present in the 0.3 M KCl fraction, as determined by two-dimensional gel electrophoresis (see Appendix). The purified 50S ribosomal proteins were then dialyzed against 1% acetic acid, lyophilized, and redissolved in binding buffer. In some experiments, the lyophilized 50S proteins were first dissolved in a small volume (0.1-0.2 ml per 15-20 mg protein) of deionized 4 M urea and then diluted 100-fold with binding buffer (Nomura and Erdmann, 1970). This procedure was found to increase the solubility of some of the higher molecular weight proteins; notably L1, L3, and L5. A small amount of both 50S and 30S protein was insoluble in binding buffer and was removed by centrifugation. These proteins were then dissolved in 8 M urea.

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard or by the modification of Geiger and Bessman (1972) when 2-mercaptoethanol was present in the protein sample.

Preparation of RNA

Ribosomal RNA was isolated either from washed, 70S ribosomes or from purified ribosomal subunits by the phenol-sodium dodecyl sulfate method of Kurland (1960) modified by the addition of bentonite
(3 mg/ml final concentration) as a ribonuclease inhibitor (Fraenkel-Conrat et al., 1961). Transfer RNA was prepared by the same procedure from the lysed cell supernatant remaining after centrifuging down the ribosome fraction. 5S RNA and tRNA were purified to apparent homogeneity on Sephadex G-100 columns using the procedure of Schleich and Goldstein (1966). Radioactive RNA samples recovered from analysis by Sephadex G-100 gel filtration were precipitated by addition of cold, 5% trichloroacetic acid, filtered through nitrocellulose filters (Millipore, 0.45 μm size), washed, dried, and counted in a Packard Tri-Carb liquid scintillation spectrometer using a toluene-based scintillation fluid containing 2,5-diphenyloxazole and 1,4-bis-[2-(5-phenyloxazolyl)]-benzene. 5S RNA was denatured by heating at 60° for 4 minutes in a 0.015 M sodium citrate buffer (pH 7.0) containing 0.15 M NaCl followed by rapid cooling to 0°; denatured 5S RNA could be renatured by heating at 60° for 5 minutes in a 0.01 M Tris-HCl buffer (pH 6.9) containing 0.01 M MgCl₂ followed by slow cooling to room temperature (Aubert et al., 1968). High molecular weight 16S RNA and 23S RNA were separated by sucrose gradient sedimentation (McConkey, 1967). RNA preparations were dissolved in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.01 M MgCl₂ and stored at -75°.

Concentrations of ribosomal RNA or tRNA in solution were calculated from the absorbance at 260 nm, assuming a value of $A_{260}^{0.1\%} = 24$.

Samples from different tRNA preparations were tested for amino
acid accepting activity using the cell-free system of Scott (1968). Typical values obtained with $^{14}$C phenylalanine were 1580 pmoles per mg tRNA as compared to 880 pmoles per mg tRNA for commercially available *E. coli* tRNA (Schwartz Biochemicals).

Preparation of Adipic Acid Dihydrazide-Agarose

Adipic acid dihydrazide was synthesized from hydrazine hydrate and diethyl adipate according to the procedure of Lamed et al. (1973). Cyanogen bromide-activated Sepharose 4B was washed with 0.001 M HCl and reacted with an excess of adipic acid dihydrazide (0.9 g per g CNBr-agarose; 0.005 mole per 0.001 mole CNBr) in 0.1 M sodium carbonate buffer (pH 9.6) at 4°C. The reaction attaching the dihydrazide ligand was conveniently carried out in a graduated glass centrifuge tube and was allowed to proceed for 12-15 hours with continuous mixing of the suspension by end-over-end rotation of the tube. The resulting dihydrazide-substituted gel was washed on a sintered glass filter with 0.2 M NaCl until all excess, unreacted adipic acid dihydrazide had been removed; that is until the filtrate gave a negative color test with sodium 2,4,6-trinitrobenzenesulfonate (Cuatrecasas, 1970). The washed dihydrazide-agarose was then resuspended in 0.1 M sodium acetate (pH 5.0) and stored at 4°C until used.
Oxidation of RNA and Coupling to Dihydrazide-Agarose

RNA, dissolved in 0.1 M sodium acetate buffer (pH 5.0) to a concentration of 1.67 mg/ml, was oxidized with a 120-fold molar excess of sodium metaperiodate added as a 0.1 M solution in 0.1 M sodium acetate buffer (pH 5.0) according to the method of Fannstock and Nomura (1972). After incubation at room temperature in the dark for 1.0-1.5 hours, the reaction was terminated by addition of 2.5 volumes of cold, 95% ethanol. The precipitate of oxidized RNA was recovered by centrifugation and washed by redissolving it in 0.1 M sodium acetate buffer (pH 5.0) and reprecipitating it with ethanol. The washed preparation was dissolved in 0.1 M sodium acetate buffer (pH 5.0) to a concentration of 1-2 mg/ml.

To couple the oxidized RNA to the dihydrazide-agarose, the two solutions were combined and mixed continuously for a period of 12-24 hours (except as noted in the kinetic binding experiments) at 4°C. The resulting RNA-agarose was washed repeatedly with 2M KCl to remove all noncovalently bound RNA, as determined by measuring the absorbance of the washes at 260 nm, and then equilibrated with binding buffer at 4°C. The amount of RNA coupled to the dihydrazide matrix was calculated by subtracting the total A_{260} units remaining in the supernatant of the reaction mixture and in the KCl washes, from the initial amount of oxidized RNA added. Typically this value was 80-90% of the input for 5S RNA and tRNA. In some cases this calculation was verified by hydrolysis of an aliquot of the RNA-agarose with 1.0 M KOH for 24 hours at 23°C. The amount of RNA
bound to the gel was determined by measuring the absorbance at 260 nm of the hydrolyzate supernatant and using a hyperchromicity correction factor of ca. 40%.

Essentially no RNA was released from the resin after six months storage in binding buffer at 4°, as determined by measuring the absorbance at 260 nm of the supernatant.

Affinity Chromatography of Ribosomal Proteins on RNA-Agarose

For affinity chromatography both batch and column procedures were used. In the batch method, ribosomal proteins and RNA-agarose, both in binding buffer, were permitted to interact at 4° for approximately 24 hours with continuous mixing. The suspension was then centrifuged in a clinical centrifuge and the supernatant removed. The gel was exhaustively washed with binding buffer by addition of 7-10 ml of buffer per gram of gel, mixing for 30-45 minutes, then centrifuging and removing the supernatant, until all unbound protein had been removed. Bound proteins were then eluted from the RNA matrix by washing the gel with a high salt-EDTA dissociation buffer containing 0.005 M potassium phosphate (pH 7.4), 2.0 M KCl, 0.005 M Na₂EDTA, and 0.006 M 2-mercaptoethanol. In column chromatographic studies, the RNA-agarose was poured to form a small column (1.0 cm x 4.5 cm per 1.0 g gel) and equilibrated at 4° with binding buffer. Ribosomal proteins dissolved in binding buffer were then passed over the column at a slow rate of 7-10
ml/hour. All unbound protein was removed by washing the column with several column volumes of binding buffer. Bound proteins were eluted from the column with a linear gradient formed from equal volumes of binding buffer and dissociation buffer. After use the RNA-agarose was re-equilibrated with binding buffer and stored at 4°C.

Electrophoretic Procedures

Disc gel electrophoresis of ribosomal proteins was carried out on 0.6 cm x 9.0 cm, 10% polyacrylamide gels at pH 4.5 according to the method of Reisfeld et al. (1962) as modified by Leboy et al. (1964). Protein samples were treated with 0.06 M 2-mercaptoethanol at pH 8.1 before electrophoresis to prevent aggregation artifacts arising from the oxidation of sulfhydryl groups (Hardy et al., 1969). Gels were stained for two hours in a 50% methanol, 9% acetic acid solution containing 0.25% Coomassie Blue (Weber and Osborn, 1969) and destained by washing with 5% methanol, 7% acetic acid.

RNA samples were analyzed on 0.6 cm x 10.0 cm, 10% polyacrylamide gels in 7 M urea by the procedure of Richards et al. (1965) as modified by Monier and Feunteun (1971). Gels were stained with 0.5% Pyronine Y in a lanthanum acetate solution for 16 hours (Marcinka, 1972).

Two-dimensional gel electrophoresis was accomplished in the apparatus described by Kaltschmidt and Wittmann (1970a). In the original procedure, 1-2 mg of ribosomal proteins were immobilized
in a 4% acrylamide sample gel placed in the center of a 0.5 cm x 18.0 cm, 18% acrylamide separation gel (pH 8.6) in 6 M urea. Electrophoresis in the first dimension was carried out in a Tris-borate buffer (pH 8.6) at 2.5 mA/gel and 4°C for 20 hours. Each first-dimension gel was then washed with a potassium acetate buffer (pH 5.8) containing 8M urea and incorporated into a second-dimension, 18% acrylamide (pH 4.5) gel slab. Electrophoresis in the second-dimension, in a glycine-acetic acid buffer (pH 4.2), was at room temperature and at 105 V for 26 hours. In some experiments (noted in the Figure legends), the apparatus was modified by addition of plexiglass inserts (see following section) to reduce the thickness of the second-dimension gel slabs. When the plexiglass inserts were used, the acrylamide gel and buffer solutions described by Howard and Traut (1973) were employed and the original procedure of Kaltschmidt and Wittmann (1970a) was modified as follows: 0.5 cm x 10.0 cm first-dimension gels (4% acrylamide, pH 8.2) were electrophoresed at 5 mA/gel for 5.5 hours at 4°C, then washed as before. Two first-dimension gels were then electrophoresed in parallel on the same second-dimension, 18% polyacrylamide (pH 4.5) gel slab for 12 hours at 140 V and at room temperature. In both procedures the slabs were stained with either 0.55% Amido Black in 5% acetic acid for 15 minutes (Kaltschmidt and Wittmann, 1970a) or with Coomassie Blue; 0.05% in 12% trichloroacetic acid for 12 hours (Fishbein, 1972), or 0.1% in 50% methanol, 7.5% acetic acid for 3 hours (Howard and Traut, 1973). The slabs were destained by washing.
Design of the Plexiglass Inserts

A plexiglass insert was developed to adapt the two-dimensional gel electrophoresis apparatus of Kaltschmidt and Wittmann (1970a) for use with reduced-size gel slabs (Fig. 23, Appendix). The insert consists of a plexiglass sheet, 8 1/4" x 7 7/8" x 1/8" with a 45° bevel, 3/16" wide, along one 8 1/4" edge. A plexiglass strip, 3/32" x 7 9/16" x 1/16", is glued flush with each 7 7/8" edge, leaving a 1/8" gap at the bottom of the sheet. The spacers allow the preparation of gel slabs 1/16" thick, as compared to 3/16" thick slabs formed in the original, unmodified apparatus. This modification also permits the use of microgram amounts of sample (as compared to milligram quantities required in the original procedure), reduces the total electrophoresis time by more than 50%, allows for two protein samples to be run on the same gel slab thus enhancing the comparison between different protein samples while doubling the capacity of the original apparatus, and results in a gel slab thin enough to permit radioautography of [14C] labeled protein samples. As can be seen in the Appendix (Fig. 24), the plexiglass insert modification retains the excellent resolution of the original Kaltschmidt and Wittmann apparatus (1970a), yet allows the individual proteins in two complex mixtures, the total E. coli 50S and 30S ribosomal proteins, to be easily identified and compared on the same gel slab.
Scanning of Stained Gels

In some cases the stained protein patterns obtained by polyacrylamide disc electrophoresis were measured by scanning the gels in a Zeiss Model M4QIII spectrophotometer equipped with the ZK3 adapter and recording the absorbance at 550 nm using a Honeywell Model 194 Lab Recorder coupled to a Series 200 Integrator (Disc Instruments).

Radioautography of Two-Dimensional Gel Slabs

For the analysis of radioactively labeled ribosomal proteins, reduced-size, two-dimensional gel slabs containing the separated proteins were dried against filter paper supports in vacuo and at 55°C using the rubber sheet sandwich method as described by Maizel (1971). The mounted, dried gels were placed in direct contact with X-ray film and exposure was allowed to take place for 1-2 weeks at room temperature. The exposed film was developed as instructed in Kodak Rapid Bath developer.

Materials

Sephadex G-100 and cyanogen bromide-activated Sepharose 4B were purchased from Pharmacia, Inc. DEAE-cellulose was the Whatman microgranular grade, DE-32. Bentonite was a Fisher product and was prepared according to the method of Fraenkel-Conrat et al. (1961). Bacteriological grade alumina was a gift of Alcoa Chemicals. [14C] L-phenylalanine was purchased from Schwartz BioResearch.
[2-\(^{14}\)C] uracil as well as the \(^{14}\)C labeled L-amino acid mixture were obtained from New England Nuclear. Polyuridylic acid was a Miles product. Ribonuclease-free deoxyribonuclease was purchased from Worthington. Acrylamide and N,N-methylenebisacrylamide were obtained from Eastman Kodak and were recrystallized before use according to the procedure of Loening (1967). Bovine serum albumin was purchased as the Fraction V crystallized powder from Pentex Corp. 5'-AMP (sodium salt) was a Sigma chemical. Amido Black and Coomassie Blue R250 stains were obtained from Canalco; Pyronine Y stain was purchased from Eastman Kodak. Ribonuclease-free sucrose (ultrapure grade) was a Schwartz/Mann product. X-ray film (Royal X-OMat RP/R2) was obtained from Eastman Kodak. 2,5-Diphenyloxazole and 1,4-bis-[2-(5-phenyloxazolyl)]-benzene were purchased from Packard Instrument Co. All other reagents used were of analytical or higher grade.

Phenol was distilled before use to remove impurities and the distillate was stored at 4° in the dark.

Dialysis tubing was pretreated by heating at 80°-90° in 0.0054 M Na\(_2\)EDTA, 0.014 M NaHCO\(_3\), and 0.007 M 2-mercaptoethanol for 30 minutes to remove heavy metal and enzyme contaminants. The tubing was then thoroughly washed with deionized water and stored at 4° in deionized water.

Urea solutions were deionized before use by stirring with 1/20 volume Amberlite MB-3 (Mallinckrodt Chemicals) mixed bed, ion exchange resin for 3 hours at room temperature, then filtering the resulting suspension.
RESULTS

Preparation and Properties of RNA-Agarose

The adipic acid dihydrazide-agarose resin is capable of binding rather large amounts of periodate oxidized RNA and other molecules containing available aldehyde groups. The coupling of such ligands, for example oxidized *E. coli* 5S RNA or benzaldehyde, exhibits a saturation phenomenon (Fig. 1). Approximately 12 mg (3.0 x 10^-7 mole) of oxidized 5S RNA can be bound per gram of dihydrazide-agarose at saturation, however this is less than 1/10 the saturating molar amount of benzaldehyde, 4.7 x 10^-6 mole per g gel (Fig. 1). A wide range of oxidized RNA species of different molecular weights can be immobilized in significant quantities by this procedure (Table 1). However steric factors evidently are important in the coupling reaction, as the molar amount linked to the gel decreases with increasing molecular weight (Fig. 1, Table 1). It should be noted in Table 1 that nonsaturating amounts of oxidized 5S RNA (approximately 50% of the saturation value, see Fig. 1), and probably also of oxidized tRNA were used. This is reflected in the moles bound/moles added ratio in Table 1 for these two RNA species; the deviation of this ratio from the theoretical value of 1.0, i.e. all of the RNA added is bound, is most likely due to experimental error. The amounts of benzaldehyde (see Fig. 1) and periodate oxidized 5'-AMP, 16S RNA, and 23S RNA coupled per g resin as shown in Table 1 should be at or near saturation as determined by the moles
Figure 1. Binding of benzaldehyde and periodate oxidized E. coli 5S RNA to adipic acid dihydrazide-agarose. The amount of 5S RNA bound was determined by alkaline hydrolysis of the reacted gel and measurement of the absorbance at 260 nm of the clear supernatant (see Experimental). Quantitation of benzaldehyde coupled was obtained by measuring the reaction supernatant at 248 nm, and the amount bound to the gel was calculated using a molar extinction coefficient of 12 x 10^3 (Robberson and Davidson, 1972)

(A) periodate oxidized 5S RNA
(B) Benzaldehyde
Table 1. Binding of oxidized and unoxidized ligands to adipic acid dihydrazide-agarose. The reaction was carried out as described in Experimental for 24 hours. The amount of sample bound to the gel was estimated by measurement of the absorbance at 260 nm of the reaction supernatant and the 2.0 M KCl wash (Experimental).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial concn. (M)</th>
<th>Vol. resin (ml)</th>
<th>Reaction vol. (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzaldehyde</td>
<td>$5.0 \times 10^{-4}$</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>$8.3 \times 10^{-4}$</td>
<td>0.3</td>
<td>1.2</td>
</tr>
<tr>
<td>tRNA</td>
<td>$1.1 \times 10^{-5}$</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>5S RNA</td>
<td>$8.3 \times 10^{-6}$</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>16S RNA</td>
<td>$9.5 \times 10^{-7}$</td>
<td>0.3</td>
<td>1.2</td>
</tr>
<tr>
<td>23S RNA</td>
<td>$4.7 \times 10^{-7}$</td>
<td>0.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Numbers in parentheses refer to the corresponding mg coupled/g adipic acid dihydrazide resin.

Benzaldehyde was not oxidized.
<table>
<thead>
<tr>
<th>Oxidized</th>
<th>Unoxidized</th>
<th>Oxidized</th>
<th>Unoxidized</th>
<th>Moles bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>4700 (0.5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.27</td>
</tr>
<tr>
<td>7700 (2.7)</td>
<td>460 (0.16)</td>
<td>10.7</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>180 (5.5)</td>
<td>15 (0.46)</td>
<td>12.0</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>150 (6.1)</td>
<td>7.3 (0.3)</td>
<td>20.5</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>8.7 (4.8)</td>
<td>2.6 (1.4)</td>
<td>3.3</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>4.8 (5.3)</td>
<td>1.7 (1.9)</td>
<td>2.8</td>
<td>0.71</td>
<td></td>
</tr>
</tbody>
</table>
bound/moles added ratios for these ligands, although this calculation may be only approximate since the efficiency of periodate oxidation for 5'-AMP, 16S RNA, and 23S RNA may not be the same as that for 5S RNA (greater than 90% oxidized; Fahnstock and Nomura, 1972).

Oxidation of the RNA is necessary for binding to the dihydrazide matrix; only a small amount of unoxidized sample is retained in the 2.0 M KCl washed gel (Table 1). Here again there appears to be a molecular weight effect as the ratio of oxidized to unoxidized sample bound, Table 1, was much greater for 5'-AMP, tRNA, and 5S RNA than for 16S RNA or 23S RNA.

It was of interest to examine the binding kinetics of both oxidized and unoxidized RNA to adipic acid dihydrazide-agarose in order to determine the effect of molecular weight on this reaction parameter and to ascertain whether or not a time point existed during the reaction where the binding of the oxidized species had essentially reached completion, but that of the corresponding unoxidized form was at a minimum. As can be seen in Fig. 2 and Table 2, the overall reaction kinetics of coupling of oxidized 5'-AMP, tRNA, 5S RNA, 16S RNA, and 23S RNA to the dihydrazide matrix were found to be essentially independent of molecular weight. The binding reactions for these aldehydic ligands were all rapid and 90% complete in 4 hours (Fig. 2). The nonspecific retention of a low level of unoxidized RNA to the gel was also rapid and exhibited similar kinetic binding patterns as that of the oxidized species.
Figure 2. Kinetics of binding of periodate oxidized ligands to adipic acid dinydrazide-agarose
Table 2. Kinetic coupling of oxidized and unoxidized ligands to adipic acid dihydrazide-agarose. The reaction was carried out as outlined in Experimental. At the time points indicated after initiation of the reaction the amount of sample bound to the gel was estimated by measuring the absorbance at 260 nm of the reaction supernatant and the 2.0 M KCl wash (Experimental)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>5'AMP</th>
<th>tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>2050</td>
<td>750</td>
</tr>
<tr>
<td>1.0</td>
<td>3080</td>
<td>650</td>
</tr>
<tr>
<td>2.0</td>
<td>4380</td>
<td>750</td>
</tr>
<tr>
<td>5.0</td>
<td>6170</td>
<td>750</td>
</tr>
<tr>
<td>9.0</td>
<td>7120</td>
<td>650</td>
</tr>
<tr>
<td>24.0</td>
<td>7750</td>
<td>470</td>
</tr>
</tbody>
</table>
Moles ($\times 10^9$) coupled/g adipic acid dihydrazide-agarose

<table>
<thead>
<tr>
<th></th>
<th>5S RNA</th>
<th></th>
<th>16S RNA</th>
<th></th>
<th>23S RNA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>0</td>
<td></td>
<td>3.4</td>
<td>0.9</td>
<td>1.9</td>
<td>0.6</td>
</tr>
<tr>
<td>98</td>
<td>3</td>
<td></td>
<td>5.4</td>
<td>2.1</td>
<td>2.6</td>
<td>0.9</td>
</tr>
<tr>
<td>120</td>
<td>8</td>
<td></td>
<td>6.9</td>
<td>1.4</td>
<td>3.3</td>
<td>0.7</td>
</tr>
<tr>
<td>142</td>
<td>8</td>
<td></td>
<td>8.0</td>
<td>1.8</td>
<td>4.1</td>
<td>0.9</td>
</tr>
<tr>
<td>150</td>
<td>13</td>
<td></td>
<td>8.4</td>
<td>2.3</td>
<td>4.4</td>
<td>0.9</td>
</tr>
<tr>
<td>152</td>
<td>8</td>
<td></td>
<td>8.7</td>
<td>2.7</td>
<td>4.8</td>
<td>1.6</td>
</tr>
</tbody>
</table>
except in the case of 5'-AMP, where the maximum amount of unoxidized sample was bound to dihydrazide-agarose within 30 minutes reaction time as compared to 9-24 hours for oxidized 5'-AMP (Table 2). Measurement of the initial coupling reaction, expressed as the time required \( T_{1/2} \) to couple one-half of the maximum amount \( A_{1/2} \) of oxidized or unoxidized RNA coupled in Table 2, showed little difference in the initial rates between oxidized tRNA, 5S RNA, 16S RNA, and 23S RNA (Table 3). However the time required for half-maximal binding of oxidized 5'-AMP was twice that required for the other oxidized RNA molecules (Table 3). In general the \( T_{1/2} \) values for the unoxidized molecules were similar to those of the oxidized species except that of unoxidized 5'-AMP which was only one-third the value for the oxidized form, while the \( T_{1/2} \) for unoxidized 5S RNA was about 3 times that of the oxidized molecule (Table 3).

**Affinity Chromatography of **E. coli** 50S Ribosomal Proteins on 5S RNA-Agarose**

The purity of E. coli 5S RNA, as well as that of tRNA, prepared as described in Experimental, was checked by polyacrylamide gel electrophoresis under denaturing conditions (7 M urea) prior to use for affinity binding in order to exclude the possibilities of cross-contamination between the two RNA preparations and of contamination by high molecular weight ribosomal RNA molecules or fragments which could result in spurious ribosomal protein binding. The preparations were essentially homogeneous when analyzed in this manner, even when
Table 3. Kinetic properties of the oxidized and unoxidized ligand coupling reaction to adipic acid dihydrazide-agarose

<table>
<thead>
<tr>
<th>Compound</th>
<th>Oxidized</th>
<th>Unoxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_2^a$</td>
<td>$T_2^a$</td>
</tr>
<tr>
<td></td>
<td>(moles x 10^9)</td>
<td>(minutes)</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>3875.0</td>
<td>93</td>
</tr>
<tr>
<td>tRNA</td>
<td>91.5</td>
<td>38</td>
</tr>
<tr>
<td>5S RNA</td>
<td>76.0</td>
<td>35</td>
</tr>
<tr>
<td>16S RNA</td>
<td>4.4</td>
<td>41</td>
</tr>
<tr>
<td>23S RNA</td>
<td>2.4</td>
<td>48</td>
</tr>
</tbody>
</table>

^a One-half the maximum molar amount coupled per g resin during 24 hours reaction time (see Table 2).

^b Reaction time required to attain one-half the maximum amount of RNA coupled, i.e. the $A_2$ (see Table 2).

^c The maximum amount of unoxidized 5'-AMP coupled was reached by the first measured time point at 0.5 hour after reaction initiation (Table 2).
the polyacrylamide gels were overloaded to detect low levels of contaminants (Fig. 3). A very small amount of a low molecular weight contaminant was noted in the tRNA sample (Fig. 3A). No high molecular weight RNA species were detected by polyacrylamide gel analysis or by analytical Sephadex G-100 filtration in representative samples of 5S RNA or tRNA preparations used for covalent attachment to adipic acid dihydrazide-agarose.

For the initial application of the affinity binding system the interaction of E. coli SOS ribosomal proteins with 5S RNA linked to agarose was studied. Ribosomal proteins from the SOS subunit, purified by chromatography on DEAE-cellulose (Experimental) and dissolved in binding buffer, were examined by two-dimensional gel electrophoresis to determine which proteins were present and whether any failed to dissolve in this buffer. All of the 5OS proteins were found to be present in solution (results not shown), however proteins L4, L5, and L21 were only marginally soluble and only small amounts were detected; the majority of these protein constituents were found in the protein fraction insoluble in binding buffer. The solubility of these three proteins could be increased somewhat by first dissolving the 5OS ribosomal proteins, lyophilized after DEAE-cellulose purification, in a small volume (0.1-0.2 ml) of 4 M urea, in which all of the ribosomal proteins are soluble, then diluting this solution 100-fold with binding buffer (see Experimental). The presence of a small concentration of urea remaining in the diluted protein solution did not affect the subsequent interaction of these
Figure 3. Polyacrylamide gel electrophoresis of *E. coli* transfer RNA and 5S RNA. Electrophoresis at pH 8.9 was performed in the presence of 7 M urea and the gels were stained with Pyronine Y as described in Experimental. Migration is from the cathode (top) to the anode (bottom).

(A) transfer RNA

(B) 5S RNA
proteins with immobilized RNA.

When the 50S ribosomal proteins in binding buffer were mixed with 5S RNA-agarose using the batch method, some protein was bound to the gel. The saturating amount of protein bound was determined to be approximately 0.25 mg per mg 5S RNA (Table 4). Affinity chromatography of the 50S ribosomal proteins on a 5S RNA-agarose column containing 11.0 mg 5S RNA coupled to 1.0 g dihydrazide-agarose also resulted in a small fraction of the total protein binding to the matrix, as evinced by its elution in the high salt-EDTA gradient (Fig. 4). The bound protein (0.96 mg) represented 6.5% of the protein input (14.9 mg). To determine whether the elution procedure had removed all of these 5S RNA-binding proteins from the substituted agarose, the high salt-EDTA washed gel was solubilized by heating at 100° in 0.4 M HCl for 0.5 hour, and the resulting solution examined for protein by electrophoresis on a polyacrylamide disc gel. No protein bands were observed on the stained gel, indicating that essentially all of the protein was removed by this elution procedure.

Examination of the 50S ribosomal proteins bound to the 5S RNA-agarose column by disc gel electrophoresis revealed two heavily stained protein bands and several lightly stained bands near the top (anodic) portion of the gel (Fig. 5B). A similar pattern was seen on polyacrylamide disc gels of the protein bound to 5S RNA by the batch method as described in Table 4. The proteins bound to 5S RNA were identified by two-dimensional polyacrylamide gel electrophoresis (Fig. 6). Two proteins, with traces of a third one were
Table 4. Affinity binding of *E. coli* 50 S ribosomal proteins to 5S RNA-agarose. The matrix contained 7.4 mg 5S RNA coupled to 1.0 g adipic acid dihydrazide-agarose and was suspended in a total volume of 5.2 ml.

<table>
<thead>
<tr>
<th>mg Protein added per mg 5S RNA</th>
<th>mg Protein bound per mg 5S RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0.06</td>
</tr>
<tr>
<td>5.0</td>
<td>0.05</td>
</tr>
<tr>
<td>12.5</td>
<td>0.25</td>
</tr>
<tr>
<td>25.0</td>
<td>0.23</td>
</tr>
</tbody>
</table>

observed (Fig. 6, left). Positive identification was made by co-electrophoresis of a mixture of the 5S RNA-binding proteins together with a small amount of total 50S ribosomal proteins (Fig. 7). The three unknown proteins were then readily identifiable against the light background of total 50S proteins as L18 and L25 (major components) and L5 (minor component). Essentially all of L18 and L25 are retained on the 5S RNA-agarose support; these proteins cannot be detected among the proteins recovered from the gel by elution with binding buffer (Fig. 6, right). Only a small amount of L5 is firmly bound to 5S RNA, as most of this protein is found in the protein fraction not bound to matrix-linked 5S RNA (Fig. 6,
Figure 4. Affinity chromatography of *E. coli* 50S ribosomal proteins on 5S RNA-agarose. 15 mg 50S ribosomal proteins in 16 ml binding buffer were chromatographed on a 1.0 cm x 4.5 cm (3.5 ml volume) 5S RNA-agarose column. The fraction size was 2.0 ml
Figure 6. Two-dimensional polyacrylamide gel electrophoresis of *E. coli* 50S ribosomal proteins fractionated on 5S RNA-agarose. Protein samples were from the affinity chromatography described in Fig. 4. The plexiglass insert modification was used as specified in Experimental.

Left: 50S ribosomal proteins which bind to 5S RNA-agarose. The dotted circle indicates the position of a faintly stained protein.

Right: 50S ribosomal proteins not binding to 5S RNA-agarose.
Figure 7. Two-dimensional polyacrylamide gel electrophoresis of the *E. coli* SOS ribosomal proteins binding to 5S RNA-agarose together with a small amount (25 μg) of total SOS ribosomal proteins.
right). These results obtained by the affinity chromatography column procedure confirmed those obtained by the batch method.

Protein L25 can be eluted to a large extent from the 5S RNA matrix using only high salt concentrations, however elution of L5 and L18 requires both high salt and EDTA. Polyacrylamide disc gel analysis of the protein fraction obtained from washing the 5S RNA-agarose, after reaction with the 50S ribosomal proteins using the batch method, with dissociation buffer containing 2.0 M KCl but without EDTA showed that only a small amount of L18 was present in this sample compared to L25, as determined from the degree of staining of the protein bands (results not shown). Washing this 5S RNA gel again with dissociation buffer containing both 2.0 M KCl and 0.005 M EDTA yielded a second protein fraction. Analysis of this fraction by polyacrylamide disc electrophoresis showed much more L18 present than in the sample obtained by washing with buffer containing 2.0 M KCl alone, as well as some L25 and a faint band corresponding in location to L5 (results not shown).

After reequilibration with binding buffer, the 5S RNA-agarose could be reused in the 50S ribosomal protein binding reaction. As tested by the batch method, all three 50S ribosomal proteins, L5, L18, and L25 were bound to the reused 5S RNA gel in approximately the same total amount as in the initial affinity reaction, although the amount of L18 binding to the matrix was reduced somewhat by reuse of the immobilized 5S RNA as determined by the intensities of staining of these proteins on two-dimensional gel slabs (results not shown).
The 5S RNA-agarose linkage was found to be quite stable when the gel was stored in binding buffer at 4°C. In a typical experiment, 1.2 A_{260} units were measured in the binding buffer supernatant in which 240 A_{260} units of 5S RNA coupled to agarose had been stored for six months at 4°C.

To examine the specificity of the binding of ribosomal proteins in greater detail, a number of parameters were studied. The possibility that the 50S ribosomal proteins were retained on the gel due to the presence of unreacted CNBr residues or unsubstituted adipic acid dihydrazide groups was investigated by examining the binding of 50S proteins to a 5S RNA-agarose gel which had been reacted with a molar excess of 0.1 M ethanolamine-HCl (pH 7.5) for 12 hours at 4°C, after the adipic acid dihydrazide coupling step, to block any remaining CNBr residues (Bartkowiac and Pawelkiewicz, 1972) and with 0.5 M acetaldehyde (20 ml per g gel) in 0.1 M sodium acetate, pH 5.0, at 4°C for 12 hours, after reaction with oxidized 5S RNA, to react with any free adipic acid dihydrazide ligands remaining. Affinity chromatography studies with this material showed that treatment of the 5S RNA gel in this fashion had no effect on the binding of ribosomal proteins; the results were the same as described above.

The implication of these results, namely that the RNA moiety is the affinity reactive center in the matrix, was tested by examining the binding of 50S ribosomal proteins to adipic acid dihydrazide-agarose containing no coupled RNA. No 50S ribosomal
proteins were observed to bind to the dihydrazide gel by either the batch or column methods; all of the proteins were recovered in the wash with binding buffer (results not shown).

70S ribosomal proteins could not substitute for the 50S subunit proteins in the binding reaction with 5S RNA; no proteins were bound when 5S RNA-agarose was reacted with 70S ribosomal proteins using the batch method (results not shown).

Further investigations on the specificity of these ribosomal protein-RNA interactions will be presented in following sections dealing with the affinity binding reactions of: (a) 30S ribosomal proteins and 5S RNA-agarose, (b) 50S ribosomal proteins and denatured 5S RNA-agarose, (c) 50S ribosomal proteins and tRNA-agarose, and (d) 30S ribosomal proteins and tRNA-agarose.

**Affinity Chromatography of **E. coli** 30S Ribosomal Proteins on 5S RNA-Agarose**

To determine whether all of the 30S ribosomal proteins were soluble in binding buffer, a two-dimensional polyacrylamide gel electrophoretogram of these 30S proteins was run (results not shown). All of the 30S ribosomal proteins were observed to be soluble in binding buffer, although protein S1 was only marginally so.

When 30S ribosomal proteins were chromatographed on 5S RNA-agarose, no proteins were bound tightly to the gel. The column chromatography is shown in Fig. 8; similar results were obtained
Figure 8. Affinity chromatography of *E. coli* 30S ribosomal proteins on 5S RNA-agarose. 16 mg 30S ribosomal proteins in 28 ml binding buffer were applied to a 5S RNA-agarose column containing 10 mg 5S RNA coupled to 1.0 g adipic acid dihydrazide-agarose. The fraction size was 2.0 ml. The following fractions were pooled for analysis by polyacrylamide gel electrophoresis.

(A) 2-6

(B) 7-15

(C) 16-17

(D) 18-20

(E) 21-25

(F) 26-28

(G) 29-34

(H) 35-56
using the batch method. Some 30S ribosomal protein appeared to be only slowly removed from the 5S RNA column during elution with binding buffer (Fig. 8) resulting in a "trailing-off" effect not seen in the column chromatography pattern of 50S ribosomal proteins on 5S RNA-agarose (Fig. 4). The disc gel electrophoretic analyses of the 30S ribosomal proteins present in various fractions, pooled as indicated in Fig. 8, from the column chromatography on 5S RNA-agarose are shown in Fig. 9. No protein was detected in the material eluted from the 5S RNA-agarose column by the high salt-EDTA gradient (Sample H, Fig. 9). However disc gel electrophoresis of the "trailing-off" portion of the 30S ribosomal chromatographic pattern on 5S RNA-agarose, represented by the pooled Samples E, F, and G in Figs. 8 and 9, showed that indeed some proteins were retarded by the matrix and thus fractionated out to a degree from the total 30S ribosomal protein mixture. The single protein band shown in the disc gel of Sample F and also very faintly in that of Sample G (Fig. 9) was identified as protein S3 by two-dimensional gel electrophoresis (results not shown). Similarly, S3 was the major component in the initial portion of the "trailing-off" region, Sample E in Fig. 8, as indicated by the darkly stained protein band in the polyacrylamide disc gel of Sample E (Fig. 9) which is present at the same position as the single band in the disc gels of Sample F and Sample G (Fig. 9); traces of proteins S6, S9, S13, S18, and S19/S20 were also detected in the two-dimensional gel of Sample E (results not shown). This retardation of a few 30S
Figure 9. Disc gel electrophoresis of the *E. coli* 30S ribosomal proteins fractionated on a 5S RNA-agarose column. The sample letters refer to column fractions pooled as specified in Fig. 8. As indicated in Fig. 5, the darkly stained band at the bottom of each gel is an artifact of the staining procedure and does not represent a protein component.
ribosomal proteins by 5S RNA-agarose evidently is specific for the RNA ligand. Using essentially the same chromatographic parameters (sample amount and volume, column dimensions, elution conditions, and sample size) as were used for the reaction of the 30S ribosomal proteins with 5S RNA-agarose (Fig. 8), the column chromatographic pattern of 30S proteins on unsubstituted, adipic acid dihydrazide-agarose showed only an initial, essentially symmetrical peak of unbound protein which terminated at fraction number 17 (see Fig. 8); no protein was detected after this point with either continued binding buffer washing or with high salt-EDTA elution (results not shown). In this respect the column chromatographies of the 30S ribosomal proteins on dihydrazide-agarose, 50S ribosomal proteins on dihydrazide-agarose, and the 50S ribosomal proteins on 5S RNA-agarose (Fig. 4) result in patterns which are similar, in that each lacks this "trailing-off" region of apparently retarded proteins seen in the chromatography of the 30S ribosomal proteins on 5S RNA-agarose (Fig. 8).

50S Ribosomal Protein Binding to Denatured
5S RNA Coupled to Agarose

To explore whether 5S RNA conformation is important in the binding reaction with 50S ribosomal proteins, the interactions of these proteins with immobilized native, denatured, and renatured 5S RNA molecules were investigated. 5S RNA was denatured by heating at 60° in a buffer containing no magnesium ions as described by Aubert et al. (1968). These authors found that the denatured 5S RNA could
be distinguished from the native form by gel filtration on a Sephadex G-100 column, from which the denatured RNA eluted first. Also denatured 5S RNA could not be incorporated into reconstituted E. coli 50S ribosomal subunits (Aubert et al., 1968). When the denatured form was renatured by heating at 60° in a buffer containing a high concentration of magnesium ions it regained many of the properties of the native form including the ability to bind to 50S ribosomal subunits during reconstitution (Aubert et al., 1968). These results suggested that a critical alteration of 5S RNA conformation could disrupt interactions with 50S ribosomal proteins.

Portions of the denatured and renatured 5S RNA, prepared as described in Experimental, were examined on Sephadex G-100 (Figs. 10, 11). Approximately 20% of the sample remained as the native form and eluted with [14C] labeled, native 5S RNA, added as a marker (Fig. 10). The remaining 80% eluted in a series of peaks well in front of the native 5S RNA. The apparent heterogeneity of the denatured form is probably due to aggregation, as reported previously by Aubert and his coworkers (1968), rather than to an irreversible degradation of the RNA since the renatured 5S RNA preparation coelutes as a single peak with native [14C] labeled 5S RNA on Sephadex G-100 (Fig. 11). It should be noted that the undenatured 5S RNA fraction of the denatured preparation (Fig. 10) and the renatured 5S RNA (Fig. 11), both identified by coelution with the same [14C] 5S RNA marker, eluted from the Sephadex G-100 columns at somewhat different locations using the same parameters.
Figure 10. Gel filtration on Sephadex G-100 of E. coli denatured 5S RNA. 0.2 mg of 5S RNA, denatured as described in the text and Experimental, was mixed with 6 μg of [14C] labeled, native 5S RNA (sp. act. 1.7 x 10^6 cpm/mg) and applied to a 1.2 x 42 cm Sephadex G-100 column at 4°. The RNA was eluted with a 0.01 M sodium acetate buffer (pH 5.0) containing 0.75 M NaCl and 1% methanol (Aubert et al., 1968). The fraction size was 1.0 ml. After absorbance measurement, the fractions were precipitated with 5% trichloroacetic acid, collected on Millipore filters, and counted in a toluene-based scintillation fluid as described in Experimental.

O---O---O Absorbance at 260 nm

•---•---• Radioactivity (cpm)
Figure 11. Gel filtration of renatured 5S RNA. 0.6 mg of *E. coli* 5S RNA, denatured then renatured as described in the text and Experimental, was mixed with 3 μg native, [14C] labeled 5S RNA (sp. act. 1.7 x 10^6 cpm/mg) and subjected to filtration on a 1.2 x 42 cm Sephadex G-100 column at 4°C. The conditions for elution and for the subsequent measurement of the fractions are detailed in Fig. 10.

O——O——O Absorbance at 260 nm

○——○——○ Radioactivity (cpm)
of column dimensions, sample size, flow rate, elution conditions, and fraction volume. The cause of this variability is not known. It is possible that the peak in Fig. 10 which elutes at the same fraction number as the renatured 5S RNA in Fig. 11 represents the undenatured 5S RNA component, rather than the smaller peak (Fig. 10) coeluting later with the native, $^{14}\text{C}$ labeled 5S RNA marker; in this case then the undenatured 5S RNA would comprise approximately 50% of the denatured 5S RNA mixture. Nevertheless, it is apparent that the heterogeneous, denatured 5S RNA preparation can be renatured into a form appearing homogeneous by gel filtration.

When 50S ribosomal proteins were allowed to interact with essentially equal amounts of each 5S RNA preparation coupled to agarose, some ribosomal protein was retained in all three instances. Disc electrophoresis showed no major qualitative differences in the binding of 50S ribosomal proteins to each of the 5S RNA forms (Fig. 12); proteins L5, L18, and L25 bound to all three preparations. However a spectrophotometric scan of the disc gels revealed that approximately twice as much protein, determined by integration of the major peak in each scan, bound to native 5S RNA compared to the denatured or renatured forms (Fig. 13). That binding of even a reduced amount of 50S ribosomal proteins to the denatured 5S RNA preparation occurs could be due to the fraction of native form
Figure 12. Disc gel electrophoresis of 50S ribosomal proteins binding to native, denatured, and renatured 5S RNA coupled to agarose. Samples containing 11 mg *E. coli* 50S ribosomal proteins in 13 ml binding buffer were reacted, using the batch method, with 0.43 g dihydrazide-agarose to which were coupled either native (1.9 mg), denatured (2.1 mg), or renatured (1.8 mg) 5S RNA. Each disc gel represents all of the protein recovered in the high salt-EDTA wash of each 5S RNA-agarose sample.

(A) 50S ribosomal proteins binding to native 5S RNA-agarose

(B) (ibid.) denatured 5S RNA-agarose

(C) (ibid.) renatured 5S RNA-agarose
Figure 13. Spectrophotometric scans, at 550 nm, of the stained, polyacrylamide disc gels containing the E. coli 50S ribosomal proteins binding to native, denatured, and renatured 5S RNA attached to agarose (Fig. 12). The scale values (0-100) represent 0-2.0 optical density units. The major peak in scan A was corrected for an off-scale reading of 2.5 O.D. units before calculation of the area under the peak. The top (anodic) portion of each gel is represented at the extreme left of the scan.

(A) 50S ribosomal proteins binding to native 5S RNA-agarose

(B) (ibid.) denatured 5S RNA-agarose

(C) (ibid.) renatured 5S RNA-agarose
present or to a slow renaturation subsequent to the coupling of 5S RNA to the dihydrazide-agarose. This possibility is supported by the reported instability of denatured 5S RNA in the presence of high magnesium ion concentrations, even at low temperatures (Aubert et al., 1968). Such a spontaneous renaturation could explain the lack of differential 50S ribosomal protein binding to the denatured and renatured 5S RNA forms. It is clear though that exposure of the RNA to denaturing conditions does affect the binding of ribosomal proteins.

Affinity Chromatography of E. coli 50S Ribosomal Proteins on tRNA-Agarose

In order to further investigate the specificity involved in the interactions between ribosomal proteins and RNA using the affinity probe, the binding of 50S and 30S ribosomal proteins to transfer RNA coupled to agarose was examined.

When 50S ribosomal proteins interacted with a tRNA matrix, in the batch procedure, only a very small amount of protein, comprising two very faint bands on polyacrylamide disc gels, was detected binding to the gel. These results were obtained in two separate experiments. However in two other experiments carried out under identical conditions no binding of 50S ribosomal proteins to tRNA was observed. The reasons for the variable results are unknown. While too little material was obtained to run two-dimensional gels, the positions of these proteins on disc gels (Fig. 14) were not the
Figure 14. Polyacrylamide disc gel electrophoresis of the 50S ribosomal proteins binding to transfer RNA-agarose. 20 mg E. coli 50S ribosomal proteins in 39 ml binding buffer were reacted with 6.7 mg tRNA coupled to 1.0 g adipic acid dihydrazide-agarose using the batch procedure (Experimental). The arrows indicate faint protein bands detected

(A) 50S ribosomal proteins not binding to tRNA-agarose
(B) 50S ribosomal proteins which bind to 5S RNA-agarose
(C) 50S ribosomal proteins binding to tRNA-agarose
same as the bands obtained with 50S ribosomal proteins which bind
to 5S RNA-agarose (Fig. 14), and they presumably represent dif­
ferent proteins.

However when 50S ribosomal proteins were chromatographed on a
tRNA-agarose column, significant amounts of protein were retained
on the matrix (Fig. 15). The protein (0.7 mg) eluted by the high
salt-EDTA gradient from the matrix, which contained 9.5 mg tRNA,
represented 3.2% of the total amount (22 mg) of 50S ribosomal pro­
tein added to the column. It is noteworthy that in contrast to the
affinity chromatography pattern of the 50S ribosomal proteins on
5S RNA-agarose, Fig. 4, there appeared to be proteins which were re­
tarded, but not strongly bound by the tRNA gel and eluted from the
column as a broad shoulder during the wash with binding buffer.
Disc gel electrophoresis (Fig. 16) of the various pooled column
fractions from the chromatography of the 50S ribosomal proteins
on tRNA-agarose, as indicated in Fig. 15, revealed that a number of
different proteins were bound to the gel (Samples H, I; Figs. 15,
16). The darkest stained bands on the polyacrylamide disc gels of
these tRNA-binding 50S ribosomal proteins do not correspond in loca­
tion to L18 or L25 (Fig. 5), the 50S proteins binding to 5S RNA,
however they are comparable in position to the upper (anodic) faint
band seen in the disc gel of the 50S ribosomal proteins binding to
tRNA-agarose using the batch method (Fig. 14C). Two-dimensional
polyacrylamide gel electrophoresis of this bound protein fraction
(Sample H, Fig. 15) indicated that the major components present,
Figure 15. Affinity chromatography of *E. coli* 50S ribosomal proteins on transfer RNA-agarose. 22 mg 50S ribosomal proteins in 21 ml binding buffer were chromatographed on a column containing 9.5 mg tRNA coupled to 1.0 g adipic acid dihydrazide-agarose. 2.0 ml fractions were collected. The following fractions were pooled for polyacrylamide gel electrophoretic analysis:

- (A) 2-4
- (B) 5-12
- (C) 13-14
- (D) 15-18
- (E) 19-21
- (F) 22-26
- (G) 27-33
- (H) 34-37
- (I) 38-48
Figure 16. Disc gel electrophoresis of *E. coli* 50S ribosomal proteins chromatographed on tRNA-agarose. Sample letters refer to column fractions pooled as indicated in Fig. 15.
i.e., the protein spots stained most heavily, were L3, L4, L5, L7/L12, L8/L9, and L21 (Fig. 17). Other proteins present, though in lesser amounts, were L1, L2, L11, L13, L15, L17, and L23; altogether approximately half of the total 50S ribosomal protein constituents were detected. Proteins L18 and L25, the two major 50S ribosomal proteins found to bind directly to 5S RNA-agarose, were either absent or present only in barely detectable quantities among the 50S proteins bound to tRNA-agarose. Sample I, Fig. 15, which corresponded to a distinct shoulder on the bound 50S ribosomal protein peak eluted from the tRNA-agarose column by the high salt-EDTA gradient, did not contain sufficient protein to permit analysis by two-dimensional gel electrophoresis. However the disc gel electrophoretic banding pattern of this sample was the same as that of Sample H (Fig. 16) which represents the major 50S ribosomal protein fraction binding to tRNA-agarose (Fig. 15). Therefore Sample I presumably contains the same 50S proteins identified in Sample H above.

An intensely stained protein spot, marked "X" in Fig. 17, present in the two-dimensional electrophoretogram of the 50S ribosomal proteins binding to tRNA-agarose, did not correspond exactly to a known 50S protein, although it did closely approximate the position of L6. This spot was not present in two-dimensional gels of the 50S ribosomal proteins in binding buffer prior to reaction with immobilized RNA nor was it seen in the two-dimensional electrophoresis pattern of the 50S proteins not binding to the tRNA matrix.
Figure 17. Two-dimensional polyacrylamide gel electrophoresis of the 50S ribosomal proteins binding to transfer RNA-agarose (Sample H, Fig. 15)
(Sample A, Fig. 15), though protein L6 was detected in these samples (results not shown). Also the quantity of this unknown protein present in samples of 50S ribosomal proteins binding to tRNA-agarose varied widely; in one sample it was the darkest stained spot present in the two-dimensional gel slab, as in Fig. 17, yet in another sample of tRNA-binding 50S ribosomal proteins from a column chromatography using different preparations of 50S proteins and tRNA, this spot was barely detectable, even though the intensities of staining of the other 50S proteins present in the tRNA binding mixtures remained relatively constant from sample to sample. Thus it is possible that this spot represents a protein aggregation artifact, yet one which seems to be specific for the affinity binding reaction with tRNA, as it does not appear in the two-dimensional gel of the 50S ribosomal proteins interacting with 5S RNA (Fig. 6).

The 50S ribosomal proteins which were retarded by the tRNA-agarose, Samples E, F, and G in Fig. 15, showed essentially the same disc gel electrophoresis pattern (Fig. 16). When these samples were examined by two-dimensional gel electrophoresis (not shown) all three were observed to contain only L2 and L18, thus indicating that these two proteins, one of which, L18, was previously shown to bind tightly to 5S RNA linked to agarose (Fig. 6, left), may interact weakly with immobilized tRNA.

In contrast to the tight affinity binding of proteins L18 and L25 to 5S RNA-agarose, each of the 50S ribosomal proteins observed in the fractions binding to, or retarded by the tRNA-agarose column
(Samples E-I, Fig. 15) were also observed among the proteins in the unbound portion (Samples A, B, and C; Figs. 15, 16) as determined by two-dimensional gel electrophoresis (not shown). This may indicate that a saturating amount of each of the bound 50S ribosomal proteins had reacted with the affinity reactive center in the tRNA matrix.

Affinity Chromatography of *E. coli* 30S Ribosomal Proteins on tRNA-Agarose

The interaction of the 30S ribosomal proteins with transfer RNA linked to agarose was next examined. In contrast to the earlier results which showed that no 30S ribosomal proteins were detected to bind tightly to 5S RNA-agarose (Figs. 8, 9), when the 30S proteins were mixed with tRNA-agarose under conditions specified for the batch method, a number of proteins were observed to bind to the gel (Fig. 18). Out of a total of 43.5 mg 30S ribosomal proteins mixed with the gel (containing 9.3 mg tRNA), 0.15 mg of protein (0.35% of the input) was eluted by the high salt-EDTA buffer. Two-dimensional gel electrophoresis of the bound protein fraction with and without a background of total 30S ribosomal proteins (Fig. 19) permitted the identification of proteins S3, S5, S6, S9, and S10. Lesser amounts of proteins S1, S2, and S4 were also detected. A few fast-migrating, faint bands seen on the polyacrylamide disc gel of the 30S ribosomal proteins binding to tRNA-agarose (Fig. 18B) could not be observed in the corresponding two-dimensional gel
Figure 18. Disc gel electrophoresis of *E. coli* 30S ribosomal protein fractions from the affinity binding reaction with tRNA-agarose. 43.5 mg 30S ribosomal proteins in 29 ml binding buffer were mixed with 9.3 mg tRNA coupled to 1.0 g adipic acid dihydrazide-agarose using the batch method.

(A) Total 30S ribosomal proteins

(B) 30S ribosomal proteins binding to tRNA-agarose

(C) 30S ribosomal proteins not binding to tRNA-agarose
The affinity binding of a 30S ribosomal protein fraction to tRNA-agarose was also examined using the column procedure (Fig. 20). Approximately 0.5 mg 30S ribosomal protein (3.5% of the total protein input) was tightly bound to the column, which contained 11.2 mg covalently coupled tRNA. Again, as with the column chromatographies of 30S ribosomal proteins on 5S RNA-agarose (Fig. 8) and 50S ribosomal proteins on tRNA-agarose (Fig. 15), a "trailing-off" effect was noted in the elution pattern, suggesting the occurrence of weaker interactions as well as the stronger ones required for a tight binding of the proteins to the matrix. As previously mentioned, this retardation phenomenon was not seen when the 30S or 50S ribosomal proteins were chromatographed on unsubstituted, adipic acid dinydrazide-agarose columns (results not shown). Polyacrylamide disc gel electrophoresis of the pooled fractions indicated in Fig. 20 showed that some 30S ribosomal proteins bind tightly to the tRNA gel (Fig. 21). Two-dimensional polyacrylamide gel electrophoresis of the 30S ribosomal proteins binding to the tRNA-agarose, eluted from the column by the high salt-EDTA gradient as two protein peaks (Samples F, G; Fig. 20), indicated the presence of one protein in the first peak (Sample F, results not shown) and five proteins in the second peak (Sample G, Fig. 22). These were identified as S3 (Sample F) and S3, S6, S9, S13, and S18 (Sample G) by two-dimensional gel coelectrophoresis of each of these samples with a small amount of total 30S ribosomal proteins (results not shown).
Figure 19. Two-dimensional polyacrylamide gel electrophoresis of the *E. coli* ribosomal proteins binding to transfer RNA-agarose using the batch method

(A) Bound 30S ribosomal proteins

(B) Bound 30S ribosomal proteins plus a small amount (20 μg) total 30S ribosomal proteins
Figure 20. Affinity chromatography of *E. coli* 30S ribosomal proteins on transfer RNA-agarose. 14.7 mg 30S ribosomal proteins in 18.5 ml binding buffer were chromatographed on a column containing 11.2 mg tRNA coupled to 1.0 g adipic acid dihydrazide-agarose. 2.0 ml fractions were collected. The following fractions were pooled for polyacrylamide gel analysis:

(A) 3-13
(B) 14-20
(C) 21-23
(D) 24-29
(E) 30-36
(F) 37-39
(G) 40-46
Figure 21. Polyacrylamide disc gel electrophoresis of the 30S ribosomal protein fractions from the affinity chromatography on tRNA-agarose (column procedure). The sample letters refer to the chromatography fractions pooled as indicated in Fig. 20.
Figure 22. Two-dimensional gel electrophoresis of the *E. coli* 30S ribosomal protein fraction binding to tRNA-agarose (Sample G, Fig. 20)
The intensities of staining of the five protein spots, S3, S6, S9, S13, and S18, on the two-dimensional gel electrophoretogram of Sample G (Fig. 22) approximated this numerical order with protein S3 stained to the greatest degree. The binding of these five proteins to a tRNA-agarose column was reproducible using several different 30S ribosomal protein and tRNA preparations. In each experiment the column chromatographic patterns were the same, although the percentage of the total 30S ribosomal protein input which was tightly bound to the tRNA matrix varied from 1%-3.5% (corrected for different amounts of tRNA used) even though the same proteins, S3, S6, S9, S13, and S18, were bound in each case. The percentage of the 30S ribosomal protein input bound to immobilized tRNA was 3-10 times greater using the column method as compared to the batch procedure.

The samples containing 30S ribosomal proteins retarded by the tRNA matrix during elution with binding buffer, and therefore ones which may show a weak affinity for either the tRNA component alone or for the tRNA-bound ribosomal protein complex, Samples C, D, and E in Fig. 20, were also analyzed by two-dimensional gel electrophoresis (results not shown). Sample C (the initial portion of the "trailing-off" region) contained the 30S ribosomal proteins S3, S4, S5, S6, S9, S13, S15/S16, S18, and S19; a small amount of S7 was also present. Sample D (the middle part of the "trailing-off" region) contained predominately proteins S3 and S6, with lesser amounts of S4, S5, S9, and S13. In Sample E (representing the end of the "trailing-off" region just before elution of the tRNA-agarose
column with the high salt-EDTA gradient; Fig. 20) protein S3 was observed to be the major component on the two-dimensional gel electrophoretogram, although small amounts of proteins S5 and S13 were also detected. All of the proteins in the retarded and bound protein mixtures from the column chromatography of 30S ribosomal proteins on tRNA-agarose (Samples C-G, Fig. 20) were also found to be present in the two-dimensional gel electrophoretic patterns of the unbound proteins (Samples A and B, Fig. 20; results not shown). Thus, as in the column chromatography of 50S ribosomal proteins on tRNA-agarose, this may indicate saturation of the affinity reactive center by the tRNA-binding 30S ribosomal proteins.

In comparing the results of the column procedure with those of the batch method for the affinity reaction of the 30S ribosomal proteins and tRNA-agarose, several points emerge. Three 30S ribosomal proteins, S3, S6, and S9 were identified as binding tightly to tRNA-agarose by both the batch (Fig. 19) and column (Fig. 22) methods. Protein S5, found in the batch method, tRNA-binding protein mixture, although not detected in the 30S ribosomal proteins binding tightly to tRNA under conditions specified for the column method, was identified by two-dimensional gel electrophoresis as a constituent in the 30S protein fractions retarded on the tRNA-agarose column (Samples C, D, and E; Fig. 20). Protein S10 was observed in the 30S ribosomal protein fraction binding to tRNA-agarose using the batch method, but consistently was found only in the two-dimensional gel electrophoretogram of the unbound 30S proteins.
(Samples A and B, Fig. 20) obtained by column chromatography on this same matrix. Although S13 and S18, two proteins binding tightly to immobilized tRNA in the column chromatography experiments (Fig. 22), were not detected in the two-dimensional gel electrophoretic pattern of the 30S ribosomal proteins bound to the tRNA matrix in the batch method (Fig. 19), several faint, fast-migrating protein bands possibly corresponding in location to S13 and S18 were seen on the polyacrylamide disc gel of this fraction (Fig. 18B). Thus these two proteins may indeed be present in the 30S ribosomal protein mixture binding to immobilized tRNA in the batch method but in quantities too small to permit positive identification by two-dimensional gel electrophoretic analysis.

As was observed in the affinity reaction of the 50S ribosomal subunit proteins with 5S RNA linked to agarose, 70S ribosomal proteins evidently cannot substitute for the 30S ribosomal subunit proteins in this interaction with tRNA-agarose. When the 70S ribosomal proteins were allowed to react with immobilized tRNA in the batch method, no proteins were detected binding to the matrix, and all of the 70S proteins were found in the reaction supernatant and binding buffer washes (results not shown).
DISCUSSION

Preparation of RNA-Agarose

The procedure described for the immobilization of RNA on adipic acid dihydrazide-agarose is simple, rapid, and results in essentially quantitative coupling of RNA. The preparation of the resin and the method of attachment of the periodate oxidized RNA are advantageous in several ways. Production of the adipic acid dihydrazide resin involves only a single step as compared to the three step method for the synthesis of ɛ-aminocaproic acid hydrazide-agarose used for the immobilization of periodate oxidized RNA described by Robberson and Davidson (1972). In addition, the Robberson and Davidson procedure evidently introduces carboxyl groups on the surface of the resin which require blocking (as amide groups) with glycaminide and a water-soluble carbodiimide. Even then the adipic acid dihydrazide matrix has a higher coupling capacity than the treated ɛ-aminocaproic acid hydrazide-agarose, as evidenced by a comparison of the amounts bound at saturation for benzaldehyde and for periodate oxidized nucleotides. The adipic acid dihydrazide-agarose can bind either $7.7 \times 10^{-6}$ mole oxidized 5'-AMP or $4.7 \times 10^{-6}$ mole benzaldehyde per g resin (Table 1); the comparable saturating values for neutralized ɛ-aminocaproic acid hydrazide-agarose were reported as $3.9 \times 10^{-6}$ mole oxidized 5'-UMP or $2.8 \times 10^{-6}$ mole benzaldehyde per g resin (Robberson and Davidson, 1972). Another advantage is that after forming amide groups, the Robberson and
Davidson method introduces positive charges which can readily and nonspecifically bind RNA.

Secondly, the method of immobilizing the RNA at a single, defined site (i.e., the 3'-terminus) would be expected to have a minimal disruptive effect on its native conformation as compared to other methods of attachment, such as direct reaction of native RNA with CNBr-activated agarose (Bonavida et al., 1970; Berridge and Aronson, 1973) which may result in the coupling of RNA to the solid support at a large number of nucleotide residues. However the possibility that a chemical modification other than oxidation at the 3'-terminal C2'-C3' atoms of ribose may occur to the RNA during periodate oxidation merits serious consideration in evaluating the biological significance of any ribosomal protein interactions with RNA immobilized in this manner. Using essentially the same parameters of periodate and RNA concentrations, pH, temperature, and reaction time as were used in these studies, Rao and Cherayil (1974) have found that the major RNA residues, adenosine, guanosine, cytidine, and uridine, are not affected by periodate treatment under these conditions (except, of course, ribose ring cleavage at the 3'-terminal nucleotide). These authors however did find that periodate reacted with the internal, sulfur-containing minor nucleotides of E. coli transfer RNA causing a quantitative desulfurization of 4-thiouridine, 5-methylaminomethyl-2-thiouridine, 2-thiocytidine, and 2-methylthio-N6-isopentenyladenosine. The desulfurized, periodate oxidized tRNA can still be recognized by the cognate
aminoacyl-tRNA synthetases (Cramer et al., 1968; Ofengand and Chen, 1972) and can still bind to the ribosomal "A" and "D" sites (Chinali et al., 1974) under nonenzymatic conditions (i.e. at high magnesium ion concentrations), thus implying a retention of the structural and conformational elements necessary for interaction with these various proteins. No such side reactions should occur during the periodate oxidation of E. coli 5S RNA since no modified bases are present in the nucleotide sequence (Brownlee et al., 1967). It is not likely that periodate reacts with the methylated bases which occur in E. coli 16S RNA (Ehresmann et al., 1972) and 23S RNA (Fellner, 1969).

The kinetic and quantitative data summarized in Tables 1-3 are consistent with a picture of a relatively rapid, molecular weight-independent, initial coupling of oxidized RNA molecules to highly exposed adipic acid dihydrazide ligands. Evidently, however, smaller molecules have access to a larger number of dihydrazide groups and therefore can react more extensively with the matrix.

Affinity Binding of Ribosomal Proteins to 5S RNA-Agarose

The identification of the E. coli 50S ribosomal proteins binding to 5S RNA covalently coupled to agarose as L5, L18, and L25 (Fig. 6, left; Fig. 7) are in agreement with results from previous
workers who have detected these proteins in *E. coli* 5S RNA-50S ribosomal protein complexes isolated by nitrocellulose filtration (Yu and Wittmann, 1973), sucrose gradient centrifugation (Horne and Erdmann, 1972; Yu and Wittmann, 1973), and gel electrophoresis (Horne and Erdmann, 1972; Gray *et al.*, 1973). It would appear that the affinity of L18 and L25 for 5S RNA is quite high under the low salt-high magnesium ion binding conditions, as all of these two proteins are removed from the initial 50S ribosomal protein reaction mixture (Fig. 6, right). These two proteins also do not elute from the 5S RNA matrix during the binding buffer wash step, since they were not detected in either the two-dimensional polyacrylamide gel of the unbound 50S ribosomal protein fraction (including any protein removed by washing with binding buffer) after reaction with 5S RNA-agarose in the batch method, or in the disc gels of the binding buffer wash fractions (in which no protein was detected by the Lowry procedure) in the corresponding column experiment, fractions 19-26 in Fig. 4 (polyacrylamide gels not shown). Thus it would appear that the binding of L18 and L25 to 5S RNA is essentially irreversible under these affinity reaction conditions of pH, ionic strength, and magnesium ion concentration, which previously had been determined to be optimal for the reconstitution of the 30S and 50S ribosomal subunits (Traub and Nomura, 1968; Nomura and Erdmann, 1970).

The situation for the binding of protein L5 is not as clear. Although a small amount binds to 5S RNA-agarose, the majority of
This protein remains in the unbound protein fraction (Fig. 6).

This is not due to the modification and subsequent immobilization of the RNA, as other workers have found only traces of L5 complexed to unmodified, native 5S RNA (Horne and Erdmann, 1972; Yu and Wittmann, 1973). It is possible that the requirements for the binding of L5 to 5S RNA or to the 5S RNA protein complex are more stringent than those required for L18 and L25 binding. Further analysis of the binding parameters, such as ionic strength, magnesium ion concentration, and temperature, should help answer this question.

It is of interest that Horne and Erdmann (1972) found traces of L20 and L30 complexed to E. coli 5S RNA; proteins not reported as binding by the other, above-mentioned authors. Although these proteins were not observed in the two-dimensional polyacrylamide gel electrophoretogram of the 50S ribosomal proteins binding to 5S RNA-agarose (Fig. 6), very faint, fast-migrating protein bands could be seen on polyacrylamide disc gels overloaded with the 5S RNA-binding, 50S ribosomal protein fraction in a region on the gels which could correspond to that where L20 and L30 migrate (Fig. 12). These faint bands can also be seen on the spectrophotometric scans of these disc gels as a small peak occurring toward the bottom (cathodic) portion of each disc gel after the main protein peak (Fig. 13). In addition, several slowly-migrating, faint protein bands, one of which is probably the protein L5, could be detected in the upper (anodic) portion of these stained disc gels (Fig. 12) and on the corresponding spectrophotometric scans (Fig. 13).
These may also represent 50S ribosomal proteins which interact with either 5S RNA or a 5S RNA protein complex. Further investigation of the 50S ribosomal protein fraction binding to 5S RNA linked to agarose, perhaps using [14C] labeled proteins and radioautography, should clarify these points.

From the experimentally determined parameters of the saturating amount of 50S ribosomal protein bound (approximately 0.25 mg protein per mg 5S RNA, Table 4) and the identity of the binding proteins (L18 and L25, Fig. 7; the molar contribution of L5 is not significant for this calculation), and their known molecular weights (14,300 and 12,000, respectively; Gray et al., 1973) one can calculate on a molar basis the amount of protein bound to 5S RNA (MW 40,000). Since previous workers found that the stoichiometric ratio of L18 to L25 was 2:1 in a reconstituted 23S RNA-5S RNA protein complex (Gray et al., 1973) and as there is evidence that in a 50S ribosomal protein mixture there is twice as much L18 as L25 (Weber, 1972), this ratio was used in computing the moles of protein bound at saturation. With these data and assumptions, a value of 0.75 mole 50S ribosomal protein complexed per mole of immobilized 5S RNA was calculated. The deviation of this value from a theoretical figure of 3.0 moles of protein bound per mole 5S RNA (again, assuming that 2 molecules of L18 and one molecule of L25 are bound per molecule of 5S RNA at saturation) could be due to denaturation or degradation of the 5S RNA binding sites for these proteins, or to steric factors limiting the accessibility
of the proteins to the specific nucleotide sequences.

The interaction between the 50S ribosomal proteins and 5S RNA coupled to agarose appears to be quite specific. One element of recognition resides in the protein component as the 30S ribosomal proteins cannot substitute for the 50S proteins in the binding reaction (Fig. 8), although it is interesting to note that the proteins identified in the 30S ribosomal protein fraction retarded by the 5S RNA matrix (Samples E, F, and G, Fig. 8), proteins S3, S6, S9, S13, and S18, are the same ones which were determined to bind tightly to immobilized tRNA (Fig. 22). It is not known whether these proteins recognize both 5S RNA and tRNA, or whether this weak interaction with 5S RNA is due to a low level of tRNA contamination in the 5S RNA preparation. However the polyacrylamide gel electrophoretic analysis of 5S RNA (Fig. 3B) tends to discount this latter possibility. The recognition process between L5, L18, and L25 also seems to be influenced by the presence of the 30S ribosomal subunit proteins, since the 70S ribosomal proteins are inactive in this reaction, probably because of strong protein-protein interactions in solution.

A second recognition element lies in the RNA, as essentially no L18 or L25 binds tightly to tRNA-agarose (Fig. 17). Conformation of the RNA also seems to be important in this binding process. Denaturation of the 5S RNA prior to covalent coupling results in 50% less 50S ribosomal protein binding to the matrix compared to that binding to immobilized, native 5S RNA (Fig. 13).
Affinity Binding of Ribosomal Proteins
to Transfer RNA-Agarose

In comparison to the interaction of ribosomal proteins with 5S RNA-agarose, the results with immobilized tRNA appear to be considerably more complicated, both in the number and amounts of proteins bound to the gel and in the strength of the binding. Binding of 30S ribosomal proteins to the tRNA matrix occurred both in the batch and column experiments, although some differences were noted. Proteins S3, S6, and S9 were tightly bound to the tRNA gel in both procedures (Figs. 19, 22). Interaction of the immobilized tRNA with proteins S5, S13, or S18 also occurred using both the batch and column methods but to different extents. Protein S5 seems to bind more tightly to tRNA-agarose in the batch method than in the column procedure (where this protein was present in the 30S ribosomal protein fraction retarded by the tRNA matrix; samples C, D, and E, Fig. 20), while the reverse appears to be the case for S13 and S18, however positive identification of these two proteins in the 30S ribosomal protein fraction binding to tRNA-agarose by the batch method was precluded due to the small amounts present, as indicated by the faint, fast-migrating protein bands in the polyacrylamide disc gel of this fraction (Fig. 18B). Only in the results with protein S10 did the batch method and column procedure with immobilized tRNA differ greatly. These variations in protein binding to the tRNA matrix may be due to the methodological differences between the batch and column procedures. The proteins are in contact with
the immobilized RNA for a longer period of time in the batch method (20-24 hours) as compared to the column technique (3-4 hours). Also the time required to wash unbound protein from the matrix by the batch method generally is two or three times longer than that required by the column method, due to a lower efficiency of removal. In addition, the increased washing time most likely is the cause of a smaller percentage of the 30S ribosomal protein input binding tightly to tRNA in the batch method compared to the column procedure. This inherent lower efficiency of removal of unbound 30S ribosomal protein could be the reason that small amounts of S1, S2, and S4 were found in the high salt-EDTA wash of tRNA-agarose in the batch procedure but not in the column technique. The presence of these residual proteins may account for the presence of S10 in the batch bound protein fraction through secondary protein-protein interactions which result in retention on the matrix. It should be mentioned for interpretation of these data that the results for the 30S ribosomal proteins binding to tRNA-agarose by the batch method represent one preparation each of 30S proteins and tRNA. Thereafter the column procedure was used because of its more efficient removal of unbound proteins, a consistently greater amount of 30S ribosomal protein binding to the tRNA matrix as compared to the batch method, and a greater sensitivity in the detection of weakly interacting proteins. Consistent binding of proteins S3, S6, S9, S13, and S18 to tRNA coupled to agarose was obtained with the column procedure using several 30S ribosomal protein preparations
and several different tRNA samples.

A comparison of the proteins found to bind to tRNA by affinity chromatography with those at tRNA binding sites on the ribosome determined by other procedures (see Introduction), reveals a number of interesting correlations and supporting evidence. Proteins S3, S6, S9, S13, and S18 are not among those 30S ribosomal proteins which bind directly to 16S RNA (Nomura, 1972), although there are conflicting reports on the existence of a weak interaction between 16S RNA and S13 (Garrett et al., 1971; Muto et al., 1974), nor are the two 30S ribosomal proteins reported to bind to 23S RNA, S11 and S12 (Garrett and Wittmann, 1973), in this group of tRNA-agarose binding proteins. There appears to be no direct relationship between these five proteins as far as cooperative protein-protein interactions which occur during the assembly of the 30S ribosomal subunit (Nomura, 1972). Neither are S3, S6, S9, S13, or S18 proximal to each other or spatially related in any sort of easily recognizable configuration in the topographical analyses of the 30S ribosomal subunit which have been performed (Visentin et al., 1973; Bollen et al., 1974). In short, these proteins which are found to bind to immobilized tRNA do not appear to have a common basis in the structure of the E. coli 30S ribosomal subunit. However a considerable amount of in situ data has accumulated correlating these proteins, S3, S6, S9, S13, and S18, with the functional binding of tRNA to the ribosome. The experiments, reviewed in the Introduction, have included protection from enzymatic digestion by proteases
upon binding of aminoacyl-tRNA to the 30S subunit, inhibition of
aminoacyl-tRNA binding to the ribosome by specific antibody frag-
ments (Fab) directed against these proteins, chemical modification
studies, and affinity labeling experiments using a reactive group
coupled to the aminoacyl-tRNA prior to binding to the ribosome.
Other 30S ribosomal proteins also implicated by the types of ap-
proaches delineated above were not observed in the 30S ribosomal
fraction binding to tRNA-agarose (see Introduction). The inhibi-
tion of tRNA binding to the ribosome by modification of these pro-
teins may reflect an indirect conformational effect; i.e., these
proteins may not interact directly with tRNA but may be important
in assuring the proper in situ conformation of S3, S6, S9, S13,
S18 at the "A" and "D" sites to permit their direct interaction
with aminoacyl- or peptidyl-tRNA.

It is also interesting to note that four of these five tRNA-
agarose binding proteins, S3, S6, S13, and S18, are present in less
than unit quantities (one mole per mole of ribosomes), only protein
S9 has unit stoichiometry (Introduction). Thus the presence of
these proteins in a 30S ribosomal subunit may govern the ability
of that subunit (or 70S ribosome) to bind aminoacyl-tRNA, and hence
may control which ribosomes are active at a given point during the
protein biosynthetic sequence. Such fractional activity in a ribo-
some population has been found by previous workers (Nomura, 1970;
Lucas-Lenard and Lipmann, 1971). Indeed in preliminary experiments
we have detected a small fraction of E. coli 30S ribosomal
subunits which binds to tRNA-agarose under the same conditions used for the reaction of the isolated 30S ribosomal proteins with this matrix. Therefore it may be possible to fractionate ribosomal subunits or ribosomes by this affinity chromatography procedure and to perhaps directly correlate tRNA binding activity during protein synthesis with the presence of these proteins in the particles binding to immobilized tRNA.

In conjunction with the preceding evidence implicating the tRNA-agarose binding proteins S3, S6, S9, S13, and S18 with the functional binding of aminoacyl-tRNA to the ribosome, it is revealing to note that iodination studies by Litman et al. (1974) show that only four 30S ribosomal proteins in E. coli, S3, S6, S9, and S18, are labeled to a significantly greater degree in the 70S ribosome than in the free, 30S subunit. This then implies that upon subunit association, a conformational change occurs which results in an increased exposure of these proteins, perhaps for the purpose of interacting with tRNA.

Since not all of these five tRNA-agarose binding proteins were bound to the matrix, in contrast to essentially all of L18 and L25 being bound to 5S RNA-agarose at approximately the same protein and RNA concentrations, it would appear that either the amounts of S3, S6, S9, S13, and S18 found in the unbound protein fraction represent inactivated forms or else that saturation of the binding site(s) for these proteins occurs at a level of bound protein which is much less than that required for the 50S ribosomal
protein saturation of 5S RNA. There are no data at present to support the first possibility, however the affinity chromatography of one 30S ribosomal protein preparation on two successive tRNA-agarose columns should indicate whether the lack of binding of these proteins remaining in the initial reaction supernatant represents an inactivation or a saturation phenomenon. Saturation of a small fraction present in the tRNA-preparation coupled to agarose may suggest binding to a single tRNA species, such as tRNA_{met}, or to a certain tRNA conformation, or an interaction with a group of tRNA molecules arranged in a favorable spatial configuration within the gel matrix. Experiments to test these hypotheses using individual tRNA species coupled to agarose are planned. Nevertheless, whatever the exact nature of the tRNA binding site(s) is for these 30S ribosomal proteins, a conclusion which can be supported from the results presented here and from those of previous workers, as discussed, is that the binding of the 30S ribosomal proteins to immobilized tRNA is specific and reflects the function of these proteins in situ.

The interaction of the *E. coli* 50S ribosomal proteins with tRNA coupled to agarose using the column procedure results in a different and a much more complex pattern of protein binding than that seen with 5S RNA-agarose. The main components of the 50S ribosomal protein fraction binding to the tRNA gel are L3, L4, L5, L7/L12, L8/L9, and L21; lesser amounts of other 50S ribosomal proteins (L1, L2, L11, L13, L15, L17, L23) were also present (Fig. 17).
Although not as much research has been done on the in situ interaction of tRNA with the *E. coli* 50S ribosomal proteins as compared to that accomplished with the 30S proteins, the 50S ribosomal proteins L3, L4, L7/L12, L11, and L15 (as well as a number of other 50S proteins) have been implicated as being involved in aminoacyl-tRNA binding to the ribosome, using similar methods of attack as were used for the 30S ribosomal proteins (see Introduction). Protein L2, which evidently shows some affinity for immobilized tRNA since it is significantly retarded by the matrix during affinity column chromatography (Samples E, F, and G, Figs. 15, 16) and is also observed in small amounts in the 50S ribosomal protein fraction binding tightly to tRNA (Fig. 17), has also been identified as being at or near the "D" site using affinity label probes (Introduction). It is noteworthy that more L5 binds to tRNA-agarose (Fig. 17) than to 5S RNA-agarose (Fig. 6, left) as judged by staining intensities, while very little, if any, L18 or L25 is found in the 50S ribosomal protein fraction binding tightly to the tRNA gel, though L18 was identified as a component in the 50S ribosomal protein fraction retarded on the tRNA-agarose column (Samples E, F, and G, Figs. 15, 16). The binding of L5 (and perhaps also L18) to both 5S RNA and tRNA coupled to agarose could be especially significant in view of earlier work by Erdmann et al. (1973) and Horne and Erdmann (1973) showing the specific association of an *E. coli* 5S RNA:L5:L18:L25 complex with the oligonucleotide, TpΨpCpGp, a sequence common to all naturally occurring, procaryotic tRNA molecules functional in protein synthesis. Also initial
experiments in our laboratory have shown that L5, L18, and L25 do not bind to 5S RNA-agarose when an amount of native tRNA, equal in quantity to that of the immobilized 5S RNA, is included in the 50S ribosomal protein solution reacted with the matrix. Further work using such multicomponent affinity chromatography systems may resolve the mechanisms and meanings of these complex, interacting ribonucleoprotein aggregates.

The presence of variable amounts of so many 50S ribosomal proteins in the fraction binding to tRNA-agarose suggests the possible occurrence of cooperative protein-protein interactions paralleling those which take place during ribosomal subunit assembly (Nomura, 1972). One possible explanation is the presence of fragments of 23S RNA in the purified tRNA preparations, and that these bind several of the proteins. Proteins L1, L2, L3, L4, L6 (identity of L6 in the 50S ribosomal protein mixture binding to tRNA-agarose is not firmly established, see Fig. 17 and Results text), L13, L17, and L23, found in the 50S ribosomal protein binding to tRNA-agarose (Fig. 17), have also been reported to bind to intact E. coli 23S RNA (Garrett et al., 1974b). Other proteins, however, which were also determined to bind to 23S RNA by these authors, namely L16, L19, L20, and L24, were not detected in the proteins binding to immobilized tRNA (Fig. 17). It must be emphasized here that some ribosomal proteins bind not to single ribosomal RNA nucleotide sequences but to several widely separated sequences presumably brought into proximity by secondary and tertiary structures, for
example protein S4 (Schaup and Kurland, 1972). This appears to be the case also for a number of the 50S ribosomal proteins binding to 23S RNA (Garrett and Wittmann, 1973; Schulte et al., 1974). Therefore a small ribosomal RNA fragment alone may not bind a ribosomal protein, even if a recognition sequence is present. The ability of a protein to bind to 23S RNA may not necessarily rule out a similar interaction with tRNA, either. An example of this situation, as discussed in the Introduction, is that of the 50S ribosomal protein L6 which evidently is involved in complexing 5S RNA to 23S RNA (Gray et al., 1972). Even so, it is quite likely that the binding of a rather large number of 50S ribosomal proteins to immobilized tRNA is due rather to cooperative protein-protein interactions with the few which bind directly to the tRNA affinity center. Additional investigations, as proposed in the previous section for the 30S ribosomal proteins and tRNA-agarose, should enable a more precise identification of the RNA reactive site recognized by these 50S ribosomal binding proteins.

The many experiments in affinity labeling, reconstitution, chemical modification, and inhibition of function by specific antibody binding as enumerated in the Introduction do not prove the direct interaction of a ribosomal protein with tRNA. These may cause other effects such as the inhibition of binding of messenger RNA or the initiation or elongation factors, or an interference with subunit association, which may be reflected indirectly as an inhibition of tRNA binding to the ribosome, thus complicating
the interpretation of the results. The affinity binding experi-
ments presented here give direct evidence for the existence of
interactions between certain ribosomal proteins and tRNA, as well
as 5S RNA. These results demonstrate the feasibility of studying
ribosomal protein-RNA interactions in *E. coli* using RNA immobilized
to agarose; a procedure which should be equally applicable for the
study of such interactions important in the structure and function
of the eucaryotic ribosome, about which much less is known. The
affinity chromatography approach developed in this research promises
to be a new and powerful tool for the ultimate understanding of the
"black box" in protein biosynthesis: the ribosome.
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APPENDIX

Design and Application of the Plexiglass Insert

Modification for two-dimensional polyacrylamide gel electrophoresis

In the original two-dimensional gel electrophoretic apparatus developed by Kaltschmidt and Wittmann (1970a), only one sample can be electrophoresed on each gel slab, thus necessitating the construction and use of a grid overlay (Kaltschmidt and Wittmann, 1970b) to compare the ribosomal proteins present in different samples electrophoresed on different gel slabs. Furthermore the thickness of the gel slabs formed in the apparatus precludes analysis of ribosomal proteins labeled with $[^{14}\text{C}]$, $[^{3}\text{H}]$, or $[^{35}\text{S}]$ radioisotopes. Our attempts to dry these thick gel slabs to permit use of these biologically important radiotracers in radioautographic analyses failed as the gels cracked and shrank unpredictably upon removal of water. A procedure for two-dimensional gel electrophoresis of ribosomal proteins on smaller-dimensioned, thinner gel slabs was introduced in 1973 by Howard and Traut which allows the electrophoresed slab to be dried without cracking or warping for radioautography. However the method requires construction of an entirely new apparatus costing several hundred dollars and, again, is limited to one sample per gel slab. Therefore we attempted to devise an inexpensive addition to the original Kaltschmidt-Wittmann apparatus which would result in reduced-size gel slabs suitable for radioautographic work
and would permit the analysis of at least two different samples on the same gel slab, thus simplifying comparison and obviating use of a grid which tends to introduce systematic errors in measuring the positions of closely spaced protein spots on different gel slabs.

The modification is a simple, removable plexiglass insert, shown in diagrammatic form in Fig. 23, which snaps easily into each two-dimensional gel chamber of the original apparatus. The design of the insert is simple, requires very little machining to produce, and costs but a few dollars. With this insert in place, gel slabs of the same dimensions as those of the original Kaltschmidt and Wittmann procedure (1970a) are formed but with their thickness reduced three-fold. Subsequent experiments also showed that two different samples could be electrophoresed on the same reduced-size gel slab without loss of resolution by using first-dimension, cylindrical gels one-half the length of those needed in the Kaltschmidt and Wittmann (1970a) method (see Experimental). For example, the separation of the total *E. coli* 50S and 30S ribosomal proteins on the same gel slab using the plexiglass insert modification is shown in Fig. 24.

It was of interest to ascertain whether the thin, two-dimensional gel slabs produced with the plexiglass insert were amenable to the analysis of ribosomal protein mixtures by radioautography. The capability for radioautography would further increase the usefulness of the insert innovation and, as applicable to the continuation of the affinity chromatography studies presented
here, would greatly increase the protein detection sensitivity, thus reducing the amount of ribosomal proteins and RNA required in the binding reaction. Fig. 25 shows the radioautogram obtained from the two-dimensional gel electrophoresis of \(^{14}\text{C}\) labeled \textit{E. coli} 50S ribosomal proteins using the insert modification. The thin gel slab was dried against a filter paper support after electrophoresis and the radioautography was performed as described in Experimental. As can be seen from a comparison of Fig. 25 with Fig. 24, left, all of the 50S ribosomal proteins were visualized on the developed X-ray film.
Figure 23. Design of the plexiglass insert modification for two-dimensional polyacrylamide gel electrophoresis
PLEXIGLASS STRIP

\[ \frac{3}{32}'' \times \frac{9}{16}'' \times \frac{1}{16}'' \]
Figure 24. Two-dimensional polyacrylamide gel electrophoresis of *E. coli* 50S (left) and 30S (right) ribosomal proteins separated on the same gel slab using the plexiglass insert modification to the apparatus of Kaltschmid and Wittmann (1970a) as described in Fig. 23 and Experimental. The 50S ribosomal proteins (prefaced with the letter "L") and the 30S ribosomal proteins (prefaced with the letter "S") are numbered according to their distance from each respective origin (Kaltschmidt and Wittmann, 1970b). Dotted circles indicate the positions of proteins stained too faintly to be seen in the photograph. Under these electrophoresis conditions, chosen to obtain maximum separation of the majority of the proteins, protein L34 migrates off the cathodic end of the first dimension, cylindrical gel and thus is not present on the second-dimension gel slab.
Figure 25. Radioautography of E. coli $^{14}$C] labeled 50S ribosomal proteins separated by two-dimensional polyacrylamide gel electrophoresis using the plexiglass insert modification. The thin gel slab, containing approximately 0.1 mg protein (sp. act. $5.3 \times 10^5$ cpm/mg), was dried against filter paper and the radioautography was performed as outlined in Experimental. The X-ray film was developed after exposure for two weeks at room temperature.