THE ISOLATION AND CHARACTERIZATION OF A LOW MOLECULAR WEIGHT RNA FROM E. COLI

by

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ABSTRACT

The extraction of soluble RNA from E. coli whole cells using the phenol procedure, yields preparations of RNA whose heterogeneity can easily be demonstrated by gel filtration analysis (Sephadex G 100). Optical density profiles of the fractionated samples reveal the presence of 4 elution regions. Elution region 4 is a mixture of the various transfer RNAs, and is present in the amount of 75%. Elution region 3 (5s RNA) is present in the amount of 15%, and is mainly isolated from the ribosomal cellular fraction. The nature of the RNA present in elution region 1 has not been determined, but on the basis of preliminary results, this material seems to be a mixture of ribosomal-like RNA, and other degraded material.

The RNA in elution region 2 is present in the smallest amount (2% of the sRNA). This RNA is found entirely in the cytoplasmic cellular fraction, when cells are divided into cytoplasmic (supernatant) and ribosomal fractions. It is a stable homogeneous species, larger in size than either 4s or 5s RNA as evidenced by (a) its molecular weight determined by ultracentrifugal analysis (54,440 daltons), and (b) its electrophoretic mobility on polyacrylamide gels.

Analysis of the nucleotide composition of elution region 2 RNA shows that this molecule possesses no unusual

bases, and is different from 4s and 5s RNA, as well as from the other soluble and ribosomal RNA species present in E. coli. Ion exchange chromatography of alkaline hydrolysates, and snake venom phosphodiesterase products show that uridine 3' 5' diphosphate is present at the 5' end, and uridine occupies the 3' hydroxyl end of the molecule.

Physical studies designed to probe the secondary structure of the molecule show that like 5s RNA, 60% of the molecule is in the helical conformation. The helices however, display a greater stability to heat denaturation (Tm 64.4°C) than the helices present in 5s RNA (Tm 54.0). These results suggest that the helices in elution region 2 RNA differ from those of 5s RNA either in length, or stability of base pairs, or both.

Elution region 2 RNA does not possess any amino acid acceptor activity when tested in an assay system containing amino acid synthetases, ATP, and a variety of ^{14}C labeled amino acids. This RNA does not bind in a complementary fashion to chain termination codons in the presence of ribosomes, suggesting its inability to participate in the chain termination events of protein synthesis.

Experiments to determine the kinetics of labeling of elution region 2 RNA show that the rate of synthesis of this RNA exceeds that of the other soluble and ribosomal RNA species over an 8 minute labeling period, this is a clear indication that elution region 2 RNA is not a degradation product of the rRNAs.

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CHAPTER ONE

Low Molecular Weight Ribonucleic Acids - A Review

INTRODUCTION

The soluble Ribonucleic acids have, for a long time, been thought of as a group of molecules with the unique property of being soluble in cold molar sodium chloride, and having the sole biological role of accepting amino acids in the translational phase of protein synthesis. Gradually, however, the term soluble Ribonucleic acid (sRNA) began to take on new meaning, as it was discovered that the sRNAs were indeed a heterogeneous group of molecules, each perhaps having a distinct role to play in the cell's metabolism.

The heterogeneity of bacterial sRNA was elegantly revealed by the work of Schleich and Goldstein (1964) who published results on the fractionation of Escherichia coli B (E. coli B) sRNA by counter-current distribution. In a subsequent paper these workers were able to show, by the use of Sephadex G 100 gel filtration techniques, that E. coli sRNA isolated by phenol, and eluted from DEAE cellulose columns using cold molar sodium chloride, consists of a variety of nucleic acids (Schleich and Goldstein, 1966). In addition to transfer RNA (tRNA), which represents approximately 75% of the composition of the crude sRNA preparations, other RNA species were found with distinct physical and chemical properties. One of these species was described as ribosomal-like, based on an analysis of its nucleotide composition; another as messenger-like; and a third species which was unable to accept amino acids

was shown to be identical with the 5s ribosomal RNA described by Rosset, Monier and Julien (1963).

The work of other investigators quickly confirmed these findings. Cannon and Richards (1967), Hindley (1967), and Loening (1967), all reported on the heterogeneity of crude sRNA preparations from E. coli, as revealed by the very sensitive technique of polyacrylamide gel electrophoresis.

The occurence of low molecular weight RNAs in other systems has also been demonstrated, and recently such RNA species have been extensively studied in mammalian systems (Dingman and Peacock, 1968; Nakamura, Prestayko and Busch, 1968). These molecules vary in size, base composition, and possibly also in biological activity, and have been found in many different cellular compartments.

An attempt will now be made to describe some of these low molecular weight RNAs, and where possible, to discuss the importance of these molecules in the cell's metabolic activities.

Small Molecular Weight Nuclear RNA (snRNA)

Recent investigations of the RNA composition of vertebrate cells have indicated the existence of a new class of RNAs of low molecular weight within the nucleus of these cells. Knight and Darnell (1967) first reported on the presence of these RNAs when polyacrylamide gel electrophoresis of nuclear RNA from HeLa cells unexpectedly revealed their existence. These nuclear RNA species are different from any other previously characterized class of nuclear RNA. The nucleoplasm and the nucleolus have been shown to contain heterodisperse RNA distributed in size from 10 to 90s, and 18 to 45s respectively. In contrast, these nuclear RNA species range in size from 4 to 6s, and appear to be stably associated with the nucleus, some with the nucleoplasmic, and others with the nucleolar fraction. A detailed characterization of these species has been presented by Weinberg and Penman (1968). Altogether they described seven RNA species of low molecular weight, found only in the cell nucleus. Named in order of increasing electrophoretic mobility on polyacrylamide gels, these are species A, B, C, D, F, G' and H. In addition, the nuclear preparations typically contained a small amount of 28s associated RNA, an RNA first described by Pene, Knight and Darnell (1968), 5s RNA, and tRNA, referred to as species E, G, and I respectively. When nuclei were

further fractionated into nucleoli and nucleoplasm, the low molecular weight RNAs were distributed as follows: species A, and the minor species B and F, were found in the nucleolar fraction exclusively. G, G', and H, were divided approximately equally between the two fractions. Species C, D, E, and I, were found principally in the nucleoplasm. Species D was present in greater amounts than any other species, while species A was the most prominent species of the nucleolar RNA fraction. Four of these RNAs were also shown to be extensively methylated when RNA from ^{3}H methyl methionine exposed cells was analyzed.

A similar pattern of snRNA was also observed by Weinberg and Penman (1968) in the nuclei of mouse fibroblast cells, and in the nuclei of developing chick embroyo brain. Rein and Penman (1969) later undertook a study of the electrophoretic mobility of these RNA species isolated from different mammalian cell sources, and reported that the mobilities were determined by the karyotype, or tumoriginicity of the cells examined. These RNA species were also present in cells of amphibians, as well as birds.

Additional reports investigating the biological properties of these snRNAs have revealed that these molecules possess no amino acid acceptor activity (Hodnett and Busch, 1968), that they have unique methylation patterns, and that they are not degradation products of other types

of methylated RNAs (Zapisek, Saponara and Enger, 1969).

The characterization of these snRNAs was more recently extended by Weinberg and Penman (1969). In their latest report, they have shown that two larger species of RNA, species K and L, are found in the nucleoplasm. These species are both methylated, and are about 260 and 220 nucleotides long, respectively. Another RNA species with an electrophoretic mobility identical to that of denatured 5s RNA, was also described. This RNA, although of the same length as 5s RNA (120 nucleotides), has 7 methyl groups per molecule, a feature that assisted in distinguishing it from 5s RNA. Some of the snRNAs were found to be extremely stable. Others, though not as stable, were decidedly different from nucleoplasmic heterogeneous RNA, and ribosomal precursor RNA which have lifetimes ranging from 10 to 30 minutes (Soeiro et al, 1968; Scherrer and Darnell, 1962). All of these snRNAs were found almost solely in the nuclei of interphase cells, and seemed to be quite loosely associated with the nucleoprotein complexes, and displayed a similar sensitivity to antibiotics as 45s nucleolar RNA.

These notable differences in the characterization of the snRNAs, as well as the differences between the many species of snRNA, all suggest that the snRNAs may differ from one another, and also from the other nuclear RNAs, in their in vivo function.

'7's RNA (28s Associated RNA)

A species of RNA somewhat larger than 5s RNA (as determined by polyacrylamide gel electrophoresis) was first observed by Knight and Darnell (1967) in cytoplasmic RNA samples which had been extracted from HeLa cells by hot phenol techniques. Pene, Knight and Darnell (1968) later showed that all of the larger ribosomal RNA component in the HeLa cell contains a small molecule of about 130 nucleotides long, which is closely associated with it. This molecule (7s RNA), is distinct from the previously recognized 5s RNA both in electrophoretic mobility, as well as in the conditions necessary for releasing it from the larger ribosomal subunit. It appears that 7s RNA does not result from enzymatic degradation of higher molecular weight RNA during the isolation procedure, since the 28s ribosomal RNA extracted directly from whole cells by cold phenol - sodium dodecyl sulfate treatment also yielded the same amount of 7s RNA upon heating, as did the 28s ribosomal RNA isolated from fractionated cells. Also, neither 16s ribosomal RNA, nor previously heated 28s ribosomal RNA yielded 7s RNA upon heating.

When the kinetics of release of 7s RNA was examined, it was found that 50% of 7s RNA is released at 42°C, at pH 5, in sodium acetate-EDTA-SDS buffer, with a maximum yield obtained after 1 minute at 60°C. Dimethyl sulfoxide, and high concentrations of urea also resulted in the

release of 7s RNA at low temperatures, indicating that 7s RNA is probably attached to the larger ribosomal RNA by non-covalent bonds.

The relatively high guanosine + cytidine content of 7s RNA is similar to that of ribosomal RNA, and ribosomal precursor RNA molecules found in HeLa cells, and different from that of the nuclear DNA-like RNA, or from the cytoplasmic RNA found in these cells.

In order to determine the origin of 7s RNA, Pene, Knight and Darnell (1968) studied the kinetics of labeling of nuclear subribosomal particles containing 28s RNA. By comparing the ratios of 7s and 28s after brief labeling, with the ratios of these species in the steady state they concluded that 7s RNA and the heated 28s ribosomal RNA molecule were part of the same polyribonucleotide chain originally. They speculated on the origin of 7s, by postulating its formation within the nucleus as a result of the larger ribosomal RNA molecule (45s rRNA) being cleaved near one end of the chain to form 28s rRNA, and a short portion, 7s RNA. They then presumed that these two molecules were subsequently bound together in the ribosome by hydrogen bonding.

Weinberg and Penman (1968) were also able to isolate 7s RNA from preparations of HeLa cell RNA. However, based on their observations of the sedimentation velocity, and the electrophoretic mobility of this molecule, they

concluded that the sedimentation coefficient was more in the order of 5.5s than 7s. To avoid confusion, therefore, they referred to this species as 28s associated (28sA) RNA. E. coli 23s rRNA was shown not to release 7s RNA after treatment with 5 molar urea, indicating the absence of such a complex in bacterial systems.

Whatever its origin, this stable low molecular weight RNA is present in isolatable amounts in HeLa cell RNA preparations. The biological role of this molecule still remains to be determined.

Membrane Associated Low Molecular Weight RNA

In 1962, Hendler proposed a general model for protein synthesis which suggested that ribosomes were membrane associated when they carried out their synthetic activities within the cell. In a series of subsequent papers (Tani and Hendler, 1964; Hendler and Tani, 1964; Hendler and Banfield, 1964), evidence was presented showing that the model for protein synthesis could be extended to the E. coli system. Schlessinger's studies (1963) showed that an appreciable fraction of the cell's total RNA is bound to membranes. Most of this RNA can be released from the membrane by simply lowering the magnesium concentration from 10^{-2} molar to 0. Similarly, 0.2% deoxycholate was capable of removing the bound RNA. These preliminary

reports served to stimulate a great deal of interest in the study of the membrane associated RNAs, an undertaking that served to shed new light on the function of these molecules.

Goswami, Barr and Munro (1962) indicated the presence of RNA in the membranes of the endoplasmic reticulum, and Chauveau et al. (1962) after base composition analysis, reported that this RNA is different from the RNAs of the ribosomal pellet, or cell sap. These studies were supported and extended by the work of Shapot and Pitot (1966), who showed that the smooth endoplasmic reticulum isolated from rat liver homogenates, has associated with it a small amount of heterogeneous RNA which contains small components with a size range of sRNA; and in addition, many higher molecular weight components varying in size up to 10s. Tulegenova and his co-workers (1968) later showed that this RNA fraction from the smooth endoplasmic reticulum is heterogeneous, not only in physicochemical properties, but also in metabolic activity, and that it is distinct from the rRNAs or the tRNAs.

Gardner and Hoagland (1968) have also reported on the presence of an RNA of approximately 3.14s, in the membranes of rat liver microsomes. This membrane RNA comprises some 20% of the total RNA, and remains in the deoxycholate supernatant after treatment to remove the ribosomes. No trace of this material is found in ribosomes

prepared from microsomes by deoxycholate treatment. A molecular weight of 22,500 daltons was reported for this RNA, and analysis of its base composition revealed a high guanosine + cytidine content. A low content of 3' terminal adenylic acid, pseudouridylic acid, and methylated bases were also found, and possibly could have resulted from slight contamination with tRNA. Control experiments clearly showed that this RNA does not result from degradation of the higher molecular weight rRNA, also present in these extracts.

Recently, King and Fitchen (1968) have published results of their efforts to fractionate membrane RNAs from the smooth endoplasmic reticulum of rat liver, utilizing polyacrylamide gel electrophoresis. These workers were able to demonstrate the presence of a 28s rRNA component, as well as an 18s rRNA, 5s RNA, and 4s RNA component in their membrane RNA preparations. They also observed a zone which migrates with a mobility slightly slower than that of 5s RNA. This zone was absent from RNA samples isolated from the ribosomes. This unusual species of RNA they called xRNA. Some evidence on the nature of xRNA was provided when turnover studies were carried out on the various membrane RNA components. A higher specific activity was observed for xRNA than was observed for 28s, 18s, 5s, or 4s RNA, ruling out the possibility of xRNA being a degradation product. King and Fitchen

have therefore speculated on the possibility of xRNA being a messenger RNA which has become unusually stabilized by virtue of its association with the membrane of the endoplasmic reticulum.

Watson and Ralph (1966) have also reported on the presence of an RNA of low molecular weight in the ribosomes of mouse liver, and sarcoma 180 cells. This RNA has a sedimentation coefficient of 7s after methylated albumin keiselguhr column chromatography. It has no amino acid acceptor activity; and based on biosynthetic studies, it incorporates ^{14}C uridine at the same rate as messenger RNA.

On the basis of these results, it seems that 7s RNA and xRNA could be very closely related, and may representative of a group of messenger RNAs which have become attached to the cellular membrane.

Histone-Bound RNA

Recently, a new class of RNA was described by Huang and Bonner (1965). This RNA was shown to be chemically linked to the native histone molecules of pea bud chromatin.

These histone molecules of the pea bud chromatin appear to be subunits of much larger structures present in native nucleohistone. Each such large structure, made of several subunits, bears an RNA molecule which is very

rich in dihydrouridylic and adenylic acid, and poor in guanylic and cytidylic acid. The chain length is approximately 38 nucleotides per molecule. Huang (1967) has shown that the peptides of the histone are linked to this RNA through an amide bond to the dihydropyrimidine nucleotides.

As far as the biological role of these complexes is concerned, Huang and Huang (1969) have shown that the presence of a protein-bound RNA complex permits *in vitro* reconstitution of a chromatin in which the same genes are functional as *in vivo*, since the reconstituted chromatin primes the synthesis of RNAs having base sequences similar to those found in intact nuclei.

Bekhor, Kung and Bonner (1969) and Huang and Huang (1969) have presented evidence suggesting that the sequence specific binding between chromosomal RNA and genomic DNA, determines the site at which the transcription inhibition chromatin binds to DNA. According to these workers, sequence recognition between specific chromosomal RNAs and the DNA specifies the pattern of the gene activity.

It seems quite likely therefore, that the histone-bound RNAs described by Bonner and his group could fall into the category of molecules described by Britten and Davidson (1969), called activator RNAs. These activator RNAs have been suggested to have the following properties: firstly they should be confined to the nucleus, and should

not be precursors of cytoplasmic polysomes; secondly when observed in their functional roles these molecules should be found in chromatin bound to DNA in a sequence specific manner; and thirdly they should often be the product of the redundant fraction of the genome. Since histone-bound RNA seems to meet all of the requirements enumerated above, it is quite possible that these molecules could fulfill an important regulatory role within the cell.

Transfer RNA (4s RNA)

The transfer RNAs (tRNAs) comprise some 10 to 20% of the total cellular RNA, and are composed of a group of relatively small molecules with molecular weights of approximately 30,000. Of all of the low molecular weight RNAs studied to date, these molecules have been the most extensively characterized. The primary biological function of tRNA is to bind, and subsequently transfer an amino acid to the ribosomal/messenger surface during protein synthesis. At least one specific tRNA serving in the binding of each amino acid.

The search for these adaptor molecules began quite early in a number of laboratories. Hultin and Breskow (1956) had obtained kinetic evidence to show that an amino acid containing intermediate is present during protein synthesis. Hoagland, Zamecnik and Stephenson (1957), and Zachau,

Acs and Lipmann (1958) were able to show by a very elegant series of experiments that a transfer RNA is linked to an amino acid by ester bond formation, and serves not only as the specific acceptor of amino acids in the activation step of protein synthesis, but also in the transfer of the bound amino acid to the ribosome/messenger surface. The tRNAs have now been shown to impart specificity to protein synthesis at essentially three levels. Firstly, in the specific recognition of the amino acid synthetases; secondly, in the interaction of the acylated tRNA with the nucleotide triplets of the messenger RNA, through the anticodon loop; and thirdly, in the interaction of the tRNA with the ribosome itself.

At present, alanine (Holley et al. 1965), tyrosine (Madison, Everett and Kung, 1966), phenylalanine (Raj-Bhandary et al. 1967), valine (Baev et al. 1967) tRNAs from Baker's yeast have been sequenced, and so have the serine tRNAs from Brewer's yeast (Zachau et al. 1966), the tyrosine Su^+ 111 tRNA of an E. coli amber mutant (Goodman et al. 1968), N-formyl methionine tRNA (Dube et al. 1968), the valine tRNA of E. coli (Yaniv and Barrell, 1969). wheat germ phenylalanine tRNA (Dudock et al. 1969), and the serine tRNA from rat liver cells (Staehelin et al. 1968).

Although the sequences of these tRNAs differ widely, they all have some interesting features in common. The well known cloverleaf model probably describes correctly

the secondary structure of tRNA. In addition a generalized model based on the cloverleaf model, has been proposed by Levitt (1969). This model is compact and predicts considerable stacking interactions.

In spite of the numerous models, it has not been possible to deduce conclusively the detailed tertiary structure of any tRNA. The crystallization of formylmethionyl tRNA from E. coli (Clark et al., 1968) has made it possible to use X-ray crystallography to determine the tertiary structure of the tRNAs. Since the first report, considerable progress has been made, and recently Blake, Fresco and Langridge (1970) have obtained crystals entirely suitable for analysis. The quality of their X-ray data is already such that it should soon be possible to test various suggested structural models.

Since much of the experimental data involving chemical modification of tRNAs suggest that the specificity of these molecules resides in the overall conformation, the outcome of the crystallographic studies will be anxiously awaited.

5S RNA

The earliest report in the literature on 5s RNA dates back to 1961 when Elson reported on the release of an RNA particle from E. coli ribosomes under conditions

of high salt. Under these conditions, Elson observed the appearance of the 5s particle, and the simultaneous disappearance of the 50s ribosomal subunit. Two years following Elson's report, Rosset, Monier and Julien (1964) carried out detailed studies on 5s RNA isolated from bacterial ribosomes under conditions of low magnesium, and reported not only on the homogeneity of this molecule, but also on its unique base composition. Studies from Comb's laboratory (Zehavi-Willner and Comb, 1966) confirmed the finding that this unique RNA is present only in the 50s ribosomal particle. Several other publications appeared in the literature, and by 1965, 5s RNA had been isolated from yeast ribosomes (Marcot-Queiroz et al. 1965), liver cell ribosomes (Galibert et al. 1965), sea urchin and amphibian ribosomes (Comb et al. 1965), giving credence to the idea that 5s RNA is a ubiquitous molecule.

Knowledge of the structure of 5s RNA has since been accumulating very rapidly. The development of a two dimensional electrophoretic technique for separating small amounts of oligonucleotides highly labeled with ^{32}P has greatly facilitated work on the nucleotide sequence of this molecule. Sanger, Brownlee and Barrell (1965), and Forget and Weissman (1968), have sequenced the 5s RNA molecules from E. coli and KB cells, respectively. The molecule is approximately 120 nucleotides long, and has a molecular weight of about 41,000 daltons.

Models for the secondary structure of 5s RNA have been proposed by a number of investigators. The model of Boedtker and Kelling (1967), based on hypochromicity measurements on E. coli 5s RNA, has 60% of the bases in hydrogen bonded regions, while the model proposed by Cantor (1968), suggests a secondary structure with 67% of the residues base paired. Lewis and Doty (1970) have recently proposed a model which like Boedtker's model proposes 60% base paired regions.

There has been much speculation as to the biological role of 5s RNA. On the basis of the cloverleaf models of 5s RNA, Raacke (1968) has predicted essentially a structural role for 5s RNA in the binding of the two ribosomal subunits. Briefly, Raacke's hypothesis proposes three basic functions for 5s RNA. Firstly, by means of a unique and universal base sequence, 5s RNA binds directly to the 50s ribosomal subunit; secondly, the bound 50s subunit becomes linked to the 30s subunit through the interaction of one of the loops of the 5s RNA with this smaller subunit in a species specific manner; and thirdly, the 5s RNA molecule then serves to orient incoming tRNA molecules by virtue of magnesium bridges formed between the phosphates in the backbone of the helical regions of the two molecules.

Kirtikar and Kaji (1968) have recently described a system in which the addition of 5s RNA stimulates the

the incorporation of labeled amino acids into protein. Since the stimulation observed was limited only to the phage/RNA-dependent protein synthesis, and did not occur in the presence of synthetic messenger RNA, it was suggested that the steps probably influenced were either initiation, release of the polypeptide chain, or both. Whatever the biological role of this low molecular weight RNA, it seems the ubiquitous 5s RNA is an essential constituent for the cell's biological function.

KB Cell Low Molecular Weight RNA

Forget and Weissman (1967) have shown that KB cells synthesize and accumulate a stable cytoplasmic low molecular weight RNA after infection with adenovirus 2. Ohe and Weissman (1970) have recently undertaken an extensive characterization of this molecule. The RNA is 156 nucleotides long, and possesses some terminal and internal heterogeneity. The 5' end is occupied by guanosine, which varies from being non-phosphorylated in some molecules, to possessing either one or two phosphate groups in others. The 3' terminus as well as the penultimate base, is uridine. In some molecules the terminal uridine is missing. The sequence obtained permits extensive base-pairing and contains prominent repeating units. Ohe and Weissman point out that some homology between this RNA and certain tRNA sequences exists, and they speculate that if the

KB RNA is a product of the virus gene, then homology between viral DNA and host DNA could be a favorable site for recombination or integration of virus DNA with the host cell genome.

6s RNA From Infected E. coli Cells

The occurence of low molecular weight RNAs in E. coli cells infected with RNA phages has been described by several workers. Kelly, Gould and Sinsheimer (1965), and Lunt and Sinsheimer (1966), have found a low molecular weight RNA in cells infected with RNA from phage MS-2. This RNA was shown to be present in greater amounts when the infected cells were treated with ultraviolet light, or actinomycin D. It was suggested that this '6s' RNA represents a device for replication of a specific portion of the viral genome, or perhaps is simply a degradation product which accumulates under conditions when the yield of phage RNA is small. Lodish and Zinder (1966) have found that in E. coli cells infected with an f2 phage mutant, a large proportion of the parental RNA is converted to a '7s' form.

Recently the presence of a low molecular weight RNA in E. coli cells infected with QB RNA has been reported (Banerjee, Rensing and August, 1969). This QB 6s RNA is the predominant nucleic acid component found in a partially

purified fraction of QB RNA polymerase, is not detected in uninfected cells, and serves as template, *in vitro*, for the QB RNA polymerase.

Chemical analysis of this RNA, or the RNA product resulting from the *in vitro* polymerase reaction shows that the two molecules are identical. Guanosine triphosphate occupies the 5' terminus, and the 3' hydroxyl terminus is occupied by adenosine. A chain length of 110 to 130 nucleotides was calculated for this molecule. This RNA was also found to be rich in guanylic and cytidylic acid residues. Both the 6s RNA synthesized *in vitro*, and the 6s RNA isolated from infected cells, are capable of serving as template in the polymerase reaction. The maximum rate of the reaction with 6s RNA, however, is about 30% of that obtained when QB RNA itself is used as template. The reaction involving 6s RNA does not require the addition of the two protein factors shown to be absolutely required when QB RNA directs the synthesis (Franze de Fernandez *et al*. 1968). The resistance of 6s RNA to pancreatic ribonuclease hydrolysis suggests protection due to extensive base pairing.

The *in vivo* role of 6s RNA still remains very puzzling. Whether this molecule is derived from QB RNA, or is present in the host cell at low levels prior to infection, is not known. So far no RNA similar to the 6s RNA described above, has been detected in uninfected cells.

A Low Molecular Weight RNA From Alcaligenes Faecalis

An RNA of sedimentation coefficient 5.5 s was isolated from Alcaligenes faecalis and characterized by Beljanski and Bourgarel (1968). This low molecular weight RNA (molecular weight 50,000 by ultracentrifugal analysis), represents some 2 to 3% of the total cellular RNA, and like E. coli 5s RNA, has uridine 3', 5' diphosphate at its 5' end, and uridine at its 3' end. Base composition analysis shows this RNA to be different from either 5s or 4s RNA. Melting temperature studies also reveal marked differences in the mid-point of the temperature transition obtained for 5.5s, 5s, and 4s RNAs.

In vitro biological studies indicated that this RNA initiates an unusual type of peptide synthesis in the presence of the four ribonucleotide triphosphates. It has been suggested that a binding occurs which involves an ester linkage between the carboxyl group of the amino acids and the 2' hydroxyl group of the RNA (Beljanski et al., 1968). Since the reaction occurs in the complete absence of ribosomes, Beljanski has speculated that 5.5s RNA could be responsible for the synthesis of a particular class of proteins - possibly structural proteins.

E. Coli Low Molecular Weight RNAs

The presence of low molecular weight RNAs in E. coli crude soluble RNA extracts has been demonstrated by a number of workers. Schleich and Goldstein (1965) have shown that sRNA prepared by the modified method of Holley (1961), and fractionated on sephadex G 100, is heterogeneous, and contains several minor RNA components. The fact that these components were previously undetected in E. coli extracts suggests that the identification of some of the minor RNAs is dependent on the method chosen for isolation.

Minor RNA components have since been observed by Cannon and Richards (1967), and Hindley (1967), when E. coli whole cell sRNA preparations were analyzed by polyacrylamide gel electrophoresis. Since no characterization of any of these minor components was carried out, nothing can be said about the nature or origin of these molecules.

One of these minor components is of particular interest. This RNA described as elution region 2 RNA by Schleich and Goldstein, elutes from sephadex G 100 ahead of both 4s and 5s RNA, and represents some 2 to 4% of the total sRNA concentration.

The presence of an RNA with a sedimentation coefficient of 6s in phage infected cells has already been

described. The appearance of this RNA following phage infection has evoked much speculation. Curiously enough, Banerjee et al. (1969) did not detect a low molecular weight RNA component in uninfected E. coli cells. This could simply be due to the limitations of the technique employed in its detection, or perhaps , as King (1967) has suggested, could be a reflection of the differences in techniques employed in the isolation of the sRNA. Whatever the reason, it seemed necessary that a thorough characterization of elution region 2 RNA be undertaken, in order to shed some light on the origin of this molecule, and also to provide some insight into the role played by this low molecular weight RNA in E. coli metabolism. The methods and results of these studies comprise this Thesis.

CONCLUSION

It is impossible in such a brief review to present a detailed account of all of the low molecular weight RNAs that have been described to date. So far, low molecular weight RNA species have been described, which have been isolated from the cytoplasm, nucleus, and from the membranes of the endoplasmic reticulum. With some exceptions, the overall picture that emerges is one in which the RNAs are intimately involved with protein molecules in the formation of nucleoprotein complexes, which is presumably the form in which these molecules carry out their respective functions. It seems, therefore, that such biologically active complexes, particularly in the case of the membrane associated RNAs, must include some lipid material. If this is the case, then it can very easily be seen why it has been so difficult to establish the biological role of many RNAs, since the procedures employed in their isolation destroy this 'active complex', and render the RNA inactive.

It is very tempting to speculate on the biological role of some of the RNAs described here, and even to group them into different categories of activity. Transfer RNA, 5s RNA, and 7s RNA can be thought of as being part of the cell's protein synthesizing machinery. These are molecules that either contribute to the stabilization of the conformation of the ribosome/messenger complex, or play an

active part in the recognition of the code on the messenger.

The second category, and perhaps the least understood, involves those low molecular weight RNAs that seem to have some template activity. These include the RNA described by Beljanski (1968), and the 6s RNA characterized by Banerjee (1969). Both of these molecules serve as templates - the 5.5s RNA of Beljanski functioning to bind amino acids in vitro, while the QB 6s RNA serves to direct the synthesis of additional RNA molecules in the presence of RNA polymerase.

The third category of activity involves the RNAs which perhaps have a regulatory role in protein synthesis. These include the RNAs described by Bonner, and possibly those described by Weinberg and Penman, and others. These are all nuclear RNAs which are closely associated with protein, and presumably recognize particular regions of the DNA in a sequence specific manner. These are the 'activator' RNAs alluded to by Britten and Davidson (1969) in their theory on gene regulation.

CHAPTER TWO

The Isolation and Purification of Elution Region 2 RNA

INTRODUCTION

The extraction of soluble RNA from E. coli whole cells using the phenol procedure yields preparations whose heterogeneity can easily be demonstrated by gel filtration analysis. Optical density profiles of such fractionated samples consistently show the presence of four elution regions. Elution region 4 is a mixture of the various transfer RNAs, and is essentially present in the cytoplasmic cellular fraction. Elution region 3 (5s RNA), on the other hand, is mainly isolated with the ribosomal fraction, and its homogeneity has already been established.

In this chapter the isolation, purification, and homogeneity of elution region 2 RNA, as established by sephadex G 100 chromatography and polyacrylamide gel electrophoresis, will be described.

MATERIALS AND METHODS

Isolation of sRNA - Whole Cells - Several methods have been employed in the isolation of sRNA from E. coli cells. Of these, the modified method of Holley (1961), involving direct phenol extraction of whole cells, was utilized.

Frozen E. coli B cells, harvested in mid-logarithmic growth, were used for all sRNA extractions. 500 grams of cells were thawed in 2 liters of deionized water, and 2 liters of 88% phenol added with constant stirring. The phenol precipitated cells were stirred continually for 8 hours before centrifuging at 12,000 x g for 30 minutes. The aqueous supernatant was carefully removed (phenol phase and interphase discarded), and 0.1 volume of potassium acetate (20%, pH 5.0), and 2.5 volumes of 95% ethanol (cold) added with constant mixing. The white, flocculent precipitate was allowed to settle overnight. The clear alcohol supernatant was carefully decanted, and the remaining suspension centrifuged for 30 minutes at 12,000 x g. The resulting pellet was then dried in vacuo, and suspended in 500 ml of 0.1 M Tris (pH 7.5). The suspended RNA was next mixed with 8 grams of washed diethyl aminoethyl cellulose (DEAE), and stirred gently overnight in the cold. The DEAE-adsorbed RNA was then rinsed 4 times with 500 ml 0.1 M Tris (pH 7.5), each time discarding the liquid layer

after centrifugation. After washing, the DEAE was suspended in 500 ml M sodium chloride buffered at pH 7.5 (0.25 M Tris), and stirred for 4 hours to remove the bound sRNA. The supernatant was recovered by centrifugation, and the RNA immediately precipitated with 95% ethanol (2.5 volumes). The precipitate was collected, washed twice with 80% ethanol, and dried in vacuo at room temperature. The yield of sRNA was usually 0.1%. All steps were performed at 4°C unless otherwise stated. A diagramatic representation of this isolation procedure is shown in Figure 1.

Isolation of sRNA - Cell Fractions - Cytoplasmic and ribosomal low molecular weight RNA preparations were obtained in the following manner. Cells (100 grams wet weight) were ground with twice their weight of washed alumina, and the resulting thick paste suspended in 3 times its volume of buffer consisting of 0.01 M Tris (pH 7.4), and 0.01 M magnesium acetate. Deoxyribonuclease (2 micrograms/ml) was added, and the suspension centrifuged twice at low speeds to remove the debris. The brownish supernatant was then centrifuged at 105,000 x g for 4 hours in a preparative ultracentrifuge. Only the upper half of the clear supernatant was used for the preparation of cytoplasmic RNA. The remainder of the supernatant was discarded, and the ribosomal pellet washed 4 times with 0.01 M Tris,

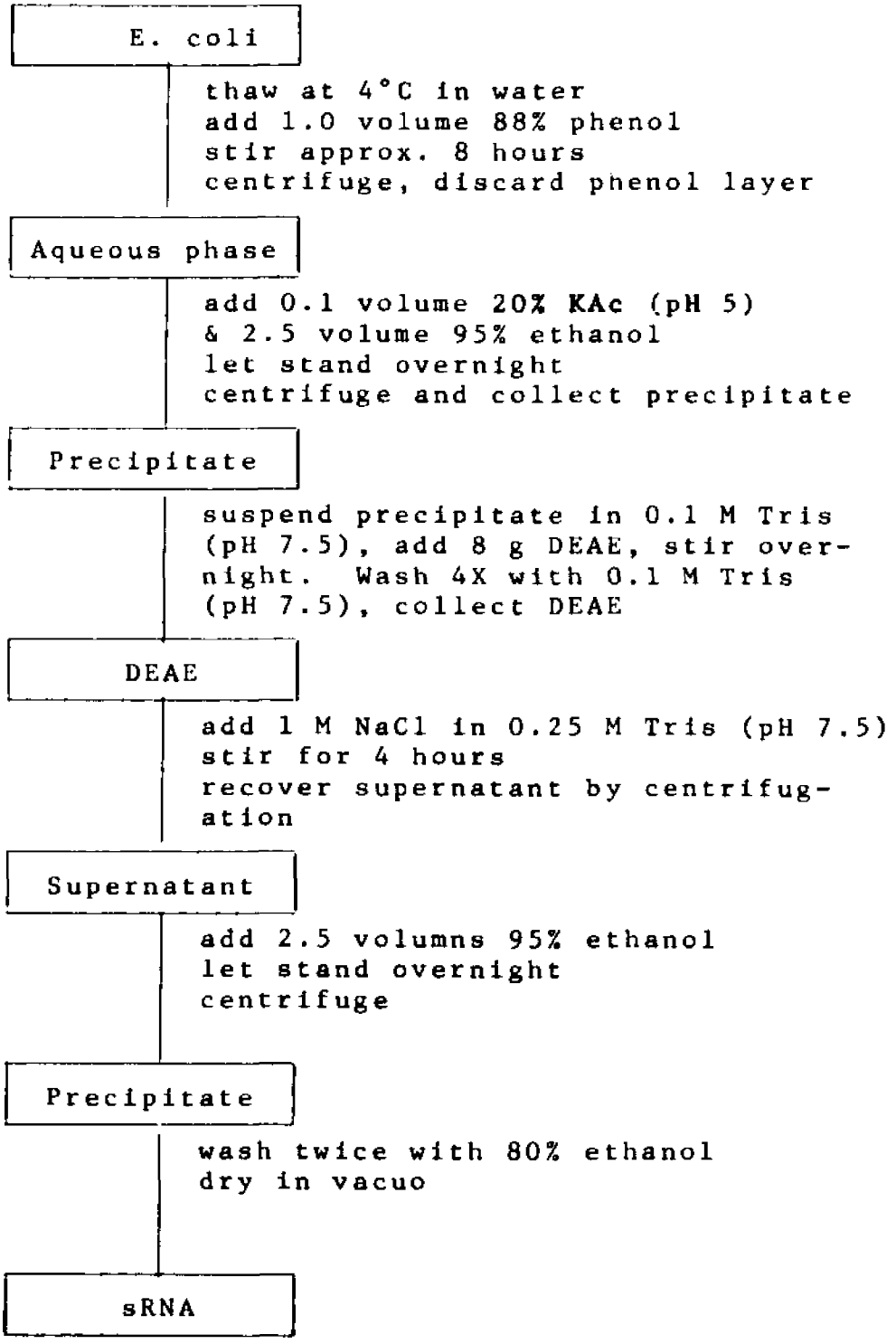

Figure 1. Flow-sheet for the preparation of sRNA from E. coli.

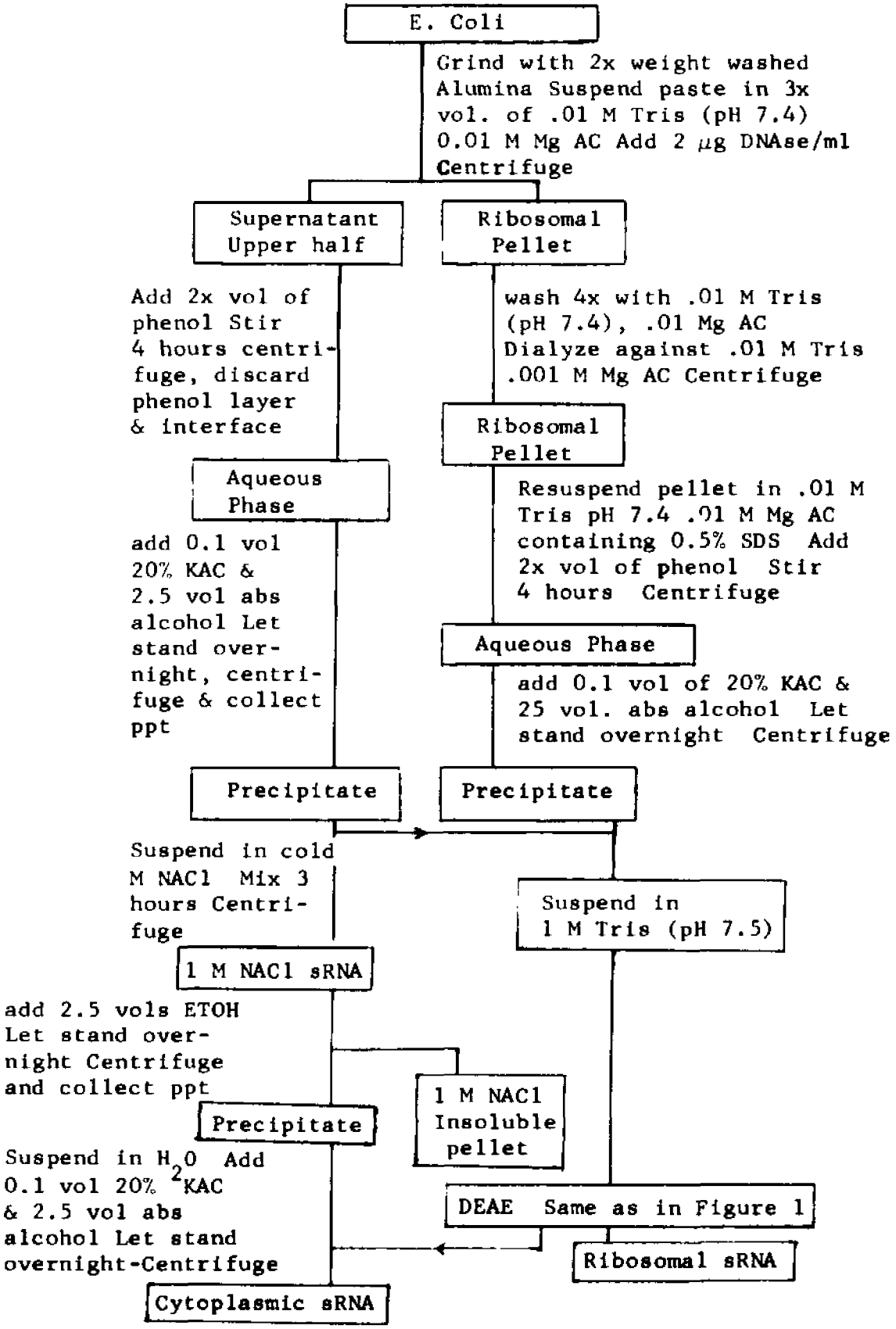

Figure 2 Flow Sheet for the preparation of Cytoplasmic and Ribosomal sRNA from E. Coli

magnesium acetate buffer (pH 7.5), resuspended in the same buffer, and dialyzed overnight against 0.01 M Tris (pH 7.5), containing 0.001 M magnesium acetate. Dialysis was continued for an additional 3 hours against the 0.01 M Tris/magnesium buffer. The dialyzed ribosomal suspension was next subjected to a low speed centrifugation, followed by another 105,000 x g centrifugation (5 hours). The pellets obtained were resuspended in 0.01 M Tris/magnesium buffer containing 0.5% sodium dodecyl sulfate, and used for the preparation of low molecular weight ribosomal RNA.

The low molecular weight cytoplasmic and ribosomal sRNAs were isolated by the phenol method in the same manner as outlined for the whole cells. The volumes used in the DEAE treatment were scaled down because of the lower RNA concentrations.

An alternate procedure was also used in the isolation of cytoplasmic sRNA. This procedure involved the use of 1 M sodium chloride to selectively remove the sRNA from the total RNA obtained after centrifuging the first ethanol precipitate; separating the sRNA from the 1 M sodium chloride insoluble fraction; and recovering the sRNA by direct precipitation with absolute alcohol (2.5 volumes). Usually a second alcohol precipitation was carried out before the RNA was dried, and chromatographed (Figure 2).

Small quantities of unlabeled, and ^{32}P labeled whole

cell sRNA were prepared exclusively by this method, since this procedure yielded sRNA preparations of similar purity to those obtained after DEAE treatment (see Figures 3a, 3b, and 4).

Growth of ^{32}P Labeled E. coli B Cells - ^{32}P labeled sRNA was prepared from exponentially growing cells by labeling cultures grown in peptone medium with 5 mc ^{32}P ortho-phosphate, for a period of approximately 3 generations. The growth was usually carried out at 37°C, and at the end of 3 generations (optical density 1.0 at 540 mu) the growth was immediately stopped by immersing the cells in an ice bath. The cells were harvested by centrifugation, washed 3 times with 0.1 M phosphate buffer (pH 7.0), and the sRNA isolated by the phenol procedure.

Sephadex G 100 Fractionation of sRNA - Sephadex G 100 column fractionation was performed on a variety of columns, depending on the size of the sample to be chromatographed, and the resolution desired. Best results were obtained with 5 x 150 cm, and 2.5 x 150 cm columns. 0.9 x 150 cm columns were used mainly for rapid analysis of the sRNA preparations.

Large scale fractionation of unlabeled, whole cell sRNA was accomplished as follows: 1 gram of crude sRNA was dissolved in 50 ml of M sodium chloride, and the dissolved sample layered on top of a 5 x 150 cm column. M sodium

chloride was used as the eluting buffer, and fractions of approximately 10 ml volume were collected (800 ml forerun).

Each fraction was analyzed for its absorbance at 260 and 280 mu. The peak regions were then pooled, dialyzed and evaporated down, and the RNA precipitated with potassium acetate (20%), and ethanol (95%).

Elution region 2 and 5s RNA prepared as outlined above, were rechromatographed on Sephadex G100 (2.5 x 150 cm columns).

Cytoplasmic and ribosomal sRNA samples were fractionated on small columns (0.9 x 150 cm). Usually 2 mg amounts were applied to the column. The effluent was analyzed for its absorbance at 254 mu, using an ultraviolet analyzer. Fractions of 2 ml were collected, and the peak regions pooled, dialyzed, and evaporated to dryness.

^{32}P labeled sRNA samples were fractionated on 0.9 x 150 cm, and 2.5 x 150 cm columns. In each case the absorbance of the sample was continuously monitored at 254 millimicrons, using an ultraviolet analyzer. The ^{32}P profile was obtained by removing 10 microliters from each fraction, and drying the aliquots in scintillation vials. 10 ml of toluene scintillation fluid (4 grams 2.5-diphenyl-oxazole (PPO), and 50 mg p-bis [2-(5-phenyloxazolyl)]-benzene (POPOP) per liter of toluene) were added to each vial, and the counts per minute obtained using a liquid scintillation counter. The remaining material in each

tube was dialyzed and evaporated to dryness, or precipitated directly from M sodium chloride with absolute ethanol.

Polyacrylamide Gel Electrophoresis - The utilization of polyacrylamide gel electrophoresis for resolving mixtures of macromolecules was first described by Ornstein and Davis (1964). The application of this technique to the study of RNAs has offered the possibility of more extensive, and precise separations of samples whose heterogeneity had previously been undetected by other methods. Richards, Coll and Gratzer (1965) have utilized this technique very effectively in the separation of low molecular weight RNAs. Cannon and Richards (1967), and Hindley (1967), have also employed this method in the fractionation of E. coli sRNA preparations. The procedure offers great versatility since fractionation is dependent on molecular filtration, and the pore size of the gels employed can be manipulated at will to provide the degree of filtration desired. Since differences in the ratio of charge to mass are usually small for RNAs, the separation is entirely determined by molecular size and shape.

The system described by Loening (1967) was utilized for all studies carried out on elution region 2 RNA, since the gels were relatively easy to prepare; gave good separations; and did not require the presence of spacer

gels for concentration of samples.

Preparation of Gels - 3% and 8% acrylamide gels (weight/volume) were used for all electrophoretic analyses of rechromatographed elution region 2 RNA. Suitable volumes of Acrylamide (Eastman Organic Chemicals, highest purity), and Bisacrylamide (5% of the acrylamide concentration for 3% gels, and 2.5% of the acrylamide concentration for 8% gels) as cross-linker, plus electrophoresis buffer (0.04M Tris (pH 7.8), 0.02 M sodium acetate, 0.002 M EDTA), and water to provide the desired concentrations, were mixed at room temperature. N N N' N' Tetramethylethylenediamine (0.033 ml), and 10% ammonium persulfate (0.33 ml) were added per gram of acrylamide present. The solution was mixed carefully, and poured into verticle glass tubes (5 mm I.D. x 100 mm) up to a height of 70 mm. Water was carefully layered over the top of each gel, and polymerization allowed to take place at room temperature (22°C). After polymerization the tops of the gels were rinsed, and the gels were pre-electrophoresed for 50 minutes at 100 volts to remove any charged material.

Procedure for Electrophoresis - The RNA samples (20 - 100 micrograms) were usually dissolved in a small volume of electrophoresis buffer (approximately 50 microliters), and mixed with 150 microliters of buffered sucrose (20% in

electrophoresis buffer). This mixture was layered on top of the gels, and electrophoresis carried out using an electrophoresis cell similar to that described by Davis (1964). Samples were normally electrophoresed for a maximum of 80 minutes at 100 volts (5 milliamps/tube). The running time was sometimes varied depending on the resolution desired. After electrophoresis the gels were removed from the tubes by carefull rimming, and immersed in a staining solution of Acridine Orange (2% weight/ volume), 1% Lanthanum Acetate, in 7% Acetic acid. All gels were left overnight in this staining solution. The unbound stain was removed by subjecting the gels to electrophoresis at 300 volts, using 7% Acetic acid as buffer.

Gel patterns were recorded by scanning the stained gels on a Joyce Loebl microdensitometer, utilizing a tungsten source, and a blue filter. Under these scanning conditions the patterns obtained provided information only on the electrophoretic mobilities of the various RNAs, and could not be used to determine relative RNA concentrations.

Scanning of Radioactivity - When ^{32}P labeled RNA samples were used in electrophoresis, the rimmed gels were fractionated on an Autogel Divider according to the procedure described by Maizel (1966). Buffalo Black was employed

to mark the tops of the gels, and fractions were collected either in scintillation vials or on planchets, dried, and the radioactivity determined. A gas flow counter was used in the analysis of the radioactivity present on the planchets.

Urea and heat treatment - Rechromatographed elution region 2 RNA (40 micrograms) was dissolved in 100 microliters 0.02 M Tris (pH 7.4) containing 0.05 M sodium chloride and 0.003 M EDTA. 100 microliters of 8 M urea was then added, and the sample thoroughly mixed. The mixture, along with appropriate controls, was incubated for 15 minutes in a reaction vessel maintained at 80°C by a thermostatically controlled circulator. At the end of the incubation period samples were removed from the vessel and rapidly plunged into an ice bath. The cooled samples were electrophoresed for 80 minutes at 100 volts.

The heat stability of elution region 2 RNA was also tested by maintaining samples at 72°C for periods up to 2 hours, and at the end of the incubation, electrophoresing the samples on 8% polyacrylamide gels.

RESULTS

The results of Sephadex G 100 gel filtration studies of the various isolated sRNAs are presented in Figure 3a. Whole cell sRNA exhibits its customary effluent profile, with the 4 elution regions being clearly resolved. A similar pattern is obtained for sRNA isolated from whole cells labeled with ^{32}P, the ultraviolet absorbance profile (not shown) coinciding exactly with the profile obtained from analysis of the radioactivity (Figure 3b). Elution region 1 represents some 8% of the total sRNA (Figure 3a), and appears in the region of the column void volume. The amount of this material present in the crude sRNA varies, depending on the method of isolation employed, and strongly suggests the possibility that this material is an artefact of the isolation procedure.

Elution region 2 is very clearly resolved, and represents 2% of the total sRNA recovered. This yield is consistently obtained for all preparations of crude sRNA examined.

Elution region 3 (5s RNA), and elution region 4 (tRNA) are the major constituents, and occur in the amounts of 15% and 75%, respectively.

The elution profile of whole cell sRNA extracted by the 1 M sodium chloride method, and chromatographed on

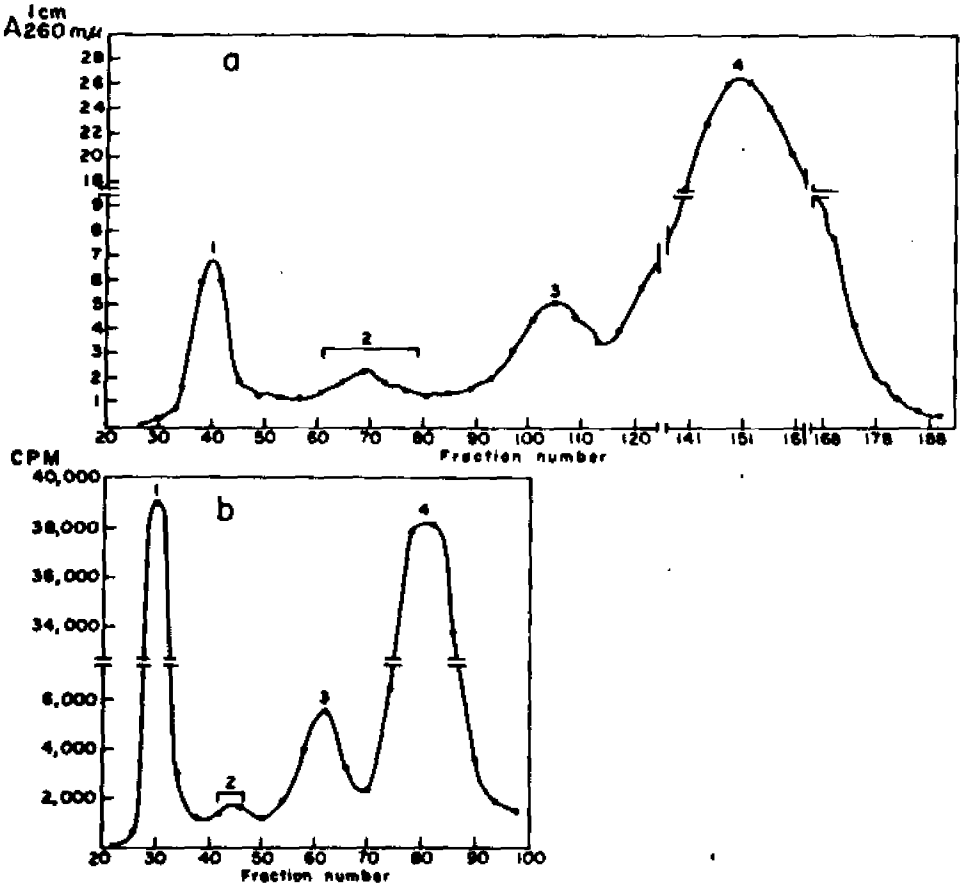

Figure 3. Elution profile (Sephadex G 100) of (a) crude soluble RNA isolated from whole cells and chromatographed on a 5 x 150 cm column. (b) Elution profile (Sephadex G 100 of ^{32}P labeled soluble RNA isolated by 1 M sodium chloride method and chromatographed on a 2.5 x 150 cm column.

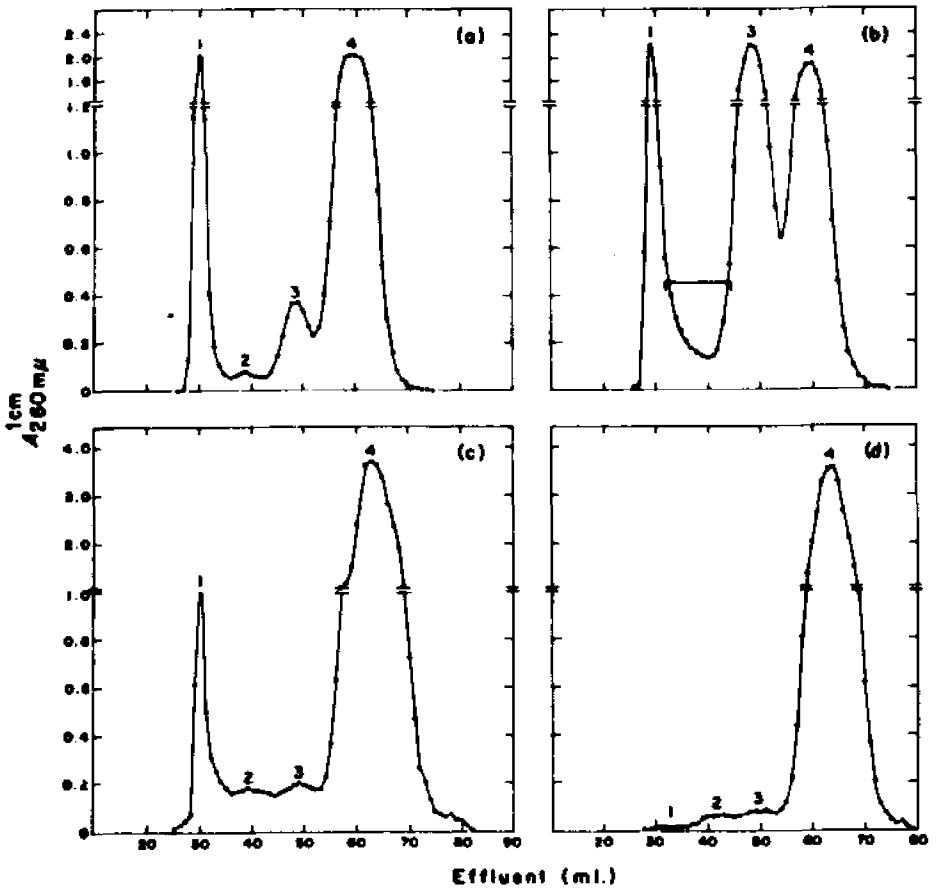

Figure 4. Gel filtration effluent profiles (Sephadex G 100) of (a) whole cell soluble RNA; (b) ribosomal sRNA; (c) cytoplasmic sRNA prepared by direct M sodiun chloride extraction; (d) cytoplasmic sRNA prepared by DEAE-cellulose treatment and M sodium chloride extraction.

the small Sephadex G 100 column (0.9 x 150 cm) is shown in Figure 4a. Once again all 4 elution regions are clearly resolved, indicating that the M sodium chloride method yields sRNA preparations that are identical to those preparations resulting from the DEAE treatment.

Cytoplasmic sRNA prepared by DEAE treatment and chromatographed on Sephadex G 100 (0.9 x 150 cm column) contains a small percentage of elution region 1 (0.3%); the usual 2% of elution region 2; a relatively small 5s RNA region (1.9%); and a major peak region of tRNA (95.8%) (Figure 4d).

The Sephadex G 100 profile obtained for cytoplasmic sRNA prepared directly by M sodium chloride extraction is shown in Figure 4c. A marked increase in the amount of elution region 1 is observed (see also Figure 3b). This is a clear indication that M sodium chloride solubilizes some high molecular weight species from the cytoplasmic cellular RNA that are selectively removed by the DEAE cellulose step. These species may vary in size over a wide range since they are completely excluded from the Sephadex. The relative amounts of the other peak regions are unchanged. The small peak of 5s RNA is consistently present in all of the cytoplasmic RNA preparations. Whether this represents 5s RNA molecules that are released during the isolation procedure, or whether it represents a small pool of free cytoplasmic 5s RNA, is not known.

The ribosomal sRNA preparations display a different elution profile on Sephadex G 100 chromatography (Figure 4b). Elution region 1 now occupies some 20% of the total sRNA recovered. Transfer RNA, and 5s RNA are evenly distributed, suggesting the presence of an equal number of these molecules on the ribosomes. This stoichiometry remains the same for all of the ribosomal sRNA preparations examined.

Elution region 2 is noticeably absent from the ribosomal sRNA chromatograms, indicating it to be a constituent solely of the cytoplasmic fraction.

When cytoplasmic or ribosomal sRNA samples were treated with Deoxyribonuclease (DNAse) for 1 hour at room temperature, prior to Sephadex G 100 analysis (10 ug DNAse/ 2 mg sRNA/ml 0.01 M Tris (pH 7.5). 0.001 M magnesium acetate), the elution profiles remain unchanged. All 4 elution regions are sensitive to hydrolysis by pancreatic Ribonuclease A, however (Schleich and Goldstein, 1965).

Rechromatography of unlabeled elution region 2 RNA (bracketed region Figure 3a) yields a major, sharp peak, with the same distribution coefficient as that obtained for the first Sephadex fractionation (Figure 5a). A small amount of material is also present in elution region 1, and elution region 3. These represent the sole contaminants detected. The fractions obtained from the Sephadex rechromatography of elution region 2 (see Figure 8a) were pooled, dialyzed, and evaporated to dryness.

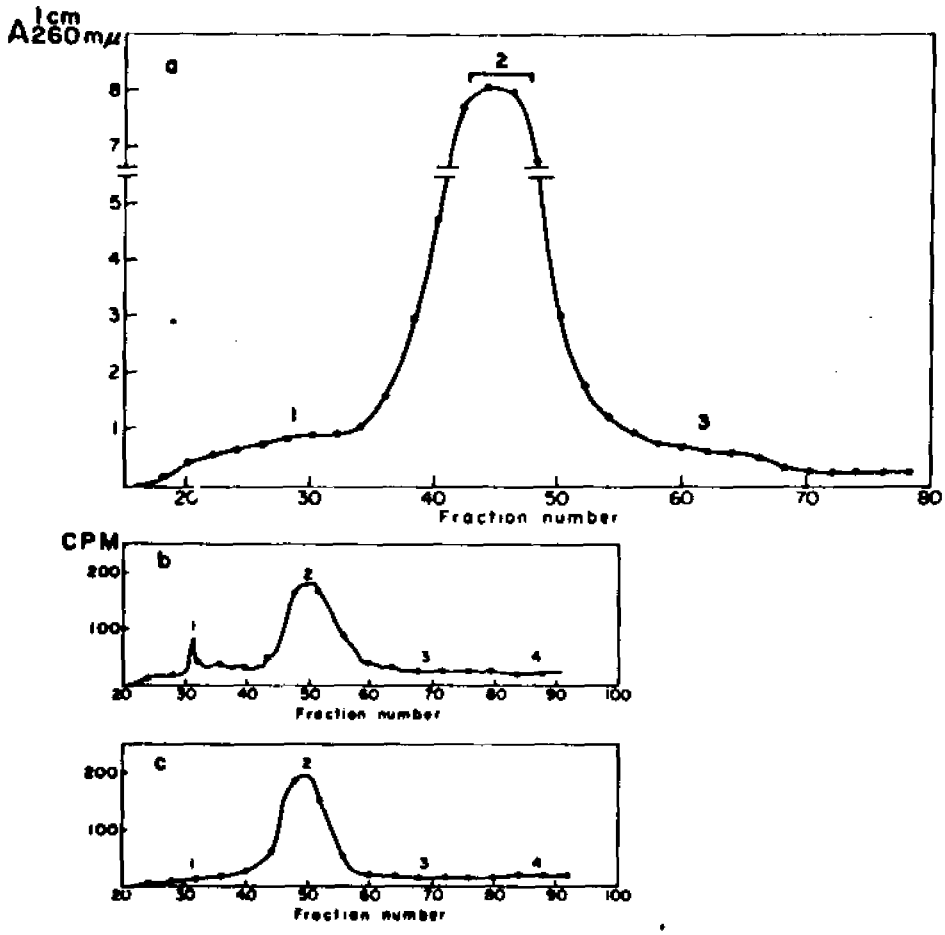

Figure 5. Elution profiles (Sephadex G 100) of (a) unlabeled elution 2 RNA from bracketed region shown in Figure 3a, rechromatographed on 2.5 x 150 cm column. (b) ^{32}P labeled elution region 2 RNA from bracketed region shown in Figure 3b, rechromatographed on a 2.5 x 150 cm column. (c) ^{32}P labeled elution region 2 (from b), third rechromatography on a 2.5 x 150 cm column.

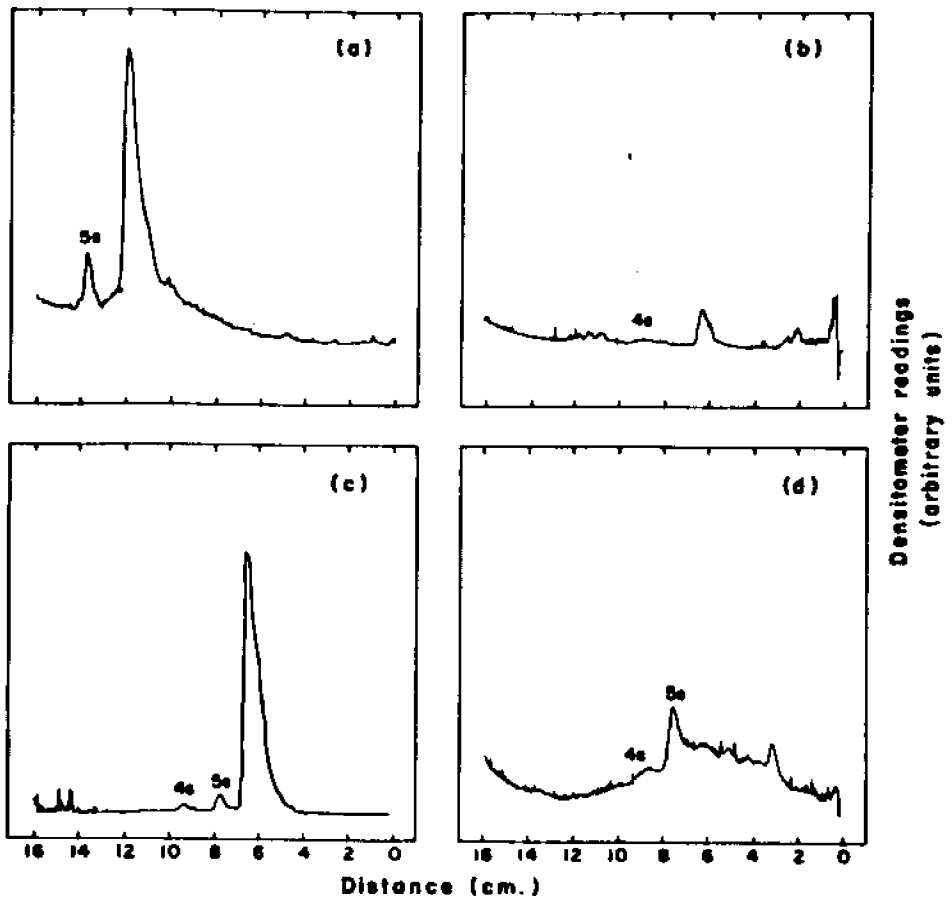

Figure 6. Polyacrylamide gel electrophoresis densitometer scans of (a) elution region 2 RNA isolated from whole cell soluble RNA; (b) elution region 2 RNA isolated from cytoplasmic RNA (105,000 g supernatant); (c) rechromatographed elution region 2 RNA from whole cell soluble RNA; (d) RNA isolated from low molecular weight ribosomal RNA (bracketed region shown in Figure 4b.

Identical conditions were used for (b), (c), and (d); RNA shown in (a) was allowed to migrate longer.

Rechromatography of ^{32}P labeled elution region 2 RNA (bracketed region Figure 3b), also yields a major peak region, and a tiny peak of elution region 1 (Figure 5b). No radioactivity is observed in the 5s region.

The stability of the ^{32}P labeled elution region 2 RNA, as well as its homogeneity on Sephadex, is demonstrated in Figure 5c. The elution profile remains the same after a third Sephadex G 100 chromatography. This stability is observed for elution region 2 samples isolated from frozen cells, or freshly grown cells. The type of medium selected for growth of the cells also has no effect on the gel filtration profiles obtained.

Although the results of the Sephadex G 100 gel filtration analysis clearly demonstrate the homogeneity, and stability of elution region 2 RNA, and also show that it is isolated with the cytoplasmic cellular fraction, it was still necessary to further examine these findings, utilizing the very sensitive technique of polyacrylamide gel electrophoresis.

Electrophoresis of unlabeled, rechromatographed elution region 2 RNA (bracketed region Figure 5a) on 8% polyacrylamide gels yields a major, well-defined peak (Figure 6c). Aside from trace amounts of 5s and 4s RNA, which appear as tiny peaks on the densitometer scans, no other RNA peaks are evident. When the electrophoretic mobility of elution

region 2 RNA is compared with that of 5s or 4s RNA, a very marked difference in mobility is observed. After 80 minutes of electrophoresis at 100 volts, both 4s and 5s RNA have moved farther down the gel than elution region 2 RNA. This difference in electrophoretic mobility of these RNA species can be directly related to the size of these molecules, and together with the Sephadex G 100 elution behavior, shows that elution region 2 RNA is a larger molecule than either 4s or 5s RNA.

^{32}P labeled whole cell elution region 2 RNA was next examined on 8% polyacrylamide gels in an attempt to show that labeled and unlabeled preparations behave identically on electrophoresis. Analysis of the radioactivity present in the fractions obtained from a gel on which elution region 2 RNA was electrophoresed (Figure 7a) reveals a major peak region with the same electrophoretic mobility as that observed for unlabeled elution region 2 RNA.

Polyacrylamide gel electrophoresis of the elution region 2 RNA obtained from cytoplasmic sRNA fractionation (Figure 4d) was also carried out in order to compare the electrophoretic behavior of these preparations with those obtained from whole cells. Densitometer scans of the stained gels resulting from these experiments (Figure 6b) clearly reveal a major component with the same electrophoretic mobility as its counterpart from whole cell extracts (Figure 6c).

Polyacrylamide gel electrophoresis (8% gels) of the material recovered from elution region 2 of the Sephadex fractionation of ribosomal sRNA preparations (bracketed region Figure 4b) shows no RNA species with the same electrophoretic mobility as elution region 2 RNA. Instead, the densitometer scan shows considerable polydispersity (Figure 6d). This result is in good agreement with the results obtained from gel filtration analysis, and provides clear proof that elution region 2 RNA is absent from the ribosomal sRNA preparations.

The homogeneity of elution region 2 RNA was further examined by subjecting a sample of rechromatographed material (bracketed region Figure 5a) to prolonged electrophoesis on 8% polyacrylamide gels. Figure 6a shows the densitometer scan resulting from such an experiment. Elution region 2 RNA still appears as a sharp, major peak, although it has now moved a considerable distance down the gel. No sign of heterogeneity of the peak region is evident.

Although the electrophoretic analysis of elution region 2 RNA was carried out in the presence of 2×10^{-3} M EDTA, the possibility of elution region 2 RNA being a partially aggregated species was not ruled out. In order to test for the presence of intermolecular aggregates in these preparations, samples of elution region 2 RNA were heated to 80°C for 15 minutes, in the presence and absence of 4M urea, rapidly cooled, and electrophoresed. Other samples

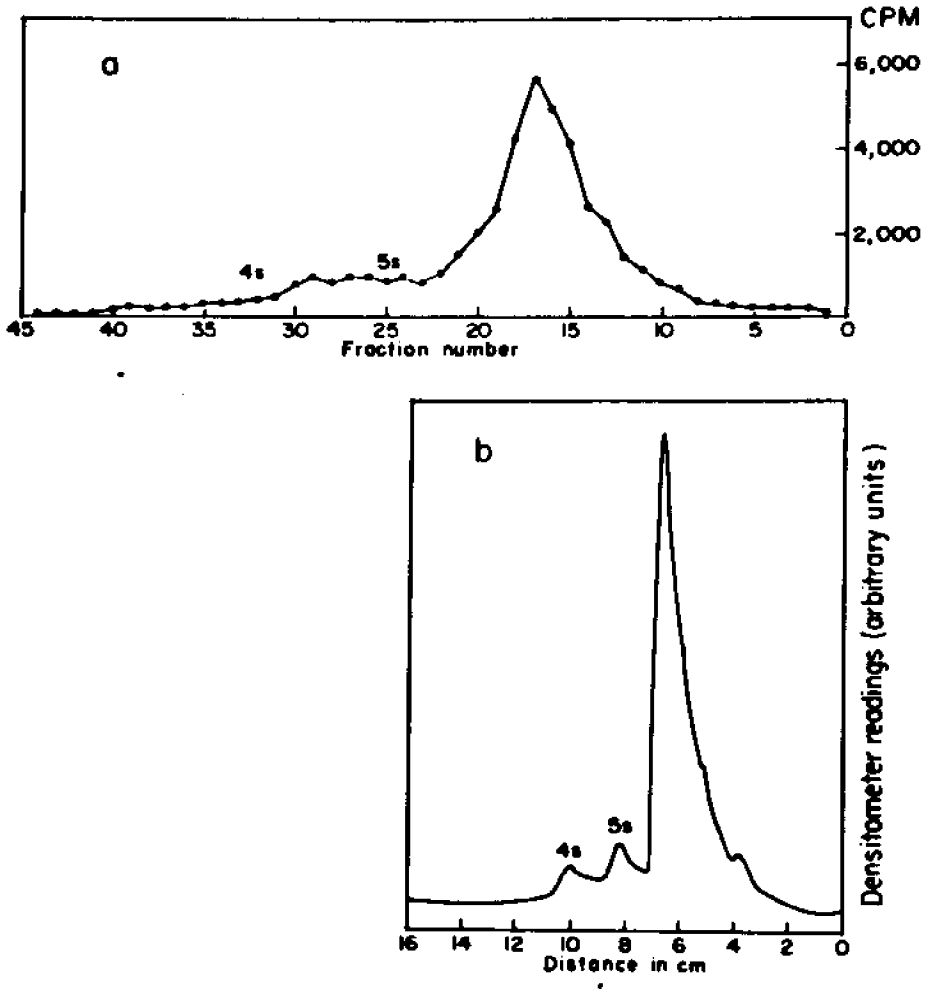

Figure 7. (a) Polyacrylamide gel electrophoresis of ^{32}P labeled elution region 2 RNA (8% gel). The rimmed gel was fractionated, and the ^{32}P counts/minute determined for each fraction.

(b) A typical densitometer scan of unheated, heat treated, or heat and 4M urea treated elution region 2 RNA (8% gel).

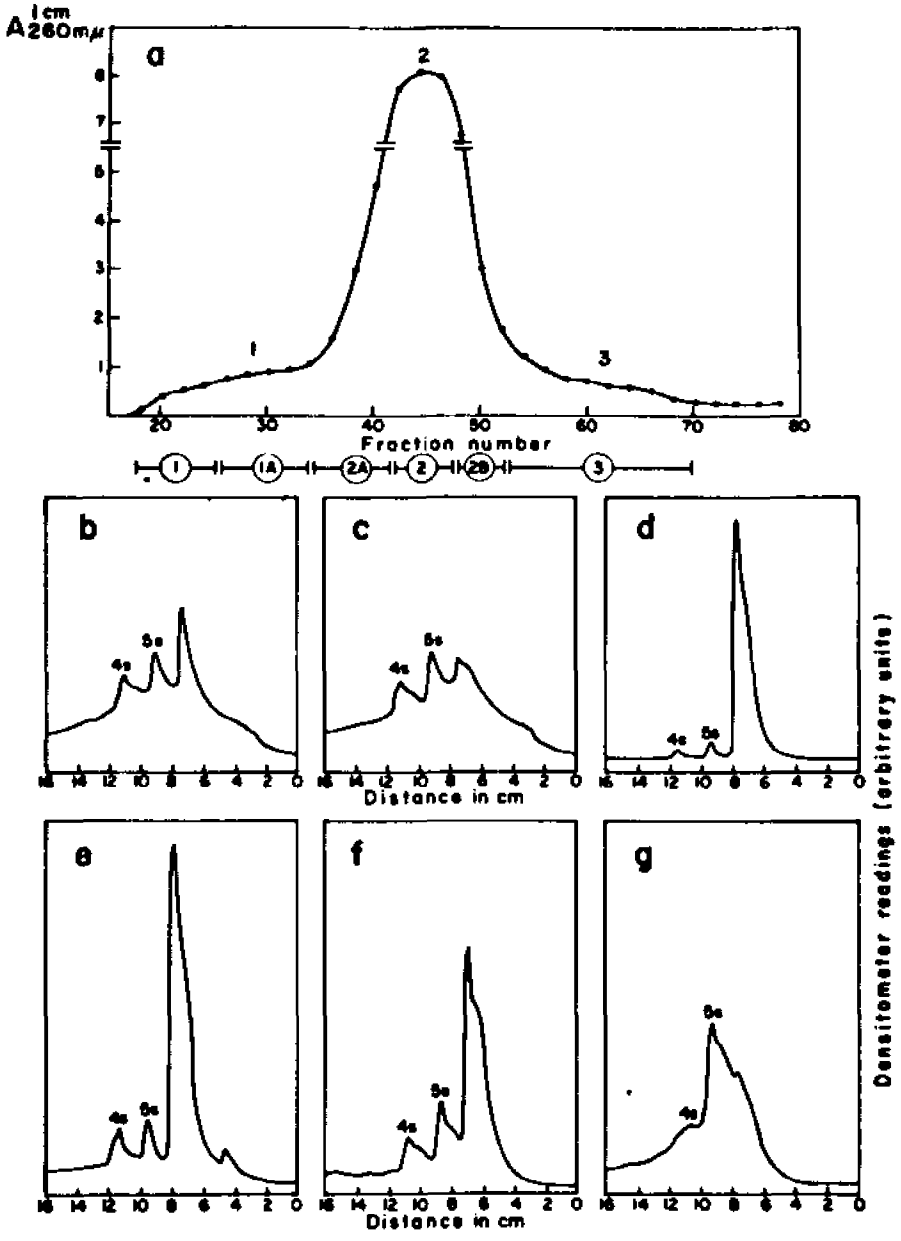

Figure 8. (a) Elution profile (Sephadex G 100) of rechromatographed elution region 2 RNA (2.5 x 150 column).
(b) Densitometer scan of region 1 (30 ug).
(c) Densitometer scan of region 1a (39 ug).
(d) Densitometer scan of region 2a (24 ug).
(e) Densitometer scan of region 2 (45 ug).
(f) Densitometer scan of region 2b (33 ug).
(g) Densitometer scan of region 3 (36 ug).

were maintained at 72°C for periods up to 2 hours before electrophoresing. Polyacrylamide gel electrophoresis of these heat and urea treated samples reveals no change in the electrophoretic behavior, regardless of the treatment, indicating that elution region 2 RNA is not an aggregated species. Figure 7b shows a typical scan obtained for all of the above samples.

The efficiency of Sephadex G 100 in resolving the various species of RNA was demonstrated by subjecting the pooled regions of the peak, obtained from the rechromatography of unlabeled elution region 2 RNA (see Figure 8a) to electrophoresis on polyacrylamide gels. Figure 8 shows the results obtained when the RNA was electrophoresed on 8% gels. Region 1 is resolved into 3 peaks (Figure 8b), and contains 4s, 5s, and elution region 2 RNA. 4s and 5s RNA peaks are also resolved when material from regions 1a, 2a, 2, and 2b are electrophoresed on 8% gels, but these species are only present in trace amounts (Figure 8 c, d, e, f) and reflect the limit of the resolution possible with Sephadex G 100. Electrophoresis of the material from region 3 (Figure 8g) shows a major peak of 5s RNA, as was expected from its position on the rechromatogram.

On the basis of these results, and in view of the precision of the separations obtained on polyacrylamide gels, it can be stated that sRNA preparations isolated from uninfected E. coli cells, contain a stable, low molecular

weight RNA, which is isolated from the cytoplasmic fraction, and is larger than either 4s or 5s RNA. This RNA is easily purified by Sephadex G 100 gel filtration.

SUMMARY

Sephadex G 100 gel filtration analysis of unlabeled, and ^{32}P labeled crude E. coli sRNA revealed the presence of 4 elution regions. These regions have been designated elution regions 1, 2, 3, and 4. Elution regions 3 and 4 are the major constituents, and occur in the amounts of 15% and 75%, respectively. Elution region 1 elutes with the column void volume, and its size varies, depending on whether the sRNA is isolated directly with cold molar sodium chloride, or with DEAE cellulose. Elution region 2 contains 2% of the total sRNA recovered, and is isolated from the cytoplasmic fraction (see Figure 2).

Polyacrylamide gel electrophoresis of unlabeled, or ^{32}P labeled elution region 2 RNA clearly showed that this RNA is a homogeneous species, displaying the same electrophoretic mobility on 8% gels; that it is larger than (has a slower electrophoretic mobility) 4s or 5s RNA; and that it does not result from intermolecular aggregation of other RNA species. Polyacrylamide gel electrophoresis was also utilized to show that rechromatography of elution region 2 RNA on Sephadex G 100 yields a preparation of high purity.

CHAPTER THREE

The Characterization of Elution Region 2 RNA

INTRODUCTION

The isolation and purification of elution region 2 RNA on Sephadex G 100 and polyacrylamide gels was described in Chapter 2. This Chapter provides a detailed analysis of the properties of this unique species of RNA, and presents evidence that this molecule is biosynthesized in vivo, and is not an artefact of the isolation procedure.

MATERIALS AND METHODS

Nucleotide Analysis - The nucleotide composition of RNA preparations have generally been determined by the separation, and spectrophotometric measurement of the 4 2', (3') ribonucleotides and other components liberated on alkaline hydrolysis. Methods used include modifications of the procedures of Cohn (1950) using columns of ion exchange resins, or of Markham and Smith (1952) using two-dimensional paper chromatography and paper electrophoresis. Irregardless of the procedure employed, knowledge of the chemical composition of RNAs has provided much useful information in the study of these molecules. Osawa (1960) has shown that the ability of sRNA preparations to incorporate amino acids was directly proportional to their pseudouridylic acid content. Dubin and Gunalp (1967) on the other hand, have suggested that the differences between E. coli ribosomal precursor RNAs and the mature species lie simply in altered conformation caused by the absence of certain methylated bases from the precursor species.

In the studies carried out on elution region 2 RNA hydrolysates the column chromatography procedure was extensively utilized. The nucleotide analysis was performed essentially according to the procedure of Osawa, Takata and Hotta (1958) using 0.9 x 10 cm and 0.9 x 30 cm columns packed with Dowex AG 1 x 2 anion exchange resin in formate

form. The Dowex resin, obtained in chloride form, was exchanged to formate by simply washing with M sodium hydroxide to remove the chloride ions, and then mixing the resin with M formic acid. Calibration of the columns was accomplished by chromatographing standard 2', 3' ribonucleotides, and determining the elution position of each nucleotide, as well as the percentage recovery based on its concentration. Concentrations were determined by summation of the optical density of each nucleotide peak, and making the appropriate calculations. The extinction coefficients of the 4 major nucleotides are listed in Table 1.

Alkaline Hydrolysis of RNA - Generally 2 mg RNA samples were dissolved in 1.0 ml of 0.6M potassium hydroxide and 1.0 ml of water added. Incubation was carried out for 18 hours, at 37°C. The resulting hydrolysate was cooled in an ice bath, and the pH adjusted to 2.0 with 6 M perchloric acid. A heavy, white precipitate of potassium perchlorate was obtained which was separated out by low speed centrifugation. The supernatant was carefully removed, and aliquots of approximately 0.2 mg used for application to 0.9 x 10 cm Dowex columns. In some cases 30 mg amounts of RNA were hydrolyzed, and the resulting hydrolysate chromatographed on 0.9 x 30 cm Dowex columns.

^{32}P labeled RNA was similarly hydrolyzed. Usually

the labeled material was coprecipitated with 30 mg of unlabeled sRNA prior to hydrolysis.

Gradient Procedure for Nucleotide Analysis - The gradients used were of 2 kinds depending on the size of the column employed. For 0.9 x 10 cm columns, elution of nucleotides was achieved in the following manner. 250 ml of water were placed in the gradient mixing chamber (a 250 ml filtering flask), and 80 ml of 0.8 M formic acid placed in the reservoir. The gradient was started immediately, and was allowed to run until cytidylic acid was eluted. The solution in the reservoir was then replaced by 4 M formic acid (150 ml), and adenylic acid eluted. Guanylic acid and uridylic acid were subsequently eluted by employing 150 ml of 0.05 M ammonium formate in 4 M formic acid, and 150 ml of 0.6 M ammonium formate in 4 M formic acid, respectively. Fractions of approximately 2.0 ml were collected, and the concentration of each nucleotide calculated based on optical density readings, fraction volume, and extinction coefficient. A positive identification of each nucleotide was made on the basis of its 260/280 absorption ratio.

Elution of nucleotides from longer Dowex columns (0.9 x 30 cm) was accomplished by employing three exponential gradients of formic acid consecutively formed by placing 160 ml of water in the mixing chamber of a linear gradient making device, and 100 ml of 0.8 M formic acid in the reservoir, and refilling the reservoir with 600 ml of

4 M formic acid, and finally with 500 ml of 4 M formic acid containing 0.6 M ammonium formate. 200 6 ml fractions were collected for analysis.

Dihydrouridylic Acid Assay - Dihydrouridylic acid was assayed for by the method of Fink, Cline, Mc Gaughey and Fink (1956), with some slight modification. Samples were dissolved in 0.5 ml of 0.6 M potassium hydroxide and allowed to incubate at room temperature for 30 minutes. 2.0 ml of color reagent (1.0 gm p dimethylaminobenzaldehyde dissolved in 100 ml of 95% ethanol, followed by the addition of 10 ml of concentrated hydrochloric acid) was then added. A positive reaction was characterized by the development of a yellow color which could be estimated by observing the optical density at 425 mu.

3' End-Group Analysis - The determination of the 3' end-group of elution region 2 RNA was carried out by utilizing the enzyme snake venom phosphodiesterase isolated from Crotalus adamanteus. The basic requirement for the activity of this enzyme is the presence of a nucleoside 5' phosphoryl residue, bearing a free, or at least non-phosphorylated 3' hydroxyl function. The hydrolysis of elution region 2 RNA was carried out according to the published procedure of Schleich and Goldstein (1966).

25 micrograms/ml of snake venom phosphodiesterase (Worthington VPH 100, of potency 0.3) were added to a

solution of RNA (usually a peak tube from a ^{32}P labeled sample diluted with unlabeled RNA) at a concentration of 1 mg/ml. The reaction mixture was buffered with a solution of 0.05 M Tris (pH 8.8), and 0.05 M magnesium acetate. Incubation was carried out at 0°C for all assays. At the desired time intervals, 1 ml portions of the reaction mixture were removed from the vessel, and immediately transferred to a hot aqueous solution containing 1.0 uM of each of the 4 major 5' nucleotides. The mixture was then heated at 100 °C for 5 minutes to terminate the enzyme action. The cooled mixture was then precipitated with 6 N perchloric acid, centrifuged at 12,000 x g, and the clear supernatant (pH 2) applied to the Dowex column (0.9 x 30 cm) for chromatography. The gradient system employed for eluting the bases was already described (see nucleotide analysis). Fractions of 6.0 ml were collected for each column run. Aliquots (5.0 ml) of each fraction were evaporated to dryness in scintillation vials, 10 ml of toluene scintillation fluid added, and analysis carried out utilizing a liquid scintillation counter. The total recovery of radioactivity in each nucleotide region was determined by making the necessary adjustments for fraction volume, and ^{32}P decay. The absorbance of each nucleotide region was measured on an ultraviolet Spectrophotometer.

Purified 4s RNA samples, hydrolyzed under identical

experimental conditions, were used as controls.

Molecular Weight Determination - Molecular weight determinations of elution region 2 RNA and 5s RNA were kindly carried out by Sister Marilyn Stephens and Dr. Kenneth Woods utilizing an analytical ultracentrifuge equipped with ultraviolet optics. The sedimentation equilibrium method was used for all studies.

Rechromatographed elution region 2 RNA (approximately 0.12 mg) was dissolved in 1.0 ml of M sodium chloride, and dialyzed overnight against molar sodium chloride. The dialyzed sample was then diluted with molar sodium chloride to yield a final concentration of 0.033 mg/ml. A sample of 5s RNA (0.031 mg/ml) purified by rechromatography on Sephadex G 100, was used as control.

Sedimentation equilibrium analysis was carried out by centrifuging the samples in a double sector cell at 12,000 rpm, for 24 hours. The chamber temperature was regulated at 293°A. The partial specific volume used for the calculations was 0.51. The density of M sodium chloride at 25°C was 1.0396.

Optical Absorbance Studies - All optical absorbance studies were carried out in standard phosphate buffer (pH 7.5) of the following concentration: 0.09 M sodium phosphate dibasic, and 0.01 M sodium phosphate monobasic.

Absorbance Measurements - Absorbance measurements were carried out on a recording spectrophotometer equipped with a thermostated cell housing. The temperature was regulated by a constant temperature circulator which was connected to the cell housing. Absorbance spectra of RNA solutions were measured by first allowing the samples to equilibrate to the desired temperature, and measuring the absorbance obtained for each 5 millimicron increment in wavelength. Absorbance/temperature profiles were obtained by raising the temperature 5°C at a time, and observing the absorbance at 260 millimicrons, 15 minutes after the desired temperature was reached. For some runs the procedure was automated by attaching a temperature programmer to the circulator, adjusted so that the temperature increment was now reduced to 1°C, and the dwell time 5 minutes. The procedure was simply reversed for the reannealing studies. Stoppered, fused quartz cuvettes were used for all absorbance/temperature studies.

Formaldehyde Reaction - 1.1 M formaldehyde solutions (37% reagent grade) in standard phosphate buffer were used for all formaldehyde studies. RNA samples were simply dissolved in the buffered formaldehyde solution and analyzed in the manner outlined above.

Nucleotide	Wavelength mu (maximum)	Extinction coefficient	pH	280/260
CMP	280	13.0×10^{-3}	2	2.09
AMP	257	15.1×10^{-3}	2	0.22
GMP	256	12.2×10^{-3}	1	0.67
UMP	262	10.0×10^{-3}	7	0.36

Table 1. Extinction coefficients of standard 2', 3' ribonucleotides (P. L biochemicals, inc.).

Table 2. Nucleotide composition of E. coli sRNA, rRNA and mRNA.

Nucleotide	Elution Region						
	2*	2(^{32}P)**	3	4[1]	16s rRNA[2]	23s rRNA[2]	mRNA[3]
cytidylic acid	25.9	25.1	28.7	28.8	22.7	22.0	24.7
adenylic acid	21.6	22.0	19.7	17.8	24.8	25.6	24.1
guanylic acid	29.2	29.3	34.5	27.4	31.0	31.2	27.7
uridylic acid	23.3	23.6	17.1	18.1	21.5	21.2	23.5
pseudouridylic acid	-	-	-	3.5	-	-	-
methylated bases	-	-	-	.9	-	-	-
A.U	.93	.93	1.15	.98	1.15	1.21	.87
G.C	1.13	1.17	1.37	.95	1.37	1.42	1.12

* performed on 0.9 x 10 cm column

** performed on 0.9 x 30 cm column

1 analysis taken from Schleich and Goldstein (1966).

2 analysis taken from Midgley (1962).

3 analysis taken from Spiegelman (1961).

Hypochromicity Studies - Samples of elution region 2 RNA, dissolved in standard phosphate buffer containing 1.1 M formaldehyde, were heated to 85°C for 15 minutes, rapidly cooled, and the absorption spectra measured at 15°C. The absorption spectra of unreacted RNA samples were also measured at 15°C and 85°C.

RESULTS

Nucleotide Composition - Analysis of the nucleotide composition of elution region 2 RNA, 5s RNA and 4s RNA shows that the distribution of the 4 major bases within these molecules is distinctly different (Table 2). The base composition of 16s rRNA, 23s rRNA, and mRNA also show no similarity to that of elution region 2 RNA. This marked difference in the nucleotide composition of elution region 2 RNA and the other species of soluble and ribosomal RNAs clearly demonstrates the unique chemical nature of this molecule.

For a more detailed analysis of the base composition of elution region 2 RNA, several analyses were carried out on alkaline hydrolysates of ^{32}P labeled samples. Figure 9 shows the Dowex column elution profile of a typical experiment. The exact position of each base was firmly established by observing the elution profile obtained from a hydrolysate of unlabeled E. coli sRNA (also shown in Figure 9). The use of longer columns (0.9 x 30 cm) for these

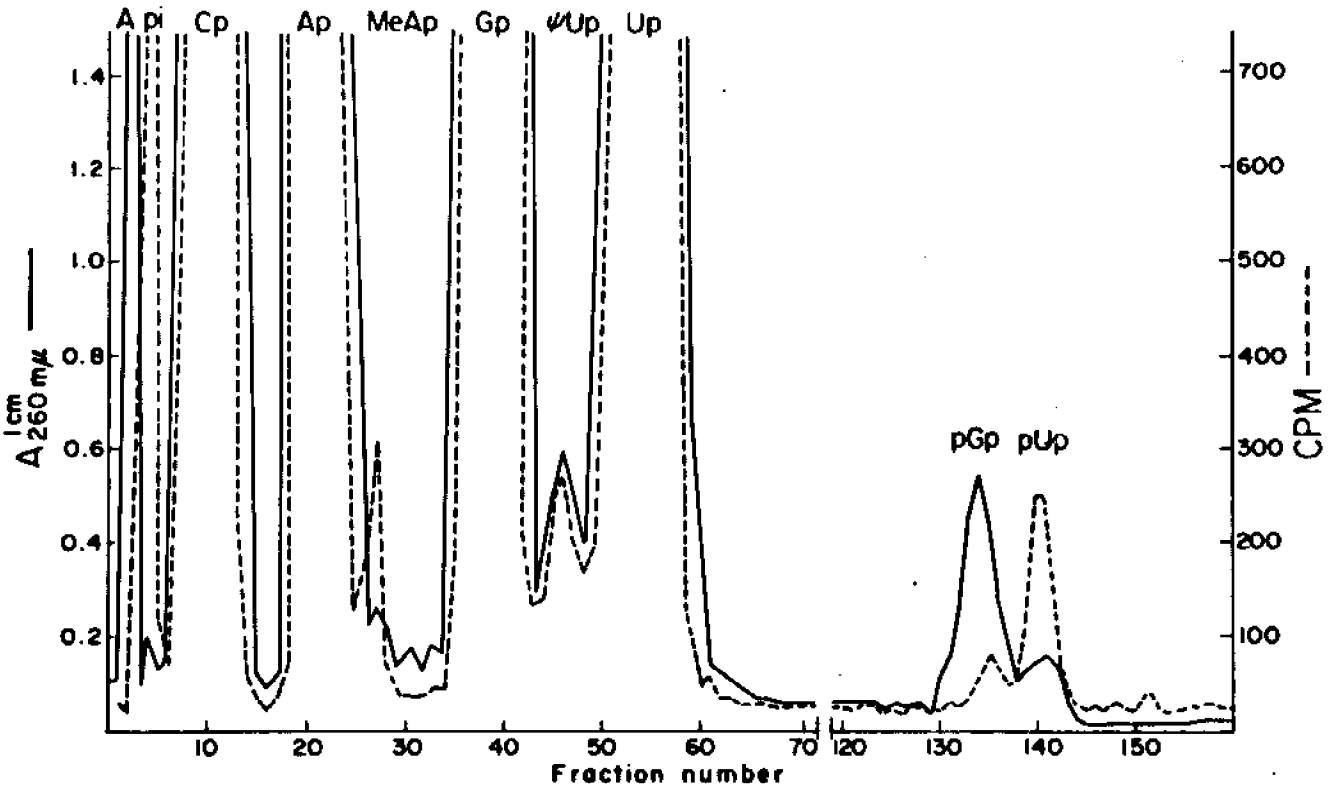

Figure 9. Dowex-1 X-2 (formate) column chromatography of an alkaline digest of ^{32}P labeled elution region 2 RNA (----); and unlabeled crude sRNA (——).

Table 3a. Calculation of chainlength of elution region 2 RNA from 5' nucleotide counts/minute recovered.

Total counts/minute recovered	"diphosphate" counts/minute	chainlength
280,400	3,777	$\frac{280,400}{3,777} \times 2 = 148 \pm 10\%$

Table 3b. Calculation of chainlength of elution region 2 RNA from 3' nucleotide release.

Total counts/minute	3' nucleotide released	chainlength
58,680	415	$\frac{58,680}{415} = 142 \pm 10\%$

analyses permitted the minor nucleotide components present in the sRNA sample to be clearly resolved.

The composition of each hydrolysate of elution region 2 RNA was determined by summation of the ^{32}P disintegrations per minute obtained from each nucleotide region. The nucleotide composition of elution region 2 RNA as determined by analysis of ^{32}P hydrolysates shows good agreement with the results obtained when unlabeled hydrolysates were analyzed on short (0.9 x 10 cm) Dowex columns (see Table 2). Methylated nucleotides and pseudouridylic acid appear to be essentially absent from the ^{32}P profile (Figure 9). Uridine 3' (2'), 5' diphosphate is the major 5' end-group recovered from the column. From the amount of 5' end-group released relative to the total amount of nucleoside monophosphate recovered (Table 3a), a chain-length of 148$\pm$ 10% is calculated for elution region 2 RNA. Schleich and Goldstein (1966) have utilized this method for calculating the chain-length of E. coli 5s RNA and have reported a chain-length of 122 nucleotides. The accuracy of this method was subsequently borne out by the work of Brownlee, Sanger and Barrell (1967) who reported a chain-length of 120 nucleotides for E. coli 5s RNA, based on sequence analysis.

The report by Huang and Bonner (1965) on the detection (by chromatographic analysis) of a high dihydrouridylic

acid content in the histone-bound RNA isolated from pea cotyledons, along with their suggestion that the dihydrouridylic acid facilitates linkage of these regulatory RNAs to the basic histone peptides, prompted an analysis of elution region 2 RNA for dihydrouridylic acid. A negative test was obtained for all assays indicating the absence of this unusual base from elution region 2 RNA. The lowest limit of detection possible with the method employed is approximately 0.2 micromoles of dihydrouridylic acid (see materials and methods).

The degree of contamination of the ^{32}P elution region 2 RNA preparations with fragments of 4s RNA can be estimated by assuming that the small peaks of 6 methyl adenosine, pseudouridine, and 3' (2'), 5' guanosine diphosphate result entirely from the presence of such fragments in these preparations. This assumption is quite valid since 5s RNA does not contain any minor bases, and does not have a 5' terminus of guanosine diphosphate (Schleich and Goldstein, 1966).

4s RNA on the other hand contains 6 methyl adenosine (approximately 2 molecules/1000 nucleotides), and pseudouridine is the most abundant minor nucleotide of E. coli 4s RNA preparations. The major 5' end-group of E. coli 4s RNA is also 3' (2'), 5' guanosine diphosphate. Finally, since the 3' (2'), 5' guanosine diphosphate counts recovered in the nucleoside diphosphate region of the column represent

a mere 0.2% of the total counts, it seems quite feasible that these counts could have resulted from the much smaller 4s RNA (approximately 74 nucleotides/molecule). The presence of 3' (2'), 5' guanosine diphosphate in hydrolysates of rechromatographed E. coli 5s RNA was also observed by Schleich (Doctoral Dissertation, 1966), and represented 2.5% contamination of the 5s RNA preparations with 4s RNA.

Besides demonstrating the differences between elution region 2 RNA and the other E. coli RNAs, the results of the nucleotide analysis also allow some speculation to be made on the in vivo origin of this unique low molecular weight RNA. Since in vivo experiments (Goldstein, Kirschbaum and Roman, 1965) have shown that the synthesis of RNA in E. coli occurs in the 5' to 3' direction, and usually results in the presence of a 5' phosphorylated terminus, the occurence of a 5' phosphorylated end-group on elution region 2 RNA molecules can be interpreted as being indicative of the in vivo biosynthesis of this molecule.

Nucleoside diphosphates have also been found at the 5' terminus of 23s rRNA (pGp...), and 16s rRNA (pAp...) (Takanami, 1967). The 5' terminus of 5s RNA is occupied by a pyrimidine nucleoside diphosphate (pUp...).

3' End-Group Analysis - Snake venom phosphodiesterase digestions of ^{32}P labeled RNA isolated from elution regions 2 and 4 show that elution region 2 RNA possesses a 3'

terminus of uridine, with a penultimate nucleotide of cytidine (Figure 10). 4s RNA as expected possesses a major 3' nucleotide of adenosine (Figure 10). A chain-length of 142$\pm$ 10% is calculated for elution region 2 RNA, based on the release of uridine from the 3' terminus (Table 3b).

Molecular Weight Determination - Computer analysis of the data obtained from sedimentation equilibrium studies yield a molecular weight of 54,440$\pm$ 199 for elution region 2 RNA. A molecular weight of 41,860 was obtained for 5s RNA run under identical conditions (Table 4). When the molecular weight of elution region 2 RNA, as determined by ultracentrifugal analysis, is compared with that approximated from the chain-length of the molecule, very good agreement is observed, considering the dissimilarity of the methods employed (Table 4).

Optical Absorbance Studies - The discovery that RNAs, like DNA, are capable of hydrogen bond formation, and as a consequence can undergo helix-coil transitions, has stimulated considerable spectrophotometric investigation of these molecules. This approach has now reached a point where it can provide useful interpretations of detailed features of these single stranded polymeric molecules (Doty, et al. 1959). In the experiments to be described, thermally induced transition or "melting" has been used almost

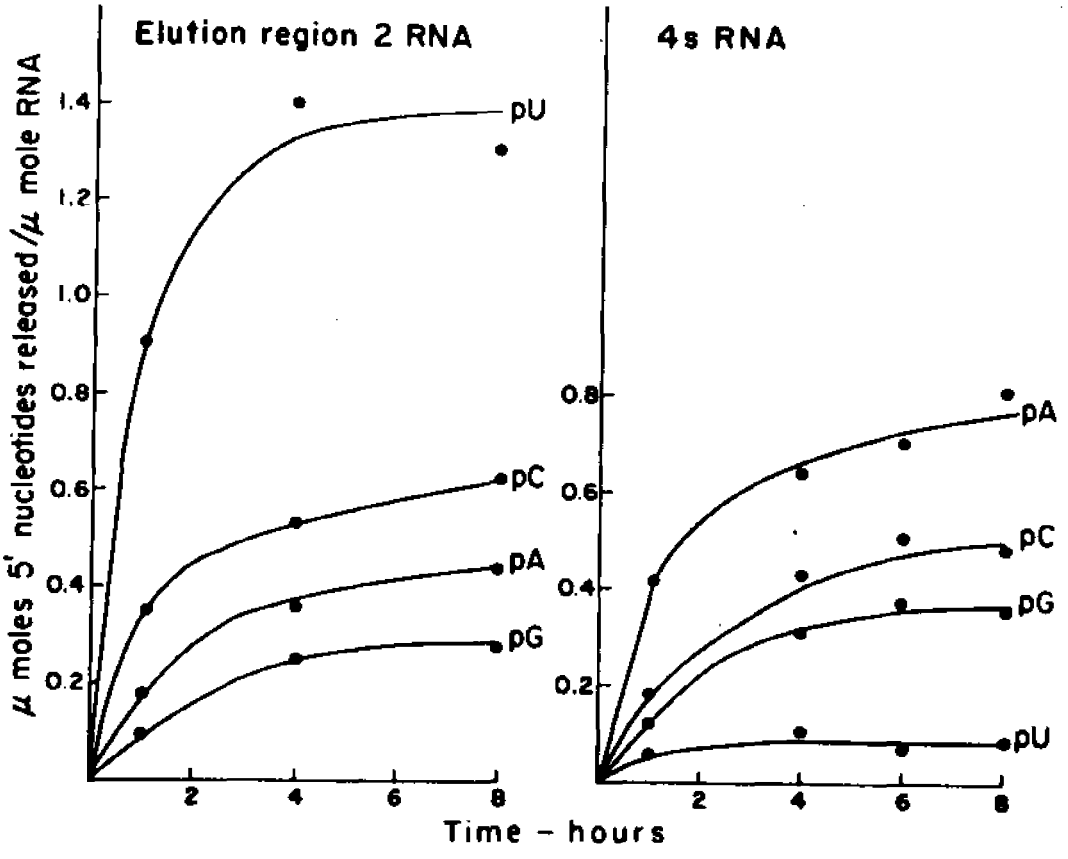

Figure 10. Kinetics of nucleotide release produced by the action of snake venom phosphodiesterase on ^{32}P labeled elution region 2 and 4s RNA. The umoles nucleotide liberated were calculated from the specific activity and the chainlength of each fraction.

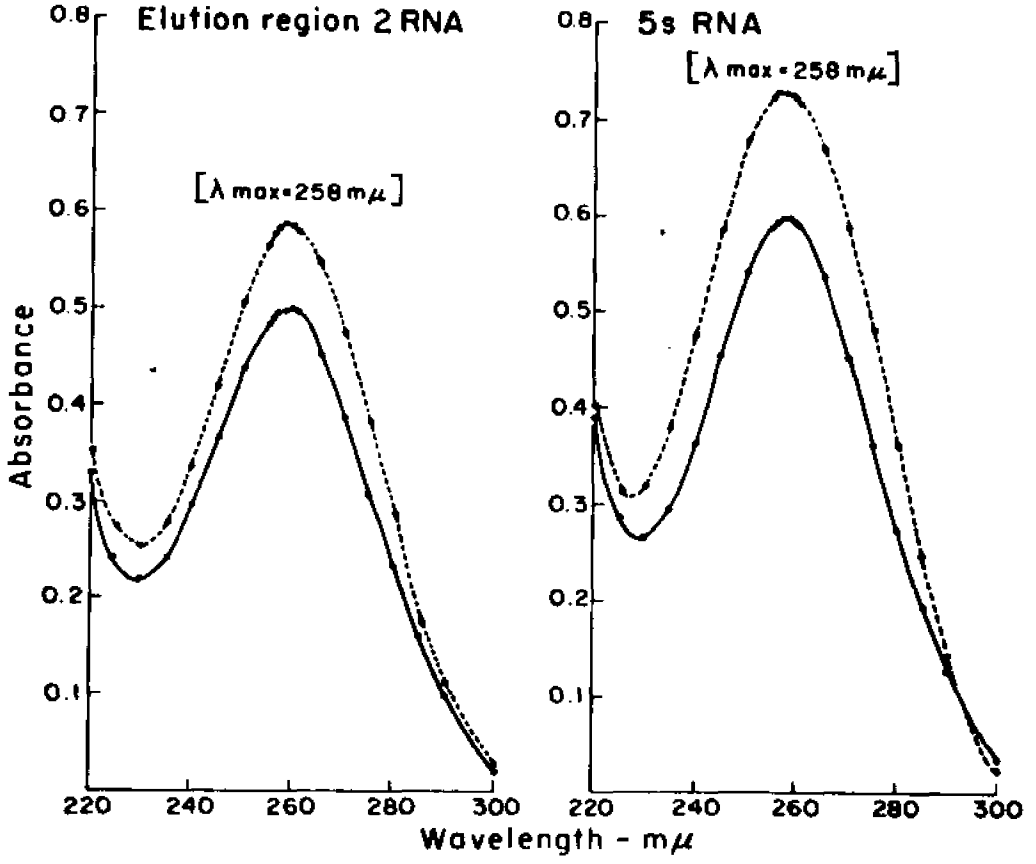

Figure 11. Absorption spectra of elution region 2 and 5s RNA at 15°C (——), and 85°C (----), in 0.09 M Na_2HPO_4 + 0.01 M NaH_2PO_4 buffer (pH 7.5).

RNA	Molecular weight (sedimentation analysis)	Molecular weight (calc. from chainlength)
Elution Region 2	54,440 $\pm$ 199	148 x 330 = 48,840 142 x 330 = 46,860
5s	41,860 $\pm$ 199	120 x 330 = 39,600

Table 4. Comparison between calculated molecular weight based on chainlength, and molecular weight as determined by ultracentrifugal analysis of elution region 2 RNA and 5s RNA.

exclusively. The helix-coil transition of elution region 2 RNA was therefore studied spectrophotometrically, using the standard phosphate buffer of Boedtker (1967) (see Materials and Methods).

The absorption spectra of elution region 2 RNA and 5s RNA were first measured at 15°C and 85°C (Figure 11). Both RNAs show the characteristic absorption maximum at 258 millimicrons. An increase in absorption with temperature is observed for both RNAs, indicating that the helical regions are shifting to random coil conformations. The shift observed for elution region 2 RNA is smaller than that observed for 5s RNA (Figure 11), suggesting a greater stability of elution region 2 RNA to heat denaturation than 5s RNA. Figure 12 shows the "melting" profiles of elution region 2, and 5s RNA. These data provide not only an accurate assesment of the mid-point of the temperature transition (Tm), and hence the stability of the helical regions within the molecules, but also display the breadth and asymmetry of the transition as well. The Tm of elution region 2 RNA is calculated as 64.4°C. The Tm calculated for 5s RNA is 54.0°C. This result agrees very well with the Tm reported for E. coli 5s RNA by Boedtker (1967).

Since Tm is generally accepted as a measure of the relative stability of a series of helices in RNAs, then it

can be concluded that the hydrogen-bonded sequences of elution region 2 RNA are relatively more stable to heat denaturation than are those of 5s RNA.

One very interesting feature about the transition obtained for elution region 2 RNA is the biphasic nature of the profile (Figure 12). This can be contrasted with the more gradual transition of 5s RNA (also Figure 12) which is compatible with an arrangement of base pairs either in short helical regions, or at random. The broad, non-cooperative transition observed for elution region 2 RNA at lower temperatures can be explained by assuming that the melting of relatively short helices that vary in average base content, is occuring. Variation of the stability of the hydrogen bonds within these helices is also assumed. The sharper transition observed for elution region 2 RNA at higher temperatures indicates that regions with stronger base pairs - possibly G.C pairs - are being melted, and suggests less "heterogeneity" (in terms of distribution and types of base pairs) in these late melting regions. For both elution region 2 RNA, and 5s RNA it is evident that the helix-coil transition is reversible, and helicity is completely restored by simply cooling the samples (Figure 12).

Formaldehyde Reaction - Fraenkel-Conrat (1954) has shown that formaldehyde reacts with the amino-groups of cytidine,

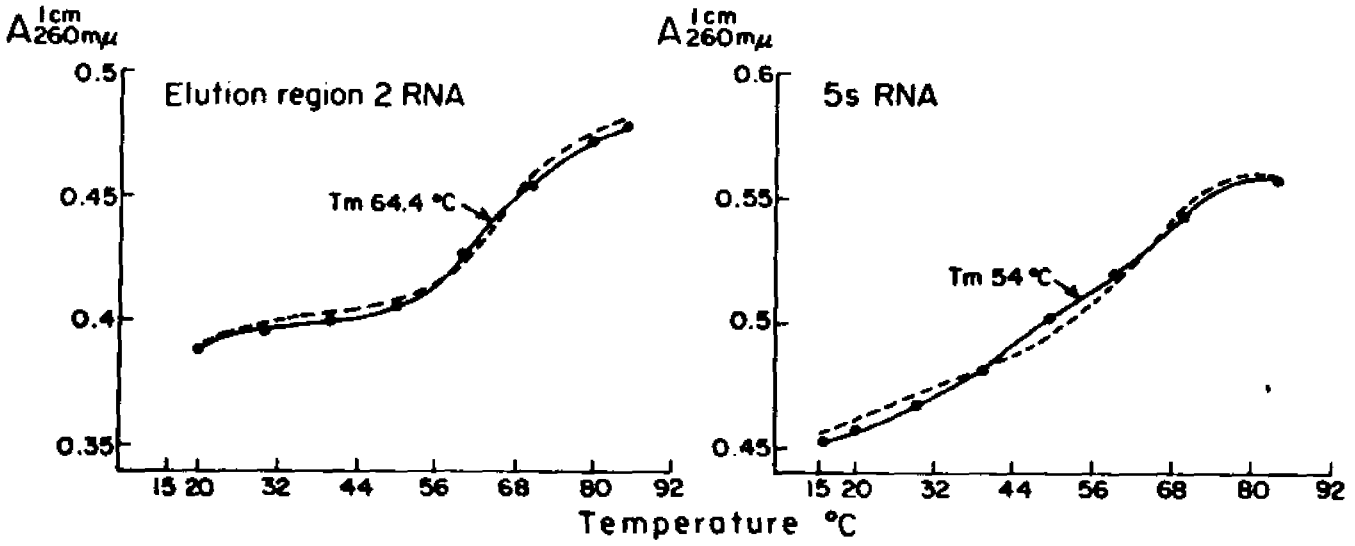

Figure 12. Absorbance Temperature profiles of elution region 2 and 5s RNA in standard phosphate buffer (——) heating profile; (----) cooling profile.

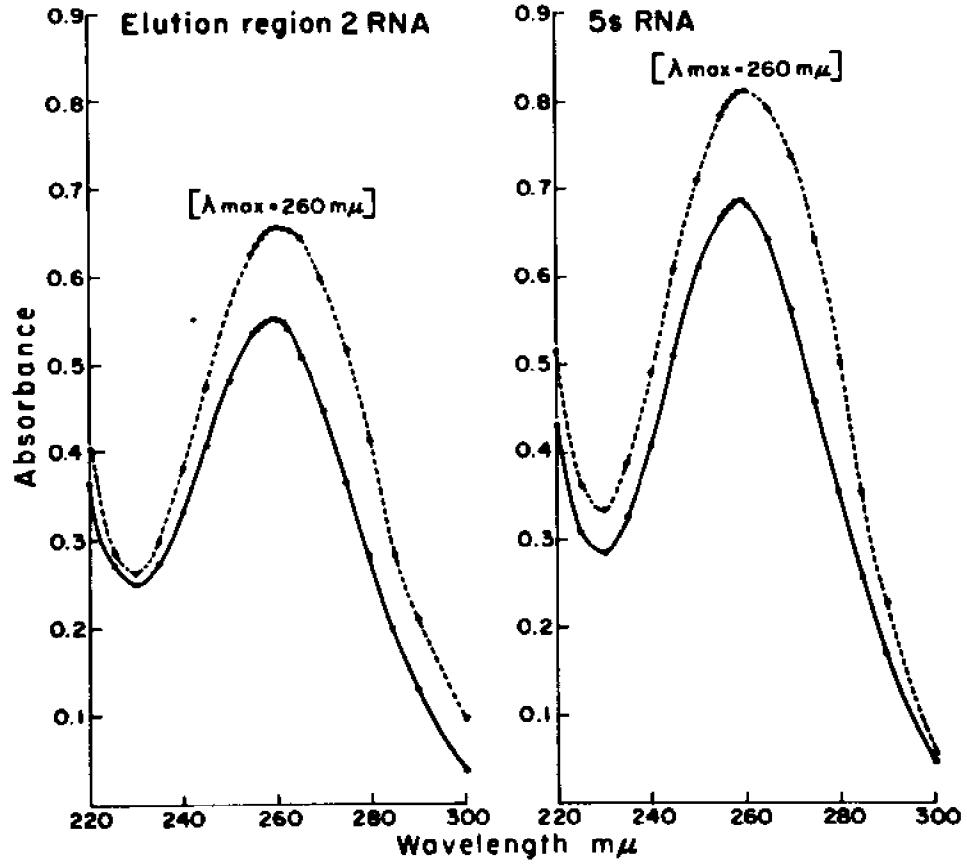

Figure 13. Absorption spectra of elution region 2 and 5s RNA at 15°C (——), and 85°C (----), in standard phosphate buffer containing 1.1 M formaldehyde.

adenosine, and guanosine, and that the reaction can be followed spectrophotometrically. Hydrogen bonds involving these amino-groups not only retard this reaction, but even limit it (Fraenkel-Conrat, 1954). Stevens and Rosenfeld (1966) have studied the reaction of formaldehyde with nucleotides and have reported on the formation of intermolecular methylene bridges as a result of formylation. However, this cross linking process proceeds at a very slow rate, and has been shown by these workers to take place far too slowly to be significant under the experimental conditions employed to study the helicity of RNAs.

Utilizing the formylation reaction, and employing conditions in which the formation of intermolecular methylene bridges does not occur (low formaldehyde concentration, short reaction time), Boedtker (1967) has carried out an extensive analysis of the secondary structure of 5s RNA. The results of her studies have been well substantiated by Cantor (1968) who employed an entirely different analytical method (Optical Rotatory Dispersion) to study the secondary structure of 5s RNA.

When elution region 2 RNA was reacted with 1.1 M formaldehyde in standard phosphate buffer (pH 7.5), and the absorbance spectrum observed, a slight shift in the absorbance maximum to longer wavelength is observed (Figure 13). This shift is accompanied by an increase in absorption

which reflects the extent of the formaldehyde reaction with the primary amino groups. A similar result was obtained when 5s RNA was reacted with 1.1 M formaldehyde (Figure 13).

Since the duration of the formaldehyde reaction is critical for minimizing side reactions, the extent of reaction of elution region 2 RNA with 1.1 M formaldehyde at 85°C was studied as a function of time. The kinetics of reaction of elution region 2 RNA with formaldehyde are shown in Figure 14. The RNA solutions were incubated at 85°C for the desired time interval, fast-cooled, and the absorbance measured at 15°C at a wavelength of 270 millimicrons, the wavelength at which the maximum total change in absorbance occurs after formaldehyde reaction (Boedtker, 1967). Although there is a small increase in absorbance at the later time points (Figure 14), the reaction is essentially complete after 15 minutes. The increment in absorbance observed at times greater than 15 minutes could have resulted from an increase in the effective formaldehyde concentration caused by the conversion of the dihydrate to the free aldehyde. Boedtker (1967) has also reported that the reaction of E. coli tRNA with 1.1 M formaldehyde is complete after 15 minutes at 85°C.

The denaturing effects of formaldehyde on elution region 2 RNA and 5s RNA was next studied by observing the relative depression of the Tm of these 2 RNAs in the

presence of 1.1 M formaldehyde. Figure 15 shows the transition profiles obtained. The Tm of both RNAs is significantly depressed. Elution region 2 RNA now has a Tm of 48.4°C, representing a depression of 16°C, while the Tm of 5s RNA is depressed 10.3°C to 43.7°C. This sharp depression of the Tm of elution region 2 RNA, compared to that observed for 5s RNA, clearly indicates that the denaturing effects of formaldehyde, as well as the subsequent reaction with the freed amino groups, occurs in a more cooperative fashion (particularly at higher temperatures) for elution region 2 RNA than for 5s RNA. This result is consistent with the earlier finding that elution region 2 RNA contains late melting regions of helicity, in which the base-pairs are less "heterogeneous", and which are now readily destabilized by formaldehyde.

Examination of the cooling profiles obtained when formaldehyde reacted samples were allowed to slow-cool clearly shows the absence of reannealing, since the formaldehyde reacted groups are incapable of reforming hydrogen bonds (Figure 15).

When elution region 2 RNA, and 5s RNA samples were heated to 85% for 15 minutes in the presence of 1.1 M formaldehyde and standard phosphate buffer, rapidly cooled, and the absorbance/temperature profiles measured, both RNAs retain the typical sigmoid curve characteristic of non-cooperative "melting", revealing the instability of the

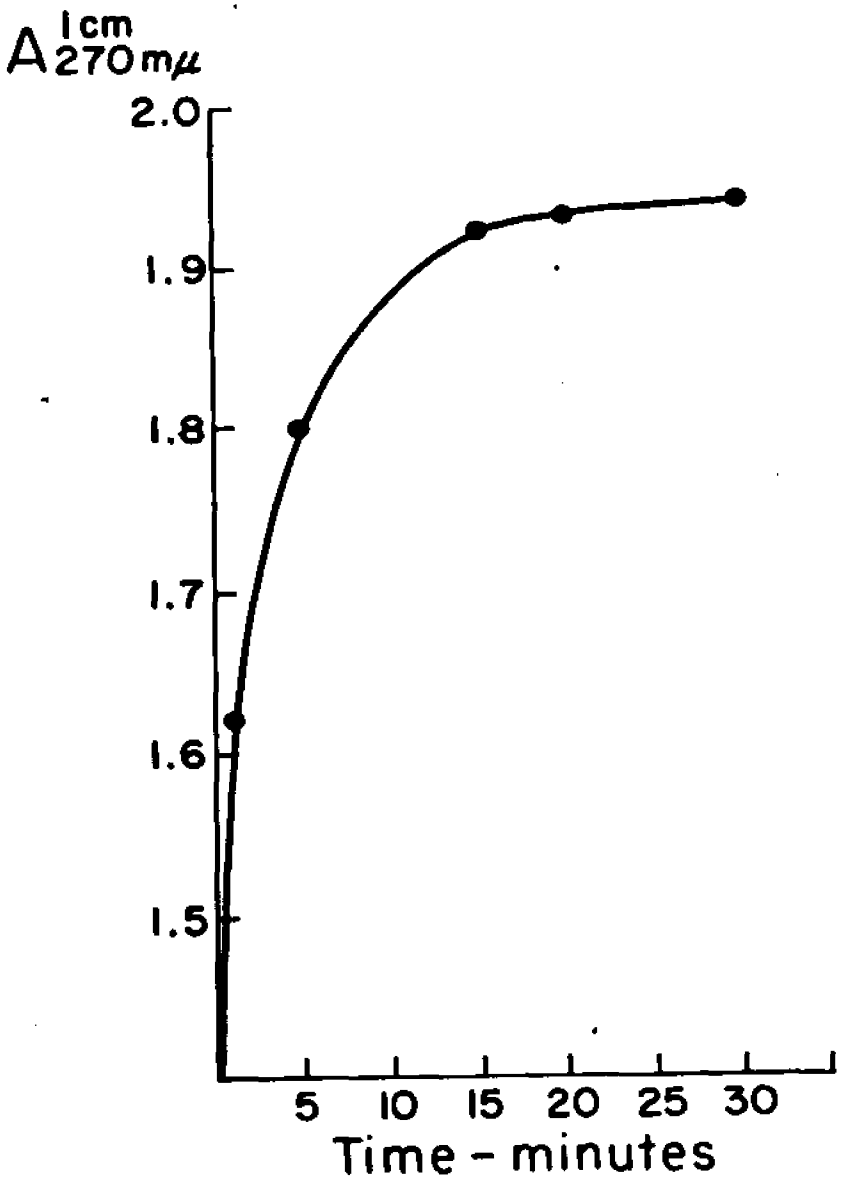

Figure 14. Kinetics of reaction of 1.1 M formaldehyde with elution region 2 RNA (85°C) in standard phosphate buffer. Absorbance measured at 15°C.

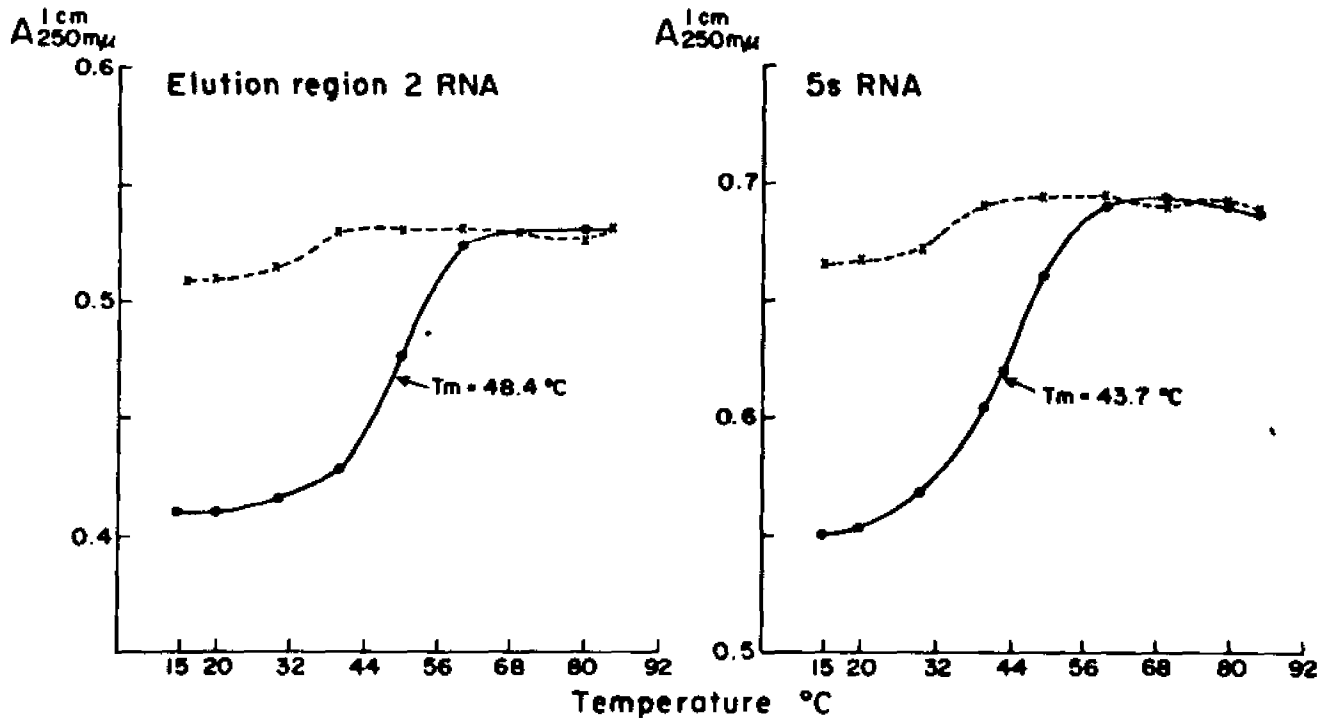

Figure 15. Absorbance Temperature profiles of elution region 2 and 5s RNA in standard phosphate buffer containing 1.1 M formaldehyde (———) heating profile; (----) cooling profile.

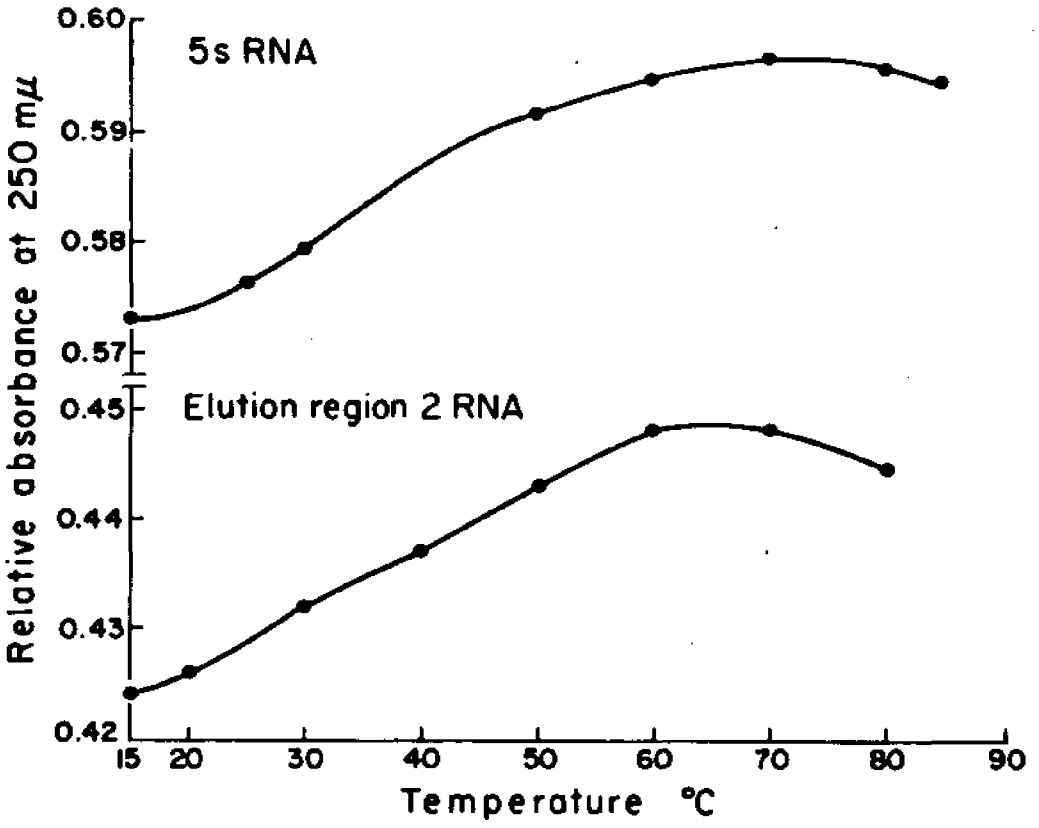

Figure 16. Absorbance Temperature profiles of elution region 2 and 5s RNA after reaction with 1.1 M formaldehyde (85°C) for 15 minutes in standard phosphate buffer.

$HT = (1 - x)Hss + x\ Hds$

HT = total hypochromicity
Hss= hypochromicity due to single stranded regions
Hds= hypochromicity due to double stranded regions
x = fraction of nucleotides involved in helical regions

Elution region 2 RNA

HT = .207
Hss= .062
Hds= .3 (Boedtker, 1967)
x = .61
Helicity = 61%

5s RNA

HT = .204
Hss= .063
Hds= .3
x = .60
Helicity = 60%

Table 5. Hypochromicity calculations for elution region 2 and 5s RNA.

"stacked" base regions at higher temperatures, after formaldehyde denaturation has occured (Figure 16). A similar observation was also made by Boedtker (1967) for E. coli 5s RNA.

Hypochromicity Studies - The double helical content of elution region 2 RNA was next examined by determining the hypochromicity of unreacted, and formaldehyde reacted RNA. Hypochromicity - defined as $1 - \frac{\text{Absorbance } 15°C(255 \text{ mu})}{\text{Absorbance } 85°C(255 \text{ mu})}$ - has been utilized by Boedtker and Kelling (1967) to determine the helical content of 5s RNA. Since the total hypochromicity observed for an RNA molecule is assumed to be the sum of the contributions from helical as well as single stranded "stacked" regions, then the fraction of the bases in helical regions can be derived from the relationship - $Ht = (1 - x)Hss + xHds$ - where Ht is the total hypochromicity of the unreacted RNA; Hss is the hypochromicity resulting from single stranded regions; and Hds is the hypochromicity due to helical or double stranded regions. The fraction of the bases involved in base-pairing is designated by x.

Utilizing the relationship given above the hypochromicity of unreacted and formaldehyde reacted elution region 2 RNA is calculated as 0.207 and 0.062 respectively (Table 5). The fraction of bases (x) involved in helical

regions in elution region 2 RNA is calculated to be 0.61. A value of 0.60 was obtained for the helicity of 5s RNA, this result agrees very well with the results obtained by Boedtker and Kelling (1967) for E. coli 5s RNA (0.63).

SUMMARY

The nucleotide composition of elution region 2 RNA has been studied by utilizing ion exchange column chromatography on Dowex resins. Analysis of alkaline hydrolysates of both labeled and unlabeled samples of elution region 2 RNA showed clearly that the distribution of the bases in this molecule is different from that observed for 5s RNA, as well as the other E. coli soluble and ribosomal RNA species.

Uridine 3' (2'), 5' diphosphate is the major 5' end-group found for elution region 2 RNA, and based on the recovery of this base from hydrolysates, a chain-length of 148 nucleotides was calculated for this molecule. This result agrees very well with the result obtained for the chain-length when the 3' end-group is released by snake venom phosphodiesterase. Uridine is also the major group released from the 3' end by the enzyme.

The molecular weight of elution region 2 RNA as determined by sedimentation equilibrium analysis is 54,440, and shows general agreement with the molecular weight

calculated from the chain-length of the molecule (Table 4).

Optical absorbance studies carried out in the presence and absence of 1.1 M formaldehyde showed that elution region 2 RNA (Tm 64.4°C) is more stable to heat denaturation than is 5s RNA (Tm 54.0), and that this added stability can be attributed to the presence of homogeneous base-paired regions within the molecule which show evidence of cooperative melting at higher temperatures (Figure 14a and 14b).

Observation of the kinetics of reaction of elution region 2 RNA with 1.1 M formaldehyde showed that the reaction is essentially complete after 15 minutes at 85°C (Figure 16).

When the Tm of elution region 2 RNA and 5s RNA was studied in the presence of 1.1 M formaldehyde, a depression of 16°C was observed for elution region 2 RNA, compared to a depression of 10°C for 5s RNA. This result clearly reflects the difference in the distribution of the helical regions within these molecules.

Finally, hypochromicity studies carried out on elution region 2 RNA showed that this molecule, like 5s RNA, has 60% of its structure in helical conformation.

CHAPTER FOUR

Metabolic Studies

INTRODUCTION

The possibility that elution region 2 RNA, shown in Chapter 2 to be isolated from the cytoplasmic cellular fraction, could play a role in protein synthesis, was tested by examining the extent of its participation in the amino acid activation, and chain termination steps of protein synthesis. A simple "charging" assay was utilized to test for amino acid acceptor activity in elution region 2 RNA. Participation of elution region 2 RNA in chain termination was studied by measuring the ability of this RNA to bind specifically to termination codons contained in the random copolymer UAG, in the presence of ribosomes.

To further explore the origin of elution region 2 RNA, and in particular to rule out the possibility of this RNA being a degradation product of the rRNAs, the biosynthesis of elution region 2 RNA was studied. This was accomplished by employing exponentially growing "fragile" cultures of E. coli B (doubling time 120 minutes) in which the stable RNA species were labeled for 2 generations with ^{14}C uracil, and the synthesis of new RNA was measured by following the flow of ^{3}H uracil into the various RNA species.

The results of these metabolic studies will be presented in this Chapter.

MATERIALS AND METHODS

Amino Acid Activation Assay

Isolation of Enzymes - The amino acid synthetases used to check the acceptor activity of Elution region 2 RNA were isolated from E. coli B cells in the following manner: E. coli B cells were ruptured in a homogenizer (500,000 rpm, 15 min. at 4°C), and 1.0 ml of the following buffer added per gram of cells present - 10 mM magnesium chloride, 25 mM potassium chloride, 35mM Tris HCl (pH 7.8 at 4°C), 0.25 mM sucrose, and 6 mM mercaptoethanol (added just before use). The broken cells were centrifuged at 12,000 x g for 30 min., and 2 ug DNAse added per ml of viscous supernatant. The supernatant was centrifuged (1 hr. at 12,000 x g) to remove cell debris, transferred to a preparative ultracentrifuge, and centrifuged for 2 hours at 30,000 x g. The resulting supernatant was divided into 2 equal fractions (approx. 20 ml each), and chromatographed on Sephadex G 25 (coarse beads, 2 x 30 cm column). The elution buffer was the same as the extraction buffer. The yellowish material, plus 10 ml after, was collected in 1.0 ml fractions, and immediately frozen. Rapid freezing was found to be necessary since slow freezing always resulted in the inactivation of some of the enzymes. The protein concentration of the preparation

was usually 11 mg/ml.

^{14}C Amino Acids - The ^{14}C amino acids used had the following specific activities: L-Aspartic acid 130 mc/mM; L-Leucine 175 mc/mM; L-Valine 142.8 mc/mM; L-Arginine 191 mc/mM; L-Phenylalanine 255 mc/mM; L-Threonine 120 mc/mM. The reconstituted ^{14}C protein hydrolysate contained the following amino acids: L-Alanine 70 mc/mM; L-Arginine 130 mc/mM; L-Aspartic acid 110 mc/mM; L-Glutamic acid 165 mc/mM; L-Isoleucine 110 mc/mM; L-Leucine 130 mc/mM; L-Lysine 180 mc/mM; L-Phenylalanine 168 mc/mM; L-Proline 110 mc/mM; L-Serine 85 mc/mM; L-Threonine 100 mc/mM; L-Tyrosine 150 mc/mM; and L-Valine 100 mc/mM.

"Charging" Buffer - The constitution of the buffer used in the "charging" experiments was as follows: 50 mM imidazole (pH 7.5); 5 mM adenosine triphosphate (potassium salt) neutralized with 1.0 M potassium hydroxide; 2 mM dithiothreitol; 20 mM potassium chloride; 13 mM magnesium chloride dissolved in 50 ml of distilled water. The pH was adjusted to 7.5 with 1 N hydrochloric acid.

RNA - All RNA samples were purified by Sephadex G 100 rechromatography. The concentration of sample used was usually 10.0 micrograms of RNA per microliter of 50 mM

imidazole buffer (pH 7.3) containing 13.5 mM magnesium chloride.

Assay Mixture - The assay mixture usually consisted of "charging" buffer, RNA, enzyme extraction buffer, enzymes and amino acids in the appropriate concentrations, all in a total volume of 0.525 ml. The samples were incubated for 30 minutes at 25°C. At the end of the incubation period, the samples were immediately placed on ice, and 50 ul aliquots removed, and pipetted (in triplicate) onto 25 mm filter paper discs (Whatman #1). The soaked discs were then immediately saturated with 50 microliters of cold trichloroacetic acid (10% solution) containing 1.0 mg/ml of the unlabeled amino acid(s) being tested. The samples, precipitated on the discs, were then stored at 4°C for 15 minutes. The discs were washed 3 times (5 minutes each time) with 5% trichloroacetic acid, and finally with an alcohol/ether solution (1:1) for 10 minutes. The dried discs were placed in scintillation vials, 10 ml of toluene scintillation fluid added, and the radioactivity measured utilizing a liquid scintillation counter.

Binding Experiments

^{14}C Phenylalanyl-tRNA Preparation - E. coli B tRNA, obtained from Sephadex G 100 column fractionation of crude sRNA

samples was used to prepare ^{14}C phenylalanyl-tRNA. ^{14}C phenylalanine with specific activity 255 mc/mM was obtained commercially. The assay mixture used for "charging" the tRNA with phenylalanine was identical to that described for the amino acid activation assay. The volume of the reaction mixture used was however scaled up two-fold.

After incubation of the reaction mixture, the samples were chilled, and 1.0 ml of water saturated phenol added. The mixture was shaken vigorously for 10 minutes, and centrifuged. The aqueous layer was pipetted off, washed 4 times with 2 volumes of ether, and after removal of the ether with nitrogen, precipitated with 0.1 volume of potassium acetate (20%), and 2.5 volumes of ethanol. The precipitate was stored overnight at -10°C. The RNA was finally collected by centrifugation, and stored until ready for use.

^{14}C Labeled Elution Region 2 RNA - The E. coli ^{14}C labeled elution region 2 RNA was prepared from cells grown to late log phase in 0.1% Difco nutrient broth containing 1.0 uc/ml ^{14}C uracil. Sephadex G 100 fractionation yielded the 4 elution regions. Only the peak tube from elution region 2 was used in these experiments.

Binding Assay - The binding assay used was essentially that of Nirenberg and Leder (1964). Each 50 ul reaction mixture

contained 0.1 M Tris-acetate (pH 7.2); 0.02 M magnesium acetate; 0.05 M potassium chloride; 2.0 A_{260} units of E. coli B ribosomes (washed 3 times); and, as indicated for each experiment, polyuridylic acid, or the random copolymer UAG, comprised of equimolar amounts of U, A, and G. ^{14}C labeled elution region 2 RNA, and ^{14}C phenylalanyl-tRNA were used as the RNA components. The reaction mixture was kept at 24°C, and the labeled RNA added last to initiate the binding. Incubation was usually carried out for 20 min. After incubation the tubes were placed on ice, diluted with 3.0 ml of buffer containing 0.1 M Tris-acetate (pH 7.2), 0.02 M magnesium acetate, and 0.05 M potassium chloride. The diluted reaction mixture was then filtered under suction, and washed 3 times with 3 ml portions of the diluting buffer, and finally dried in an oven. The radioactivity of each disc was determined on a liquid scintillation counter, utilizing toluene scintillation fluid.

Kinetic Studies

Growth of Cells - Fragile cultures of E. coli B were produced by growing cells in the following medium: potassium monohydrogen phosphate, 1.12 g: potassium dihydrogen phosphate, 0.48 g: ammonium sulfate, 0.5 g: magnesium sulfate, 7 H2O, 0.49 g; Ferric chloride. 0.51 g; and glucose, 2 g. 0.5 M sodium sulfate was used to induce fragility.

Peptone Medium - The peptone medium used consisted of 10.0 grams of peptone (Difco), 5.0 grams of sodium chloride, and 1.2 grams D-glucose per liter of water.

Nutrient Broth - Nutrient broth had the following composition per liter: 8.0 grams of nutrient broth (Bacto, dehydrated), 5.0 grams of glucose, and 18.0 grams of agar (Difco).

Monitoring The Growth - Growth of cultures was monitored by recording the change in optical density at 540 mu, as well as by determining the viable cell count, obtained by plating cells on nutrient broth.

Labeling of RNA - 180 ml of "fragile" medium were inoculated with E. coli B cells from a freshly grown culture as follows: 20 ml of cells grown in peptone were spun down at 6,000 x g for 10 minutes, and resuspended in 20 ml of fresh peptone medium. The suspended cells were immediately added to the flask containing the "fragile" medium. 1.0 ml of ^{14}C uracil (100 uc) of specific activity 0.489 uc/ug, was added to the flask, and the cells allowed to grow for two generations (4 hours approximately). After 2 generations of growth, 3.2 ml of ^{3}H uracil, of specific activity 183 uc/ug, were added (1.6 mc). Aliquots of the culture were removed at the desired time intervals, and the flow of ^{3}H

label into the various RNA components determined.

Removal of Aliquots - 25 ml aliquots were removed from the culture at the following time intervals: zero time (immediately prior to ^{3}H uracil addition), 20 seconds, and 1, 2, 4, 6, and 8 minutes. For each time point, growth of the cells was completely arrested by transferring the aliquot of cells to a flask containing crushed ice, and 0.25 ml of M potassium cyanide.

RNA Isolation - The cells were harvested by centrifugation, and mixed with approximately 300 grams of unlabeled cells. Total RNA was isolated by the phenol procedure described in Chapter 2. Cold M sodium chloride was used to separate the sRNA from the high molecular weight rRNA. The sRNA fractions were precipitated twice with ethanol (95%) prior to Sephadex G 100 fractionation (see Figure 17).

Sephadex G 100 Fractionation of sRNA - The sRNA samples were fractionated on Sephadex G 100 columns (2.5 x 150 cm), using M sodium chloride as the eluting buffer. 4.0 ml fractions were collected for each analysis.

Fractionation of 1 M Sodium Chloride Insoluble RNA - 1 M sodium chloride insoluble RNA preparations were fractionated

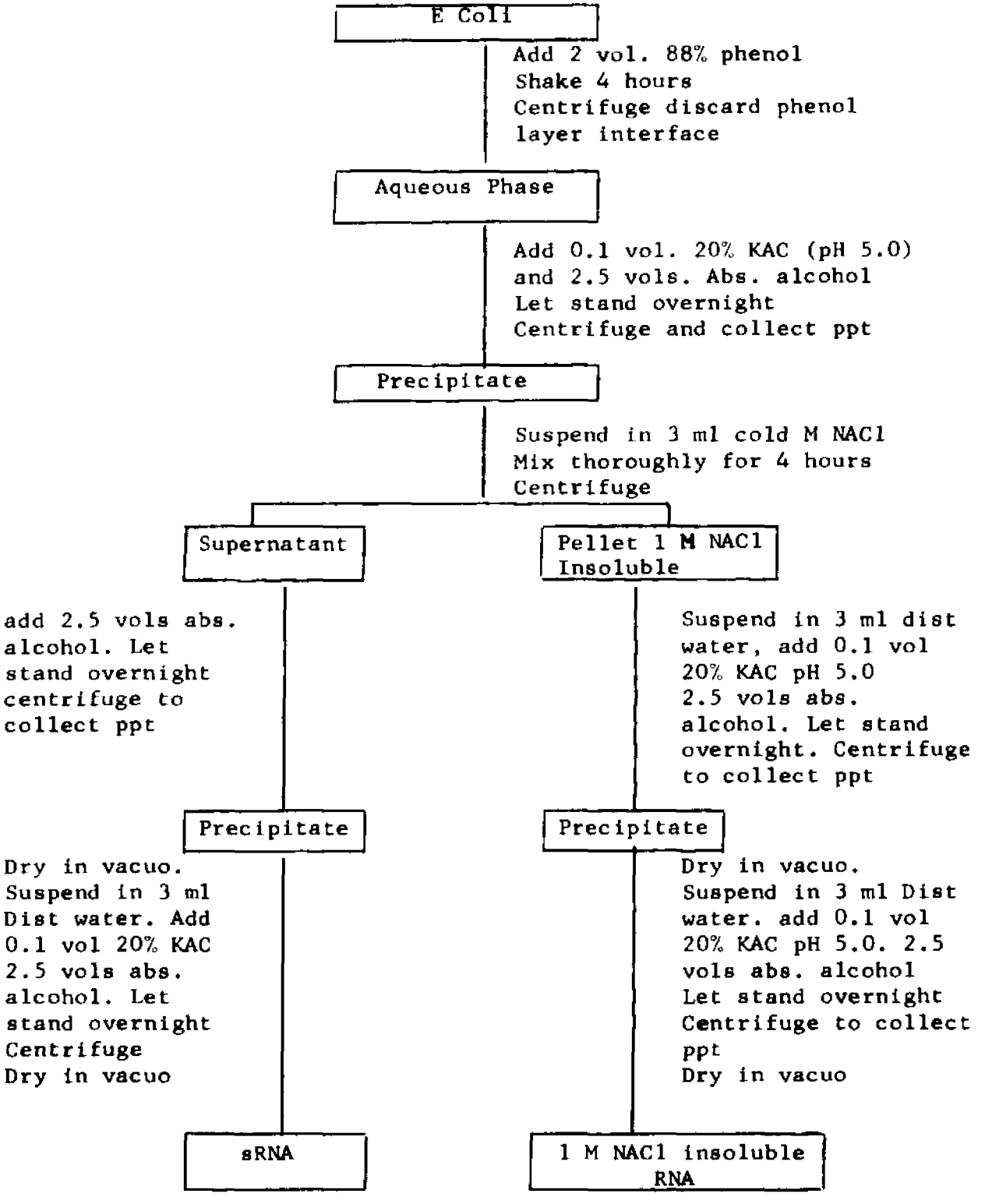

Figure 17. Flow Sheet for Preparation of sRNA and 1 M NACl Insoluble RNA From Fragile ^{14}C ^{3}H labeled E Coli Cells

on methylated albumin keiselguhr (MAK) columns, utilizing the procedure of Dr. Goldstein (unpublished), and with the expert technical assistance of Miss R. Sipl.

Gradient Procedure Employed in MAK Column Fractionation - A gradient maker containing 180 ml of 0.4 M sodium chloride, 0.05 M sodium phosphate monobasic buffer (pH 6.7) in the mixing chamber, and 180 ml of 1.2 M sodium chloride, 0.05 M sodium phosphate monobasic buffer (pH 6.7) in the reservoir, was used to produce a linear gradient. The flow-rate of the column was adjusted to 78 ml/hr with a peristaltic pump. Fraction volumes of 2 ml were collected for analysis. The ultraviolet absorption of each RNA preparation was monitored on an analyzer at 254 mu.

Assay of Radioactivity - For analysis of the ^{3}H and ^{14}C radioactivity in the column effluent, the following procedure was used: 2 ml aliquots were taken from each fraction, and 0.5 mg of bovine serum albumin added, followed by 0.5 ml of trichloracetic acid (20%). The precipitate was allowed to settle (20 minutes) before filtering onto a glass filter disc. Each disc was washed 3 times with 5% trichloroacetic acid, dried in an oven at 90°C, and counted in a liquid scintillation counter using 10 ml of

toluene scintillation fluid. The ^{3}H and ^{14}C activities were determined simultaneously. Quenching was found to be uniform throughout the counting ranges examined. The counting conditions used gave efficiencies for ^{3}H and ^{14}C of 43% and 71%, respectively, with 9% of the ^{14}C activity contaminating the ^{3}H channel. Less than a half of 1% of the ^{3}H activity appeared in the ^{14}C channel.

RESULTS

Amino Acid Activation Assay - In order to test the activity of the amino acid synthetases, ^{14}C labeled amino acids were first reacted with tRNA. The components of the charging reaction mixture are given in Table 6. The data presented in Table 7 demonstrate the ability of tRNA to accept amino acids under these experimental conditions.

When elution region 2 RNA was tested under the same conditions, no incorporation of radioactivity into the trichloroacetic acid precipitable material is observed.

The use of a ^{14}C protein hydrolysate permitted elution region 2 RNA to be tested with a wider variety of amino acids. When the assay was carried out with such a hydrolysate, no activity is again observed for elution region 2 RNA (Table 7). These findings clearly show that elution region 2 RNA is unable to participate in the "charging" reaction of protein synthesis.

Binding Studies - The termination of protein synthesis in E. coli has been studied in numerous laboratories. Results of genetic (Brenner et al. 1965) and biochemical (Bretscher, 1968) studies show that the synthesis of a peptide chain is terminated, upon translation of the mRNA codons UAA, UAG, or UGA. Capecchi (1967) has shown that release (R) factors are required for the recognition of these terminator codons. Although the substrate of the R factors seems to be the ribosome-mRNA-peptidyl tRNA complex, yet it is not clear whether the mechanism of action of the R factors involves the participation of another molecule. Such a molecule would read the codon, but would be unable to carry an amino acid. Scolnick et al. (1968) have, in fact, suggested that other molecules, possibly RNAs, could interact with the R factors, which could then act as translators of the terminator codons.

Since many investigators have searched in vain for a tRNA species that could fulfill the above role, it seemed worthwhile to investigate the ability of elution region 2 RNA to "recognize" the terminator signals. A simple binding experiment was therefore carried out, involving ribosomes, elution region 2 RNA, and a synthetic messenger - poly UAG - containing terminator codons in random distribution.

The assay used measures the binding of ^{14}C labeled

"charging" buffer	RNA	Enzyme	Enzyme buffer	Amino acid
0.25 ml	0.25 ml (250 ug)	1.0 ml (1.6 mg)	-	0.15 ml (50 uc)

Table 6. Components of the reaction mixture used in the amino acid activation assay.

Amino acid	Specific activity	Total CPM accepted	
		Elution region 2	tRNA
L aspartic acid	130 mc/mM	-	31,060
L leucine	175 mc/mM	-	74,880
L valine	142 mc/mM	-	9,580
L arginine	190 mc/mM	196	20,180
L phenylalanine	255 mc/mM	-	71,810
L threonine	120 mc/mM	76	21,030
Protein hydrolysate	(see materials and methods)	-	67,240

Table 7. Amino acid activation studies on elution region 2 and tRNA.

Oligonucleotide	^{14}C phe-tRNA bound to ribosomes (uu M)	^{14}C elution region 2 RNA bound to ribosomes (uu M)
None	0.83	.038
UAG	1.18	.040
UUU	2.10	.040

Table 8. The binding of ^{14}C phenylalanyl-tRNA and elution region 2 RNA to polyuridylic acid, and the random copolymer UAG, in the presence of ribosomes. ^{14}C phe-tRNA (9.38 uuM) or elution region 2 RNA (9.1 uuM), 25 muM poly U or poly UAG, and ribosomes (2.0 A_{260} units) were incubated at 24°C for 20 minutes. Components of the reaction mixture are described in materials and methods page 91.

RNA to the ribosomes simply by determining the amount of radioactivity retained by the filters. Since cellulose nitrate filters have been shown to retain ribosomes, then the formation of a complex between ribosomes, messenger, and labeled RNA would be indicative of specific recognition of trinucleotide sequences by the RNA. Such specificity of binding has already been amply demonstrated by Nirenberg and Leder (1964).

The data of Table 8 show that when ^{14}C phenylalanyl-tRNA is incubated with ribosomes in the absence of polynucleotide, little radioactivity is retained by the filters. What little is retained represents non-specific binding of the RNA to the ribosomes. When the experiment is performed using the UAG copolymer , an increase in the amount of radioactivity bound to the filters is observed, indicating some association of the phenylalanyl-tRNA with trinucleotide sequences within the UAG chain. In the presence of polyuridylic acid however, a marked stimulation of the binding of ^{14}C phenylalanyl-tRNA to the ribosomes is observed.

The binding of elution region 2 RNA to the ribosomes was next tested in an assay system containing either random copolymer UAG, or polyuridylic acid. Table 8 summarizes the results obtained from these experiments. No significant retension of radioactivity by the filters is observed for either of the polynucleotides, demonstrating the inability

of elution region 2 RNA to bind to nucleotide sequences in these messengers.

The nature of the R factors had not been reported when these experiments were performed. It therefore could be argued that these proteins are necessary to promote the interaction of elution region 2 RNA with the terminator codons. However, the more recent experiments of Caskey et al. (1968) negate any role for an RNA molecule (particularly of the size of elution region 2 RNA) in chain termination.

Kinetic Studies - Most studies on the kinetics of RNA synthesis have been carried out on cultures whose growth was interrupted by the use of inhibitors. Under these conditions a wide variety of ribonucleoprotein particles accumulate. However, it has not been clearly established whether the RNA species contained in these particles represent a form of precursor that naturally exists within the cell, or whether they are anomalous by-products of the growth conditions.

The more direct studies of RNA biosynthesis carried out by Mc Carthy and Britten (1962), and Mangiarotti et al. (1968) simply follow the flow of a radioactive precursor into RNA chains, and have provided much information on the biosynthesis of E. coli ribosomal RNA. One attractive feature of this approach is that it allows for the separation

of precursor rRNA species from the mRNA fragments that had previously obscured much of these precursor species in sedimentation profiles.

Utilizing this approach, and employing "fragile" cultures of E. coli (doubling time 120 minutes), Mangiarotti et al. (1968) has shown that ribosomal precursor RNAs represent an array of incomplete RNA chains which sediment in a broad region between 4s and 16s on sedimentation analysis. These workers have also shown that the newly formed rRNA remains in the cytoplasm for at least 6 minutes prior to being incorporated into polysomes.

Since elution region 2 RNA is found in the cytoplasmic fraction (2% of the sRNA), it is quite possible that it could be a ribosomal precursor RNA which appears in the cytoplasm prior to being incorporated into ribosomes. In order to test this possibility, a thorough study of the kinetics of synthesis of elution region 2 RNA, and the other stable sRNA and rRNA species was undertaken.

"Fragile" cultures were used for these studies, and Figure 18 (inset) shows the growth profile observed from measuring the optical density (540 mu) of such a culture. The viable cell count (Table 9), measured by plating aliquots of the culture on nutrient broth, verifies that under these conditions of growth the cells are dividing exponentially (see also Mangiarotti and Schlessinger, 1966).

Sephadex G 100 and MAK column chromatography of the sRNA and rRNA samples isolated from cells grown for 2 generations in the presence of ^{14}C uracil (zero time) shows the typical distribution of the sRNA and rRNA species, respectively. The distribution of the 4 peak regions of sRNA (Figure 18a) is consistent with the distribution observed for unlabeled sRNA isolated from cells grown in non-synthetic medium (see Figure 4a). MAK column chromatography of the ^{14}C 1 M sodium chloride insoluble RNA (Figure 18b) shows the characteristic peaks of 16s and 23s rRNA. These species cochromatograph with unlabeled 16s and 23s rRNA (not shown).

The introduction of ^{3}H uracil to the culture immediately following the sampling of the zero time, allowed the synthesis of RNA chains to be followed over an 8 minute period in the presence of this isotope. This was done by simply calculating the ratio of ^{3}H to ^{14}C for each species of RNA. Figure 19a shows a plot of the data obtained when $^{3}H/^{14}C$ ratios of the crude sRNA ans 1 M insoluble RNA samples representing the various time intervals were calculated. It is quite evident that the rate of the ^{3}H uracil uptake by the 1 M sodium chloride RNA exceeds that observed for ths sRNA, throughout the labeling period. These data are consistent with the data of Mangiarotti et al. (1968) who showed similar labeling kinetics for E. coli sRNA and rRNA.

When the sRNA and rRNA preparations were resolved into

Time-hours	OD 540 mu	Cell count
0	.18	$.3 \times 10^8$
2	.40	$.7 \times 10^8$
4	.85	1.8×10^8

Table 9. Viable cell count obtained for "fragile" growing E. coli B (doubling time 120 minutes at 37°C).

Time	Elution regions sRNA				Elution regions rRNA	
	1	2	3	4	16s	23s
20 sec.	.209	.298	.181	.186	.245	.282
1 min.	.333	.733	.305	.238	.536	.643
2 min.	.615	1.393	.421	.347	1.369	.997
4 min.	1.529	3.226	1.050	.710	1.895	2.066
6 min.	3.266	6.130	2.028	1.174	3.922	3.831
8 min.	3.064	5.924	2.552	1.513	4.406	4.271

Table 10. $^{3}H/^{14}C$ ratios calculated for the various stable sRNA and rRNA species over an 8 minute labeling period.

Slope	Elution Regions sRNA				Elution Regions rRNA	
	1	2	3	4	16s	23s
Log/Log	.77	.88	.68	.52	.79	.75

Table 11. Slopes (log/log) of the various stable sRNA and rRNA species. Values derived from data in Table 10.

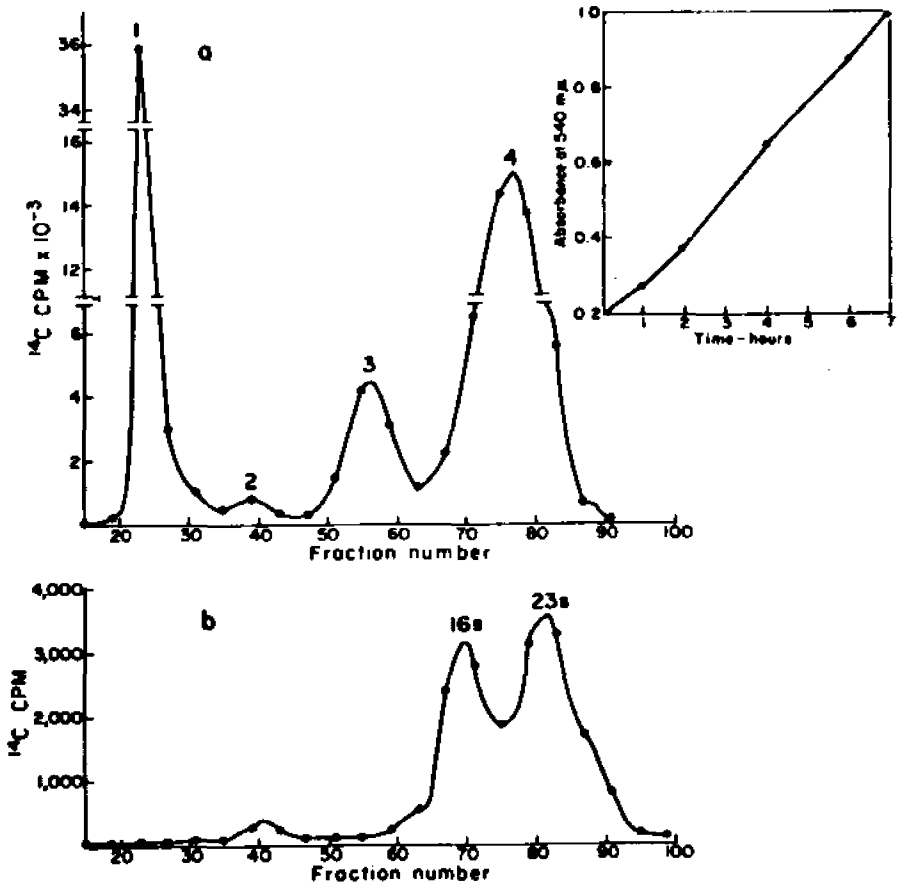

Figure 18. (a) Elution profile (Sephadex G 100) of ^{14}C labeled sRNA isolated from fragile E. coli cells.

(b) Elution profile (MAK) of ^{14}C labeled 1 M sodium chloride insoluble RNA isolated from fragile E. coli cells.

(inset) Typical growth curve obtained for fragile growing cultures.

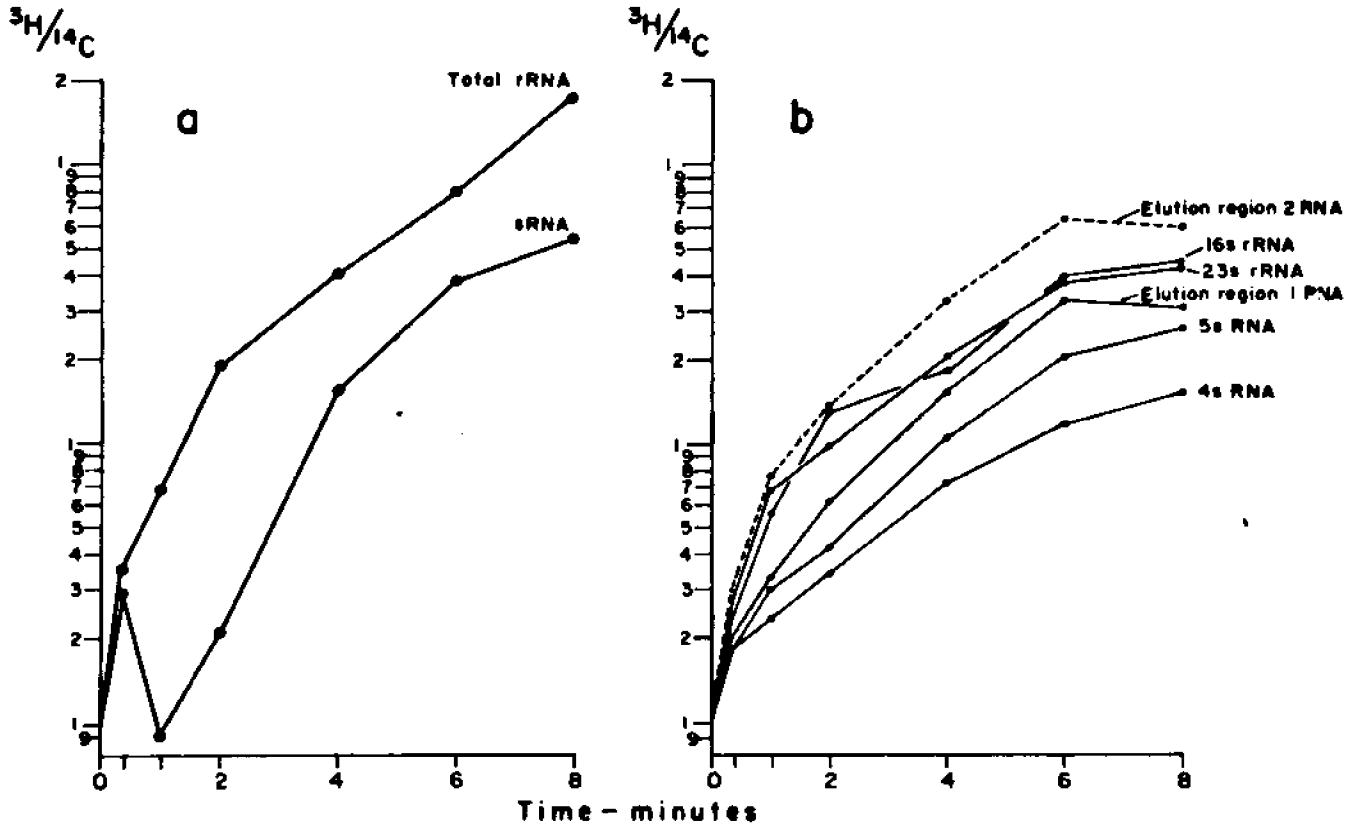

Figure 19. Plot of $^3H/^{14}C$ ratios calculated for RNA samples isolated from fragile cells in which the "old" RNA was labeled for 2 generations with ^{14}C uracil, and the "new" RNA labeled with ^3H uracil over an 8 minute period. (a) ratios obtained for unfractionated ribosomal and soluble RNA; (b) ratios obtained for the various ribosomal and soluble RNA fractions.

their constituent RNAs, a very marked variation is observed in the rate of synthesis of the different RNA species (Figure 19 and Table 10). These rates were determined by calculating the $^{3}H/^{14}C$ ratios for the peak tubes of each elution region. The curves obtained (Figure 19b) show that 20 seconds and 1 minute after the introduction of the ^{3}H uracil label, the rate of synthesis of elution region 2 RNA slightly exceeds that of 23s RNA, but is much greater than that of the other RNAs shown. After 2 minutes of labeling with ^{3}H uracil 16s RNA synthesis closely approaches that of elution region 2 RNA, but does not overtake it. The rapid rate of synthesis of elution region 2 RNA persists throughout the labeling period. The relative rates of synthesis of the other RNA species increasing in the following order: 4s RNA (which has the slowest rate), 5s RNA, elution region 1 RNA, 16s RNA, and 23s RNA.

Calculation of the slopes of the curves shown in Figure 19b yields values which again verify elution region 2 RNA to be the most rapidly synthesized RNA species (Table 11).

Since elution region 2 RNA displays the most rapid rate of synthesis throughout the ^{3}H labeling period, this RNA cannot be a degradation product of the higher molecular weight rRNAs. This conclusion is also supported by the results obtained from end-group analysis of this molecule.

Since the rate of RNA synthesis in bacterial cells is a product of the number of RNA chains under construction, and the rate at which nucleotide residues are added to each chain (Winslow and Lazzarini, 1969), then it must be assumed that either more chains are being initiated for elution region 2 RNA than for the other RNA species, or that the rate of addition of nucleotides to the growing chains of elution region 2 RNA exceeds that of the other RNA species. Much of the published data on RNA synthesis, however, are consistent with the notion that the rate of movement of RNA polymerase on DNA is similar from one cistron to another (see Mangiarotti et al. 1968). This then would favor the former possibility.

Pigott and Midgeley (1968) have shown, based on results obtained from hybridization experiments, that 70% of the rapidly labeled RNA in E. coli is rRNA precursor molecules. The other 30% in mRNA which is usually associated with the polysomes.

Although no definite precursor/product relationship can be established for elution region 2 RNA and the rRNAs (see curves Figure 19b), it is still tempting to conclude that elution region 2 RNA is a precursor to rRNA. This conclusion is based on two lines of evidence; the rapid rate of labeling observed for elution region 2 RNA, as well as the data obtained in Chapter 2 which suggest that elution

region 2 is a cytoplasmic RNA.

All of the ribosomal precursor RNAs observed in E. coli systems to date (Dahlberg and Peacock, 1970; Adesnik and Levinthal, 1969), are molecules with molecular weights similar to those of the mature rRNA species (based on electrophoretic mobility on polyacrylamide gels). Elution region 2 RNA, because of its size (54,440 daltons) could therefore be representative of a much earlier stage in the synthesis of the rRNAs.

On the other hand the possibility remains that elution region 2 RNA could be a species of mRNA which is soluble in cold M sodium chloride, and has somehow become unusually stabilized. Rapidly labeled low molecular weight RNAs have been isolated from other systems (King and Fitchen, 1968; Gardner and Hoagland, 1968), and it has been suggested that these molecules are possibly mRNAs which have become unusually stabilized by association with the cellular membrane.

Whether elution region 2 RNA is such a stable messenger released from the cellular membrane by the isolation procedure, is not known. However, its rapid rate of synthesis, coupled with its marked stability, certainly suggests a biological role for this molecule.

Although the labeled sRNA isolated from the "fragile" cells used in these experiments displays the same Sephadex

G 100 elution profile as that observed for sRNA isolated from cells grown in non-synthetic media, it nevertheless had to be clearly demonstrated that these RNA species behave the same electrophoretically. Peak tubes were therefore pooled from each elution region, and the RNA precipitated directly from M sodium chloride using absolute alcohol. The dried RNA was then dissolved in water (50 ul), and applied to 8% polyacrylamide gels for electrophoresis. Elution region 2 RNA migrates as a single peak (Figure 20a) displaying the same electrophoretic mobility as unlabeled preparations. The labeled 4s and 5s RNA samples also display their usual electrophoretic behavior on 8% polyacrylamide gels (Figure 20 b and c).

Electrophoresis of elution region 1 RNA (2.7% gels) reveals RNA peaks which appear to have similar mobilities to those observed for 16s and 23s rRNA, run under identical conditions (Figure 20 d and e). A similar result was obtained for unlabeled elution region 1 RNA by Valenti (unpublished data) who noticed that electrophoresis of freshly prepared, unlabeled elution region 1 RNA (material precipitated immediately following Sephadex G 100 chromatography) yields 2 RNA bands which display similar electrophoretic mobilities to the rRNAs. Valenti also observed that the stability of these 2 high molecular weight RNA species varies with the manner and duration of storage. If, for example, elution region 1 fractions were allowed to stand

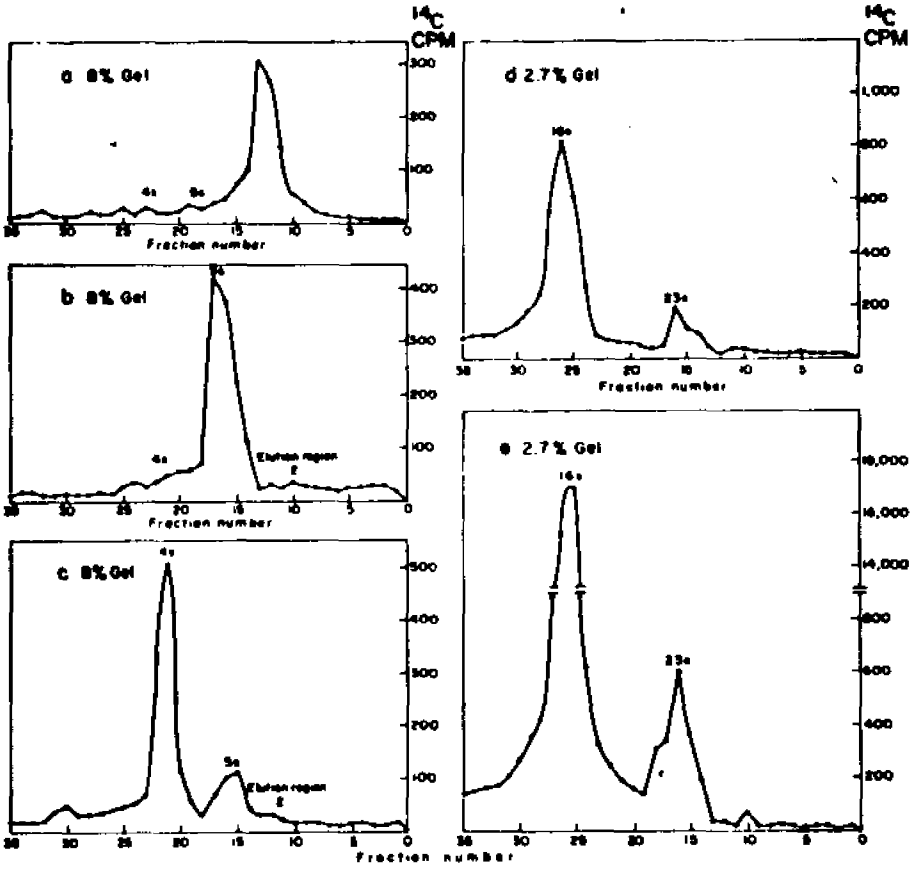

Figure 20. Polyacrylamide gel electrophoresis of ^{14}C labeled sRNA fractions. (a) elution region 2 RNA; (b) 5s RNA; (c) 4s RNA; (d) elution region 1 RNA. ^{14}C labeled 16s and 23s rRNA (obtained commercially) are shown in (e).

The rimmed gels were fractionated and the ^{14}C counts/minute determined for each fraction.

for a few days at 4°C in M sodium chloride, the material was completely degraded. On the other hand, immediate precipitation of the fractions after gel filtration, followed by storage of the dried precipitates at -10°C, slows up the degradation process, although it does not prevent it. The exact nature of the 2 high molecular weight components observed in elution region 1 RNA preparations remains to be determined.

SUMMARY

The ability of elution region 2 RNA to participate in the "charging" reaction of protein synthesis was investigated using a variety of ^{14}C labeled amino acids. No activity was detected for elution region 2 RNA, indicating that this RNA is unable to participate in the "charging" reaction.

The investigation of the possible involvement of elution region 2 RNA in polypeptide chain termination was next tested, using the simple binding assay of Nirenberg and Leder (1964). In this assay ^{14}C labeled elution region 2 RNA was tested for its ability to "recognize" specific terminator signals present in the random copolymer UAG. The results of these experiments showed that elution region 2 RNA does not bind in a complementary fashion to the UAG copolymer in the presence of ribosomes. Such complementarity

was clearly demonstrated when phenylalanyl-tRNA was mixed with ribosomes and polyuridylic acid. These results do not completely rule out a role for elution region 2 RNA in chain termination, although such a role seems less likely in view of the recent reports on the characterization of the release factors (Caskey et al. 1968).

The kinetics of labeling of elution region 2 RNA, and the other stable sRNA and rRNA species was next examined by introducing ^{3}H uracil to an exponentially growing "fragile" culture already labeled with ^{14}C uracil for 2 generations, and observing the flow of ^{3}H label into the various stable (^{14}C) RNA species. Elution region 2 RNA was shown to be the most rapidly labeled species of RNA, with its rate of synthesis exceeding that of all of the other RNAs over an 8 minute labeling period. It is therefore concluded that elution region 2 RNA is not a degradation product of rRNA.

Although no definite precursor/product relationship can be established for elution region 2 RNA and the rRNAs, it has been suggested that this RNA (elution region 2) is a precursor to rRNA. This conclusion is based on two lines of evidence; the rapid rate of labeling observed for elution region 2, and the data (Chapter 2) which suggest that it is a cytoplasmic RNA.

However the possibility of elution region 2 RNA being a stable mRNA was not ruled out by these experiments.

Polyacrylamide gel electrophoresis of the sRNA species isolated from the "fragile" cells showed that these RNAs are homogeneous, and have the same electrophoretic mobilities as their counterparts isolated from unlabeled cells, grown under different culture conditions.

SUMMARY

The detection of elution region 2 RNA in sRNA preparations isolated from uninfected E. coli cells, has added yet another RNA to the list of low molecular weight RNA molecules present in this organism. The list already includes 5s RNA, and 4s RNA, both stable species which have been extensively studied. Since 5s and 4s RNA have been shown to be integral components of the translational machinery, it is tempting to think of elution region 2 RNA as another molecule which participates in the translational process.

The studies reported in this thesis have clearly established the homogeneity, and uniqueness of this molecule, and in addition, have shown that elution region 2 RHA is a stable species.

Gel filtration analysis (Sephadex G 100), and polyacrylamide gel electrophoresis have both shown that elution region 2 RNA is isolated in its entirety from the supernatant cellular fraction. This RNA is easily purified by Sephadex G 100 rechromatography; it migrates as a discrete, homogeneous band on polyacrylamide gels; and has a base composition which is distinctly different from that of the other E. coli RNAs. Elution region 2 RNA has a molecular weight of 54,440 daltons (chain length of 142 to 148$\pm$ 10%), bears a 5' phosphorylated end, and a 3' hydroxylated end

of uridine, and does not contain any minor nucleotides.

Physical studies designed to probe the secondary structure of the molecule, have shown that like 5s RNA, 60% of the molecule is in the helical conformation. The helices however, display a greater stability to heat denaturation (Tm 64.4°C) than the helices present in 5s RNA (Tm 54.0°C). These results suggest a completely different distribution of the helical regions within the two molecules, with the helices in elution region 2 RNA differing from those of 5s RNA either in length, or stability of the base pairs, or both.

Elution region 2 RNA does not possess the ability to participate in the amino acid activation, or chain termination events of protein synthesis.

The results of kinetic experiments have shown that the rate of synthesis of elution region 2 RNA exceeds that of the other sRNA and rRNA species over an 8 minute labeling period. This is a clear indication that elution region 2 RNA is not a degradation product of the ribosomal RNAs.

Since the rapidly labeled RNA in E. coli has been shown to be either ribosomal precursor RNA (70%), or mRNA (30%) (Pigott and Midgley, 1968), it is quite possible that elution region 2 RNA could be a member of one of these groups of RNA. If it is a messenger RNA, then it would be representative of a type of messenger which has become

unusually stabilized. Alternatively, assuming it to be a ribosomal precursor RNA, then it seems that the processes involved in the synthesis of mature rRNA molecules in E. coli are different from those operating in mammalian cells. The transition from precursor to mature form of ribosomal RNA in E. coli would therefore involve the extension of a precursor molecule, rather than the cleavage of a larger molecule.

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