ABSTRACT

THE 5' TERMINI OF RIBOSOMAL RIBONUCLEIC ACID FROM HIGHER PLANTS

By

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This thesis is based on a study of higher plant ribosomal ribonucleic acid (rRNA). The objectives were (a) to determine the identity of the 5' rRNA termini, that is, to ascertain whether these groups consist of specific nucleoside 3',5' diphosphates, and (b) to determine whether the terminal groups are the same in different higher plant species. A monocotyledon, wheat (Triticum aestivum, L.), and a dicotyledon, cauliflower (Brassica oleracea, L. var. botrytis), were the two species studied.

RNA was isolated by one of three methods, depending on the characteristics of the tissue to be extracted. Essentially, the techniques used were extraction and deproteinization with either (a) phenol-sodium dodecyl sulfate solution, (b) phenol-phosphate solution, or (c) phenol-cresol-naphthalene 1,5 disulfonate. The

procedures were developed to the extent that consistent quantitative yields were obtained from a given plant source.

The purified rRNA was hydrolyzed in 0.3 N KOH at 37 C for 48 hours, and the solution was subsequently neutralized by the addition of a sulfonic acid resin (Dowex 50-$H^+$ or Rexyn 101). Components of the hydrolysate were separated by ion exchange on DEAE-cellulose or by gel filtration on Sephadex G-15. Nucleoside 3',5' diphosphates released from the 5' terminus by alkaline hydrolysis were identified by having identical properties as standards in paper chromatography, electrophoresis, as well as in anion exchange chromatography, and by comparison of absorption spectra.

Adenosine 3',5' diphosphate was the only 5' terminal group obtained following alkaline hydrolysis of unfractionated (25S + 16S) cauliflower rRNA extracted by phenol-sodium dodecyl sulfate, and also from rRNA extracted by phenol-phosphate from one source of wheat embryo. Similarly, this was the predominant group obtained from wheat embryo rRNA extracted from another source. In addition, traces of cytidine 3',5' diphosphate

and guanosine 3',5' diphosphate were also found in the RNA hydrolysate from the latter source.

These observations suggest that a common mechanism may control the initiation of rRNA synthesis in the species studied.

Satisfactory methods for the synthesis of nucleoside 3'(2')5' diphosphate standards, with which the 5' rRNA termini could be compared for identification were lacking at the beginning of this study. The synthesis of these standards, catalyzed by nucleotide phosphotransferase in a low energy phosphate transfer from p-nitrophenyl phosphate to 5' nucleotides, is also documented in this thesis.

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This thesis is dedicated to my wife Lidia, our son Buck, and to my father, each for their unique inspiration.

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# INTRODUCTION

That ribosomes function in protein synthesis is well established. It is now apparent, however, that knowledge of ribosomal organization is necessary in order to further elucidate the synthetic mechanism (1). Base sequence analyses of ribosomal ribonucleic acids (rRNA) and studies of ribosomal structural proteins may be useful in determining the mechanism of RNA-protein interactions which result in the formation of biologically active ribosomes. Little is known of the base sequences or functions of rRNAs; however, progress has been made on the identification of ribosomal proteins (2-8). Investigations of high M. W. rRNA synthesis have demonstrated that formation of the ribosome begins in the nucleolus by the transcription of a high molecular weight precursor rRNA. Portions of this precursor molecule are methylated before it becomes associated with ribosomal structural protein. Two ribosomal subunits are subsequently formed by cleavage at specific points in the molecule; concurrent with this process, part of the precursor RNA is lost (9).

The 5' end of rRNA was chosen as the subject of the present investigation for several reasons. Synthesis of RNA on a DNA template has been shown to occur from the 5' to the 3' end of the RNA molecule (10,11). Identification of the 5' terminus, the first nucleotide of the growing chain, may therefore be important in studying the initiation of RNA synthesis. Knowing the identity of end groups may also be useful in determining RNA heterogeneity, the chain length of the molecules, and in elucidating the mechanism of ribosomal assembly.

The objectives of this investigation were (a) to determine the identity of the 5' termini of ribosomal RNA, i.e., to learn whether these groups consist of specific nucleoside 3',5' diphosphates, and (b) to determine whether the same rRNA 5' termini occur on different plant species. Satisfactory methods for the synthesis of nucleoside 3',5' diphosphate standards, with which the 5' termini could be identified, were lacking at the beginning of this study. Nucleotide phosphotransferase was isolated from Escherichia coli cells, which had been commercially grown in high peptone medium and harvested in late log phase, and used for the synthesis of standards. The

synthesis of these standards by enzymatic low energy transfer of phosphate from an organic substrate to the 3'(2') position of nucleoside 5' monophosphates is also documented in this thesis.

# LITERATURE REVIEW

## Ribosomal Structure

There are several types of ribosomes; most bacterial ribosomes are of the 70S type, but in yeast, higher plants and animal cytoplasm, the ribosomes are of the 80S type, containing 40-50% RNA (20).

Ribosomes are composed of two ribosomal subunits; the larger having a sedimentation constant of 50-60S, the smaller an S value of 30-40. The molecular weights of subunits from plant or animal tissue are respectively about 1.5 and 2.5 x $10^6$ daltons, while those from bacteria are 1 x $10^6$ and 2 x $10^6$ (12). The ribosomes contain 40-60% protein, depending on the organism from which they are extracted. The average molecular weight of the protein, reported on the basis of N-terminal amino acids, was estimated to be 2.5 x $10^4$ (21,22).

Early models for protein synthesis postulated that ribosomes were passive assembly plants for translation. This concept fit into the models since specific protein

synthesis is programmed by a specific messenger RNA (mRNA). Messenger RNA (13) and transfer RNA (14) bind to the smaller subunit during protein synthesis, while the growing polypeptide chain is bound to the larger subunit through the attached RNA (15,16). Some support for the concept of the ribosome as a passive structure or non-specific workbench was provided by findings that ribosomes could be used interchangeably between species of bacteria. Additionally, no new ribosomes are formed subsequent to T2 bacteriophage infection of bacteria, suggesting that pre-existing bacterial ribosomes become the site of attachment of the newly synthesized T2 mRNA (17). Ribosomes which are homogeneous in structure have been proposed in support of the concept that ribosomes are a non-specific worktable for protein synthesis. Reports citing the presence of ribosomal proteins in the amount of one copy per ribosome (4) appeared to support the concept that homogeneity exists among ribosomes. More recent evidence based on the results of acrylamide gel electrophoresis and reconstitution of ribosomal subunits, however, indicates a distinct heterogeneity of ribosomal proteins (5, 18,19).

Analysis of purified ribosomes from E. coli yielded 21 ribosomal proteins from the 30S subunit and 34 proteins from the 50S subunit (18). The ribosomes from E. coli therefore have about 55 different proteins (6), whose molecular weights range from 10,000-65,000 (23).

Thirty unique 40S subunit proteins and thirty-nine 60S subunit proteins were found in mammalian ribosomes. Preparations of 80S ribosomes contained three proteins that were absent in each of the individual subunits. Eukaryotic ribosomes were estimated then to contain 68-72 different proteins (5).

The proteins isolated were found to yield unique peptides when hydrolyzed with trypsin, indicating a heterogeneous population (4). This was also supported by immunological experiments in which no cross reactivity was indicated for antibodies to the proteins (7). The heterogeneity of proteins in ribosomal subunits is therefore strongly supported. Preliminary results indicate that ribosomal proteins from eukaryotic cells have the same general characteristics as those from prokaryotic organisms (5).

Several lines of evidence now indicate that in addition to a major group of proteins occurring to the

extent of one per ribosome, there are some "fractional" proteins present in much lower frequency (i.e., less than one per ribosome) (6,18). Two models representing different interpretations of the heterogeneity of the 30S particles are present in the literature. Both models assume that a ribosomal "core" composed of "unit" proteins is formed, to which the fractional proteins are added. The "static" model assumes no exchange of proteins while the "steady-state" model predicts that the fractional proteins are exchanging from one ribosome to another during the protein synthetic cycle. These fractional proteins are suggested to determine the functional capabilities of the ribosome. They could influence the initiation of protein synthesis, chain propagation or termination by the orderly exchange of fractional proteins (8,18). A high rate of protein exchange in ribosomal proteins was demonstrated in double labeling experiments using $^{14}C$ leucine and $^{3}H$ leucine; protein components rapidly exchanged with similar ribosomal proteins in the cytoplasm (24). The <u>in vitro</u> exchange of free ribosomal proteins with proteins of the intact ribosome has also been reported (8). The steady state model is further supported by activation of 30S ribosomal subunits. "Inactive" 30S ribosomes were

incubated with unfractionated 30S protein and 50S subunits, resulting in an 80% increase in poly-U-directed polyphenylalanine synthesis.

Ribosomal structure has been further studied through the use of proteolytic enzymes. Disk electrophoresis on polyacrylamide gels was used to study the effects of enzymes (trypsin, pronase, chymotrypsin), on proteins of the ribosome. Protein banding patterns on the gels were altered as enzymatic cleavage of the proteins occurred. The effects on intact ribosomes were very selective, with the pattern of attack very similar for the different enzymes. Free ribosomal proteins, on the other hand, were rapidly broken down without the selectivity found for the same proteins in the intact ribosomes (25). This suggests that the proteins are not accessible to the enzymes while in the intact structure. The susceptibility may therefore reflect the structural arrangement of the proteins or the degree of binding to RNA. Shielding of mRNA, tRNA, and nascent polypeptides against enzymic breakdown has also been observed when these substances are ribosome bound. RNA itself may interfere with the specific functions of added proteolytic enzymes such as

trypsin, which is highly dependent on positively charged side chains (25).

A model of ribosomal structure based on dye stacking studies, has been proposed in which the rRNA exists in a non-helical single stranded configuration. This configuration in the ribosome would then be determined by interactions between nucleotide bases and ribosomal proteins, resulting in the exposure of nucleotide phosphates to the surrounding medium (26). The negatively charged phosphate groups may then interact with positively charged magnesium ions in forming linkages to other nucleotides. The association-dissociation of the ribosome appears to be governed by the $Mg^{++}$ concentration, higher concentrations favoring association. This model suggests exchange by means of $Mg^{++}$ salt bridges between RNA-phosphates of the different particles. Loosening of the ribosomal particles may then occur by dissociation of protein interactions upon the removal of $Mg^{++}$, rather than by disruption of the secondary structure of RNA. The same principle may be applied to the binding of mRNA to ribosomes.

Another ribosomal model presented in the literature suggests that considerable double helical rRNA structure exists in the ribosome, as well as in aqueous

solutions of rRNA. This conclusion is based on the close coincidence of the melting curves of rRNA and of the ribosomes. This same evidence suggests that ribosomal protein binds to single stranded regions of the molecule. Proteins bound to nucleic acids in the double helical state result in molecular stabilization as suggested by an increase (15-20 C) in the melting temperature (100).

Experiments in which the ribosome is broken down to its component parts and subsequently reassembled should yield information valuable to the structural knowledge. Reconstitution experiments in which the ribosomal subunit is re-formed from the completely dissociated respective proteins and rRNA have, in fact, been performed. Purified proteins derived from the 30S ribosomal subunit, sequentially added to purified rRNA resulted in the reconstitution of functional 30S ribosomal subunits (98). This latter study and others suggested that specific sequences of rRNA nucleotides recognize their protein counterparts (28). It was reported further that 16S rRNA from yeast or 18S rRNA from rat liver cannot replace E. coli 16S rRNA in the formation of functional 30S particles. Active hybrid 30S particles were formed, however, when 16S rRNAs from distantly related bacterial genera, such as

Azotobacter vinelandii or Bacillus stearothermophilis, were used to replace E. coli 16S rRNA (19). These three genera of bacteria were reported to have portions of 16S rRNA in common, suggesting that a requirement for specific base sequences in portions of rRNA may exist.

The comparison of rRNA sequences of several bacterial species revealed a correlation between species and rRNA sequence. Definite differences were found in nucleotide distribution of rRNAs among species unrelated taxonomically, while no differences were found between E. coli and its related species (39). Sugiura and Takanami (40) reported similar 5' terminal nucleotides from three species of Bacillus. Hydrolysis of rRNA from these species with T1 RNase and pancreatic RNase respectively produced fragments identical in length and in 5' termini. Alkaline hydrolysis also released the same 5' termini from each of the species. The results differed from those obtained when other genera were studied.

## Ribosomal RNA

The ribosomal RNAs have been postulated to have a single stranded linear chain arrangement, composed of four primary nucleosides, and occasionally modified nucleosides, connected by phosphodiester linkages. The 16S and 23S RNA molecules from E. coli contain respectively, about 1500 and 3000 nucleotides each, the larger molecule breaking down into two smaller chains under certain experimental conditions (31,32). The 5S rRNA of E. coli has been found to contain 120 nucleotide residues (33).

High molecular weight rRNAs vary in size among ribosomes of different organisms. The larger ribosomal (50-60S) subunit contains a single molecule of 23-28S rRNA. Lower eukaryotic organisms and some plant cells, for example, contain 25S RNA with a molecular weight of about $1.3 \times 10^6$. The smaller (30-40S) subunit, on the other hand, contains a single molecule of 16-18S rRNA with a molecular weight of 0.5 to $0.7 \times 10^6$ (1). Another rRNA with a sedimentation coefficient of 5S is bound to the larger subunit (33,43-49). Several lines of evidence including nucleotide distribution, 5' terminal nucleotide sequences and 3' terminal nucleotide sequences, indicate

that 23-28S and 16-18S RNAs are independent high molecular weight species. This is further supported by genetic evidence for distinct cistrons for these classes of molecules (16), although both 23-28S and 16-18S RNAs are derived from the same precursor ribosomal RNA molecule (1). The 5S rRNA, on the other hand, is transcribed separately from the high molecular weight rRNAs (93).

The role of ribosomal RNA in the function of the ribosome has not yet been resolved. Ribosomal reconstitution experiments indicate a direct involvement of the rRNA, however, chemical studies on the primary structure of rRNA are incomplete; hence it is impossible to draw meaningful conclusions.

Methylation studies have been used to investigate the relationship of some aspects of rRNA synthesis to ribosome formation. Evidence that methylation is a highly specific process which results in the production of E. coli 16S and 23S RNAs, that are homogeneous with respect to methylated sequences, suggests that a special functional significance may be attached to this specific modification (methylation) of the basic polymeric structure (34). When HeLa cells were grown under conditions of methionine "starvation," undermethylation of the 45S

ribosomal precursor RNA resulted and functional ribosomes were not formed under these conditions. The methyl-deficient 45S precursor RNA was found, however, to yield 32S RNA. The failure to form functional ribosomes was attributed to the lack of methylation.

The degree of methylation in ribosomal RNA of plant, animal, and bacterial cells studied is similar. The 16S and 23S RNAs of E. coli contain, respectively, about 17 and 11 methyl groups per 1000 nucleotides (36), while the 16S and 28S RNAs of HeLa cells contain 19 and 13 methyl groups per 1000 nucleotides (37,38). A major difference between these organisms is found in the pattern of methylation. In E. coli, methylation occurs principally in the heterocyclic bases; in wheat germ and HeLa cells, the methylation occurs predominantly in the 2' position of the ribose moiety.

The only positively identified function of the 16S rRNA is its role in the assembly of the ribosomal particle; however, it is conceivable that some parts of the 16S rRNA are involved in the binding of mRNA and or tRNA. Moore (41) studied the effects treatment with several chemical reagents (formaldehyde, nitrous acid, dinitrofluorobenzene and perphthalic acid), had on the

ability of ribosomes to bind to mRNA. Formaldehyde, which reacts with free primary amines in nucleic acids, did not affect amine groups involved in hydrogen bonding. Formaldehyde treatment of ribosomes prevented binding of polynucleotide mRNA, inactivated the 30S subunit, prevented 70S dissociation and subunit reassociation, and prevented incorporation of amino acids. Nitrous acid, used to test the hypothesis that amino groups might be involved in binding, affects compounds of the general type $R\text{-}NH_2$, by converting them to the corresponding alcohols. Treatment with this acid resulted in inhibition of polynucleotide (mRNA) binding and failure of 30S and 50S subunits to reassociate, suggesting that amino groups might be involved in the maintenance of the 70S structure. Dinitrofluorobenzene was used to determine if protein groups are involved in binding, since it reacts with a wide range of protein groups, but is unreactive with RNA. Ribosomal binding of mRNA was unaffected and no effect was found on ribosomal structure. Conversely, a reagent which selectively oxidizes the $N_1$ nitrogens of cytosine and adenine but does not affect amino groups of protein was used. Thus, perphthalic acid treatment of ribosomes confirmed the idea that rRNA amino groups are necessary for binding,

as mRNA was inhibited by the reagent. Perphthalic acid also prevented 30S-50S reassociation. The conclusion drawn from these experiments is that rRNA amino groups are directly involved in messenger binding, and that mRNA is bound by its backbone via hydrogen bonds. The results of these experiments could be strengthened by repeating the study in conjunction with reconstitution experiments to more specifically examine the functional groups of the respective ribosomal components. Specific streptomycin binding to E. coli 16S rRNA has very recently been shown to prevent ribosomal reconstitution (42). This binding also occurs on 30S ribosomal subunits of E. coli streptomycin sensitive strains in the same amounts as on the isolated 16S rRNA. The availability of the rRNA streptomycin attachment sites appear to be controlled by the binding of a protein, P10, which is present in streptomycin resistant strains (42). This further serves to strengthen the concept of a functional role of rRNA in "control" of protein binding.

A suggestion of a possible function of rRNA which persists in the literature is as a template in the formation of ribosomal proteins. During the recovery period from methionine starvation, or conditions where protein

synthesis has been inhibited, cells make ribosomal protein preferentially and much of the accumulated rRNA is converted to mature ribosomes (51), even under conditions of inhibition of RNA synthesis with actinomycin D (50). Ribosomal RNA accumulated during inhibition of protein synthesis is thus suggested to have template capabilities in subsequent preferential synthesis of ribosomal protein. The literature also contains reports suggesting contamination with non-ribosomal RNA as the reason for template activity for polypeptide synthesis during in vitro experiments (52,53).

Very recently, the influence of the secondary structure on the translation of rRNA was studied (101). Thermal denaturation of rRNA resulted in increased template activity, with temperature (40-65 C) when the antibiotic neomycin was included in the reaction mixture. Neomycin (or streptomycin) interacts with protein P10, a core protein of the 30S ribosomal subunit. Once the primary block to template activity is overcome with neomycin the remaining influence of secondary structure on the template activity was demonstrated.

## 5' Terminal Residues of Various RNA Species

The most completely analyzed RNA species, tRNA, in most cases yields the purine pGp at the 5' terminus. At least 15 tRNA species have been completely sequenced; others have been partially sequenced (102).

A breakthrough in the endgroup analysis of high molecular weight RNA of bacterial origin came with the development of a method for specifically labeling 5' terminal groups by alkaline phosphatase hydrolysis, followed by enzymatic phosphorylation with polynucleotide kinase and $\gamma$ - $^{32}$P ATP (54). The label at the 5' terminus aids in identifying the terminal group following fragmentation of the molecule.

The 5' terminal RNA residue has been found in several cases to contain a nucleotide triphosphate. RNA synthesized *in vitro* with *E. coli* DNA-dependent RNA polymerase contained predominantly purine triphosphate on the 5' terminals (10,11). The nucleoside triphosphate pppG, has been found as the 5' terminus of RNA isolated from bacteriophage R17 (55), R23 and QB (56), and F2 (57). RNAs from tobacco mosaic virus and turnip mosaic virus have been shown to contain predominantly adenosine bearing a free OH

as the 5' terminus (57,58,59). The 5' termini of these viruses are summarized in Table 1. The viral RNAs in each case exhibited purines as the 5' terminal residues.

TABLE 1.--Partial 5' terminal sequences of viral RNA.

| Source of RNA | 5' terminal sequence | Reference |
|---|---|---|
| Bacteriophage | pppGpGpGpUp... | 96 |
| MS2 | pPupPupPy... | 84 |
| | pppPupPupPy... | |
| Variant | | |
| QB + strand | $ppp(Gp)_4(Ap)_2(Cp)_2$... | 85 |
| - strand | pppG... | |
| QB (6S) | pppGpGpGpApUp | 99 |
| QB *in vitro* | $pppGpGpGpGpAp(Cp)_2$... | 86 |
| QB | $pppGp(Gp)_{3-4}(Ap)_2Cp$... | 87 |
| R17 *in vivo* | pppGpPupPyp... | 88 |
| Coliphage | pppApUpGp... | |
| fd | pppGpUpUp... | 89 |
| | pppGpUpAp... | |
| f2 | pppG... | 94 |
| TYMV | | 57 |
| | ApPyp... | |
| STNV | ppApGpUp... | 90 |
| | pppApGpUp... | 97 |
| | ApGp... | 59 |
| TMV | Ap... | 58 |

Takanami (54) found the 5' terminus of E. coli rRNA to be phosphorylated. E. coli rRNA treated successively with alkaline phosphatase and polynucleotide kinase ($\gamma^{32}$P ATP) yielded p*Ap at the 5' terminus of 16S RNA, and p*Gp at the 5' terminus of 23S RNA upon alkaline hydrolysis. Although purine groups form the 5' termini of E. coli high molecular weight rRNA, a subsequent survey revealed that a pyrimidine makes up the 5' termini of Bacillus and Saccharomyces rRNA (40,54), Table 2.

TABLE 2.--Partial 5' terminal sequences of rRNA from microorganisms.

| Source | RNA Species | 5' Terminal sequence | Reference |
|---|---|---|---|
| Yeast | 5S | (p)ppGpGpUpUpGp... | 106 |
| E. coli | 5S | pUpGpCpCpUp... | 91 |
| E. coli | 16S | pApApApPypGp... | 54 |
| E. coli | 23S | pGpPupPyp... | 54 |
| Bacillus cereus, B. subtilis, B. stearothermophilus | 23S | pUpXpXpXpGp... | 40 |
| | 16S | pUpXpXpXpXpGp... | |
| Sarcina lutea | 23S | pApApGpPyp... | 40 |
| | 16S | pUpXpXpXpGp... | |
| Saccharomyces cerevisiae | 28S | pUpUpGp... | 40 |
| | 18S | pUpXpXpXpXpGp... | |

Only a few terminal sequence determinations at the 5' end of high molecular weight rRNAs from eucaryotic organisms have been reported. The only example, to date, of an RNA on which no preferential termination has been observed at either end, is wheat germ rRNA which had been hydrolyzed with 1M alkali (30). The results, in this case, are consistent with internal cleavages or phosphate transfer reactions during hydrolysis with 1M alkali.

Some results of 5' terminal sequences of rRNA from eucaryotic organisms are reported in Table 3. The 5S rRNAs derived from mammalian cells were found to be generally homogeneous, and the 5' terminus for each was found to be preferentially guanosine.

## Nucleotide Separation Techniques

Analyses of high molecular weight RNA species have been pursued as methods were developed for handling the large and quite fragile molecules. Certain intrinsic molecular characteristics aid in the identification of the terminal residues, and separation and isolation of the remaining oligonucleotides. Nucleic acids, nucleotides

TABLE 3.--Partial 5' terminal sequences of rRNA from eucaryotic organisms.

| Source | RNA species | 5' terminal sequence | Reference |
|---|---|---|---|
| KB cells (Carcinoma) | 5S | pGpUpCpUpAp... | 103 |
| HeLa cells (Cultured) | 5S | (pp)pGpUp... | 104 |
| Potorous (Kangaroo rat) cells | 5S | pGpUp... | 105 |
| LS mouse cells | 5S | pGp... | 107 |
| Xenopus laevis | 5S | (pp)pGpCpCpUpAp... | 108 |
| L cells (Cultured) | 28S | pCp... | |
| | 16S | pUp... | 38 |
| Wheat germ | 18S + 28S | pAp...(9.75) | |
| | | pGp...(37.8) | |
| | | pCp...(24.4) | |
| | | pUp...(28.1) | 30 |
| Novikoff hepatoma ascites cells | nucleolar precursors | | |
| | 45S, 35S, 28S | pAp...(10) | |
| | | pCp...(10) | |
| | | pCmpUp. (80) | 92 |

*Numbers in parentheses indicate the relative percentage of 5' terminals.

and nucleosides, for example, contain a variety of ionizable groups. The presence of these groups permits the relative mobility of RNA hydrolysate components to be predicted at a given pH (62). This knowledge may be utilized in the separation and identification of the various hydrolysate components by electrophoretic or chromatographic methods. Electrophoretic separation is based on the net charge of the molecules and the resistance to movement due to the size and shape of the molecule. Therefore the relative mobilities of molecules may be determined by the degree of ionization and the approximate resistance to flow. Chromatographic separation in the simplest form is based on the degree of attraction to either a stationary or a mobile phase of the system.

Three classes of ionizing groups are important in separating nucleotides and polynucleotides: i) the primary and secondary phosphate groups, ii) the amino groups of adenine, guanine, cytosine or their derivatives, and iii) the enol groups of guanine, cytosine, or uracil. The effect of the primary and secondary phosphate groups and of the amino groups on migration are most important, since the enol groups have pK values of 9.5 or above. This is

significantly higher than the useful values for most nucleic acid separations (62).

In aqueous solution each of the nucleic acid bases has a unique 220-300 nm spectrum which varies characteristically with pH (63). This absorption of light due to the presence of the conjugated double bond systems of purines and pyrimidines also aids in their identification (63,64).

Cleavage of RNA molecules by any of the hydrolytic processes in which an intermediate cyclic phosphate linkage is formed (chemically or enzymatically), produces characteristic products. Alkaline hydrolysis, for example, produces a 3',5' nucleoside diphosphate at the 5' terminus, intermediate nucleoside monophosphates, and a nucleoside at the 3' terminus. Interruptions of hydrolysis occur when specific ribose moieties are methylated in the 2' position, and results in the production of di- or tri-nucleotides. The different extent to which the 5' and 3' terminals are phosphorylated, compared with the rest of the chain, allows their separation based on charge differences.

The oligonucleotides produced by hydrolysis of RNA may be separated by ion-exchange chromatography on DEAE

cellulose or DEAE Sephadex. This process is affected not only by ionic interactions between the oligonucleotides and the exchanger, but also by secondary non-ionic interactions such as hydrophobic and hydrogen bonding (65). These secondary forces are minimized by the incorporation of 7M urea, although all secondary binding forces are not completely eliminated, especially with DEAE cellulose (66).

The nucleoside monophosphates, dinucleotides, trinucleotides and nucleoside 3',5' diphosphates have net charges of -2, -3, -4, and -4 respectively, at pH 7.8 (37). This charge difference constitutes the basis of separation by batch elution with dilute salt, or by a linear salt gradient. Fractionation of RNA hydrolysate eluted from columns of DEAE cellulose has a practical limit of oligonucleotides containing ten residues or less.

# MATERIALS AND METHODS

## Experimental Materials

Wheat (Triticum aestivum, L.) embryo was a gift of Quaker Oats Co., Saint Louis, Mo. Cauliflower (Brassica oleracea, L. var. botrytis) and mung beans (Phaseolus aureus, Roxb.) were purchased from commercial sources. Broad beans (Vicia faba var. Windsor) were purchased from Vaughn's Nurseries. Most seeds were surface sterilized briefly with a 1% sodium hypochlorite solution before allowing them to imbibe water; broad beans required a 35-minute exposure in order to prevent fungal contamination.

Escherichia coli W cells were obtained from General Biochemicals, Chagrin Falls, Ohio. Frozen E. coli B cells were kindly supplied by Dr. John Boezi. DEAE cellulose (medium) was purchased from Sigma Chemical Co., St. Louis, Mo. Triisopropyl naphthalene sulphonic acid and m-cresol were purchased from Eastman Kodak Co., Rochester, N.Y.

Other chemicals used were reagent grade and were available locally.

## Extraction of RNA from Plant Tissue

RNA was extracted by one of three methods, depending on the characteristics of the tissue to be extracted. Considerations taken into account were: i) large quantities of RNA were needed for detection of terminal residues, ii) noticeable degradation of the rRNA could not occur in the extraction process, and iii) the isolated RNA had to be free of interfering substances.

### Extraction with Phenol-Phosphate

The method of Singh and Lane (37) was modified for the preparation of rRNA from wheat embryo. The plant material (60 gm) was added to 360 ml of 0.05 M phosphate buffer, pH 7.0, and an equal volume of water saturated phenol. This suspension was shaken at high speed on an Eberbach 60 cycle shaker for 20 minutes at room temperature, cooled on ice for 30 minutes, and subsequently

centrifuged at 12,000 x g for 10 minutes in a Sorvall refrigerated centrifuge. The aqueous phase was siphoned off and the RNA precipitated by the addition of two volumes of redistilled absolute ethanol by allowing the solution to stand 24 hours at 4 C. The precipitate was collected by centrifugation, washed two times successively with 80% ethanol, and suspended in 1 M NaCl by stirring with a magnetic stirrer for three hours. The high molecular weight RNA was collected by centrifugation after allowing the solution to stand for 24 hours. It was washed two times with 80% ethanol and again suspended as before. The resulting high molecular weight RNA was washed with 80% ethanol, dissolved in 0.05 M phosphate buffer, centrifuged, and examined spectrophotometrically before running other tests for integrity of the material. The material noted above that was soluble in 1 M NaCl was precipitated with two volumes of absolute ethanol and saved for isolation of sRNA.

Extraction with Phenol and Sodium Dodecyl Sulfate

This method of preparing ribosomal RNA by direct extraction was used on cauliflower florets and other fresh

plant tissues. The procedures of Halloin (60) as adapted from McCarthy and Hoyer (67) were used. The tissue was homogenized in two volumes (w/v) of water saturated phenol and two volumes (w/v) of solution A, which consisted of 5% sodium dodecyl sulfate, 0.28 M lithium sulfate, 0.1 M sodium acetate, and 0.001 M $MgCl_2$, pH 5.0 (60,67). After siphoning off the upper aqueous layer which separated by centrifugation, the phenol deproteinization was repeated. Following the addition of two volumes of absolute ethanol, nucleic acids which precipitated were collected by centrifugation. The original method of separating low molecular weight RNA and DNA from high molecular weight RNA was modified in these experiments by using two washes with 1 M NaCl rather than using 3 M sodium acetate. The precipitated nucleic acids were suspended in 200 ml of 1 M NaCl and stirred with a magnetic stirrer for three hours before allowing to stand overnight. High molecular weight RNA was again collected by centrifugation, and washed two times with 80% ethanol.

## Extraction with Phenol and Naphthalene 1,5-Disulfonate

The method of Hastings and Kirby (68) as modified by Loening (69) was used for extracting RNA from germinated tissue. Plant tissues were homogenized in a Waring blendor at 70% line voltage for one minute in five times their weight of grinding solution containing 30 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% sodium naphthalene 1,5 disulfonate (NDS) plus an equal volume of phenol-cresol solution. The phenol-cresol contained water saturated phenol (650 ml), m-cresol (70 ml), and 8-hydroxyquinoline (0.5 gm). The homogenate was centrifuged at 3,000 x g for 15 minutes in a refrigerated centrifuge. The aqueous layer was removed and made 1% in sodium triisopropyl naphthalene sulfonate and 6% in sodium 4-aminosalicylate. The aqueous solution was homogenized with an equal volume of phenol-cresol and recentrifuged. The interphase and aqueous layer were removed and made 0.45 M in NaCl, homogenized with phenol-cresol and recentrifuged. RNA was precipitated from the resulting aqueous layer by adding two volumes of absolute ethanol and allowing the solution to stand overnight in the cold. Low molecular

weight RNA and DNA were removed by treating with 1 M NaCl, as in the previous RNA extraction methods.

## Tests for Purity and Integrity of the Isolated RNA

### Optical Purity

Spectra were taken with a Beckman DB spectrophotometer in the range from 220-300 nm. The ratio of the readings at 260 nm/280 nm ranged from 1.95 to 2.15 for purified RNA. Estimations of RNA were based on the assumption that 1 mg of RNA yields 24 optical density units (O.D.) at 260 nm.

### Sucrose Density Gradient Centrifugation

Samples of RNA (2 mg) were routinely sedimented on linear 4-20% sucrose gradients prepared in either saline acetate (0.05 M NaCl, 0.05 M sodium acetate, 0.001 M $MgCl_2$, pH 5.3), or saline citrate (0.015 M NaCl, 0.015 M sodium citrate, pH 7.0). Centrifugation was performed at 53,000 x g with a Spinco 25.1 rotor for

16-17 hours at 2 C. The bottoms of the centrifuge tubes were punctured, and seven drop fractions collected which were then diluted with 1.5 ml of the buffer used to prepare the gradient. The samples were read on a Beckman DB spectrophotometer.

## Polyacrylamide Gel Electrophoresis

Samples of RNA were examined for structural integrity prior to alkaline hydrolysis. Purified acrylamide and bisacrylamide were used for preparation of the gels. Gels used in this study contained 4.6% acrylamide since both high and low molecular weight RNAs could be distinguished at this concentration. Acrylamide and bisacrylamide (respectively, 4.6 and 2.3 gm/100 ml buffer solution), were mixed and degassed at room temperature under reduced pressure. NNN'N" tetramethylethylenediamine (0.033 ml) and 10% (w/v) ammonium persulfate (0.033 ml) were added per gram of acrylamide present. The solution was mixed, rapidly pipetted with a propipet into 5 cm vertical tubes to within 1.5 cm from the top and water layered over the solution to ensure a flat gel surface. The buffers used contained: 0.04 M Tris, 0.02 M sodium acetate, 0.002 M

sodium EDTA; acetic acid was used to adjust the pH to 7.8 at 5 C. The sodium acetate was added to maintain the secondary structure of the RNA (70). Current was applied for one hour to remove excess polymerization catalysts from the gels prior to application of the samples. The RNA sample, 0.5 O.D. units, was dissolved in 50 microliters of the buffer containing 5% sucrose and layered over the gel. Electrophoresis was carried out at about 5 C in a cold room, with up to 10 v/cm applied. The amounts of the respective nucleic acid species were determined by reading the gels directly on a Gilford spectrophotometer with an attachment for reading gels.

Dische Diphenylamine
Test for DNA

One ml of .1% nucleic acid solution was mixed with two ml of diphenylamine reagent and heated for 10 minutes in a boiling water bath. The optical density was read at 600 nm and compared with a standard curve relating O.D. to micrograms of DNA (71).

## Alkaline Hydrolysis

RNA was dissolved in a small amount of water and hydrolyzed in 0.3 N KOH (one ml/mg RNA) for 48 hours at 37 C. The hydrolysate was cooled on ice before adjusting to pH 7.0 with a sulfonic acid, strong acid cation exchange resin (Dowex 50 W-$H^+$, or Rexyn 101). The neutralized hydrolysate was filtered with a sintered glass funnel and the resin thoroughly rinsed with distilled water before applying the diluted RNA hydrolysate to a DEAE cellulose column.

## Chromatography and Electrophoresis

### Fractionation of RNA Hydrolysate by DEAE cellulose (Formate) Ion Exchange Chromatography, Using Batch Elution with Increasing Concentrations of Tris-formate

DEAE cellulose was prepared by allowing the dry powder to settle in 1 N NaOH, thereby minimizing the occlusion of air. Fifteen ml of alkali were used for each gram of dry adsorbent (72). The cellulose suspension was poured on a coarse sintered glass funnel and

washed with 1 N NaOH until the filtrate was colorless. It was then washed successively with 1 N HCl and 1 N NaOH and distilled water. Fines were removed by suspending the cellulose in large volumes of distilled water and allowing it to settle for one hour before decanting the supernatant solution. The pH of DEAE cellulose was adjusted by adding the buffer with which the column was to be equilibrated.

DEAE cellulose was diluted with buffer until a suspension was made which was thin enough so that the rising surface of the bed could be seen while pouring the column. After filling the column to the level desired, 0.025 M Tris-formate, pH 7.8, was passed through it until equilibrium was reached. This served also to compact the column by gravity, before the neutralized dilute RNA hydrolysate was applied.

Nucleosides were collected during the loading of the column with the RNA hydrolysate and during subsequent washing with 0.025 M Tris-formate buffer, pH 7.8. Nucleoside monophosphates and dinucleotides were eluted with 0.085 M and 0.17 M Tris-formate buffer, respectively. The buffers of the last two concentrations were made 7 M in

urea to minimize non-ionic interactions. Trinucleotides and nucleoside 3',5' diphosphates were eluted with 1 M ammonium formate, pH 9.2, or with 1 M pyridine formate, pH 5.4.

## Chromatography of Nucleosides and Congeners from RNA Hydrolysate on DEAE Cellulose ($Cl^-$) Using a 0.0-0.3 M NaCl Linear Gradient

DEAE cellulose, prepared as above (materials and methods), was suspended in 1 M NaCl solution before packing columns. The packed columns were then washed with 4 M NaCl until the eluate produced a reading of zero at 260 nm. It was then rinsed with distilled water until the excess salt was removed (as indicated by a negative test with $AgNO_3$). The column was equilibrated with 0.02 M sodium acetate (pH 5.4) containing 7 M urea, before applying the diluted RNA hydrolysate. The elution was effected with a linear gradient of 0.0-0.3 M NaCl in 0.02 M sodium acetate (pH 5.4) buffer at a rate of 60 ml per hour.

## Chromatography by Sephadex Gel Filtration

Sephadex was poured by allowing a measured amount of dry beads to swell in water (e.g., for G-15 Sephadex the swelling time was five hours), prior to pouring a column. A thin slurry was prepared and added in a single batch to a 2.5 x 45 cm column which had been filled to one-third of its volume with 0.025 M phosphate buffer, pH 7.0. Elution of nucleosides was effected with the same buffer at a flow rate of 20 ml per hour.

## Desalting

Samples containing excess inorganic salt from being eluted from columns or chromatographic paper were desalted by one of two methods. The first was by adsorption of the sample to acid-washed Norit-A charcoal in water solution. Each sample was washed 14-18 times by mixing the slurry with a Vortex mixer and centrifuging at 10,000 x g to pellet the charcoal. The samples were eluted from the Norit A charcoal twice with eight ml of 80% ethanol containing 1% ammonia, and once with eight ml of 50% ethanol also containing 1% ammonia, the charcoal

being pelleted by centrifugation. The ethanol eluates were combined and the sample dried under reduced pressure at 40 C.

The second method used was column desalting, using DEAE cellulose (carbonate). Columns (1.0 x 2.0 cm) were packed with DEAE cellulose suspended in 1 M ammonium carbonate and subsequently washed with 2 M ammonium carbonate. Excess salt was removed by rinsing the column with distilled water. The samples were layered onto the columns in dilute solutions containing less than 0.05 M salt, before washing with 50 ml of 0.02 M ammonium bicarbonate. Elution was effected with 2.0 M ammonium carbonate. The eluate was evaporated to dryness under reduced pressure at a temperature less than 30 C with a flash evaporator. Ammonium carbonate was volatilized by repeating the latter step, usually three times.

## Chromatography on Dowex-1 Anion Exchange Resin

Columns of Dowex-1 (X 2, 400 mesh, $Cl^-$) were packed with the suspended resin in 1 M NaCl and subsequently washed with 4 M NaCl in 0.01 N HCl to remove

residual substances absorbing at 260 nm (54). The columns (0.6 x 15 cm) were washed with 0.01 N HCl to remove residual NaCl. Nucleoside 3',5' diphosphate samples were applied to the resin in 0.01 N HCl solution and elution was effected by a continuous gradient of 0.0-0.3 M NaCl, also in 0.01 N HCl (pH 2.0). Nucleoside diphosphates were collected at an elution volume characteristic for each nucleotide. The columns were reused, but the washing procedure was repeated and the resin backwashed before each use to prevent compaction.

## Paper Chromatography

All paper chromatography was of the descending type on Whatman no. 1 or 3MM filter paper unless otherwise indicated. Chromatograms were developed in the following solvent systems: System A) ethanol 75, water 25, paper impregnated with 10% ammonium sulfate solution (73); system B) 1-propanol 11, ammonia 7, water 2; system C) saturated ammonium sulfate solution 79, 0.05 M phosphate buffer 19, isopropanol 2; system D) isopropanol 70, concentrated $NH_4OH$ 10, $H_3BO_3$, 20 (74).

Systems B and C are listed in the 1967 catalog of Schwartz BioResearch Inc., Orangeburg, N.Y.

## Paper Electrophoresis

Separation of nucleosides and congeners was achieved in a Beckman RD-2 electrophoresis unit. The solvent system of Chandra and Varner (75) as modified by Becker (76) was used. It contained 0.01 M EDTA (tetra-sodium salt) and 0.34 ml of pyridine per liter, and was adjusted to pH 3.5 with glacial acetic acid. A constant potential of 400 volts was applied across Whatman 3MM paper (3.0 x 30 cm) to achieve separation.

## Isolation of Nucleotide Phosphotransferase from E. coli

The enzyme was isolated from frozen cells of E. coli B and E. coli W. The latter had been grown on a high peptone medium and harvested in late log phase. Methods patterned after those of Brunngraber and Chargaff (77) were used to purify the nucleotide phosphotransferase. All operations were carried out in the cold. Frozen cells

were ground with alumina and 0.1 M acetate buffer (pH 6.0) and the homogenate centrifuged one hour at 20,000 x g. The supernatant solution was made 30% of saturation in ammonium sulfate and the precipitate resulting from centrifuging at 20,000 x g for 30 minutes was discarded. The supernatant solution was then made 90% saturated in ammonium sulfate and allowed to stand one hour prior to centrifuging at 20,000 x g for 30 minutes. The resulting precipitate was dissolved in 0.001 M sodium acetate buffer (pH 6.0) and dialyzed against two changes (one liter each) of this buffer.

The dialyzed partially purified enzyme from above was applied to a DEAE cellulose column (2.5 x 20 cm) previously equilibrated with 0.001 M sodium acetate (pH 6.0). Elution was effected with a continuous gradient of 0.005 M to 0.3 M sodium acetate pH 6.0, at a flow rate of 20 ml per hour. The eluate was collected in ten ml fractions.

The enzyme was assayed at each step of the purification. Aliquots (0.1 ml) were tested for total protein (Lowry-Folin), phosphatase (formation of p-nitrophenol from p-nitrophenylphosphate), and for nucleotide phosphotransferase. Assay for the latter enzyme was based

on the transfer of phosphate from p-nitrophenylphosphate to uridine. Paper chromatography or paper electrophoresis was used to determine uridine monophosphate formation.

# RESULTS

## Isolation of RNA from Plant Tissues

The procedure for isolating RNA was developed to the extent that consistent quantitative yields were obtained from a given plant source (Table 4). In a typical preparation, 200 grams of cauliflower florets yielded approximately 180 mg of RNA. However, variation in RNA yields did occur between "batches" of cauliflower. As indicated in Table 1, RNA yields from two sources of wheat embryo were approximately 300 mg/60 gm of tissue.

TABLE 4.--Yields of rRNA from different plant tissues.

| Plant tissue | Amount | Type of isolation | $\frac{A260\ nm}{A280\ nm}$ | RNA Yield |
|---|---|---|---|---|
| Cauliflower florets I | 200 gm | phenol-soln. A | 2.10 | 181 mg |
| Cauliflower florets II | 200 gm | phenol-soln. A | 2.11 | 181mg |
| Wheat embryo I | 60 gm | phenol-phosphate | 2.05 | 310 mg |
| II | 60 gm | phenol-phosphate | 2.05 | 319 mg |
| III | 60 gm | phenol-phosphate | 2.12 | 306 mg |

The molecular integrity of the extracted RNA was determined by means of sucrose density gradient centrifugation and acrylamide gel electrophoresis. Analysis of fractions collected from the sucrose density gradient tubes (A260 nm), following centrifugation, indicated a bimodal distribution of rRNA for both cauliflower and wheat (Figure 1). In each case, the area of the profile of the 25S peak was approximately twice that under the 16S peak. RNA used for the analysis of terminal groups contained neither DNA nor low molecular weight RNA as evidenced by sedimentation profiles or by acrylamide gel electrophoresis (Figure 2). Negative diphenylamine tests also indicated that the RNA used was free of DNA. Degradation of the RNA species sedimenting in the 25S region was observed when the phenol-phosphate solution was used to extract tissue by prolonged shaking (50 minutes). No such problem occurred when the time of shaking was decreased to 20-30 minutes.

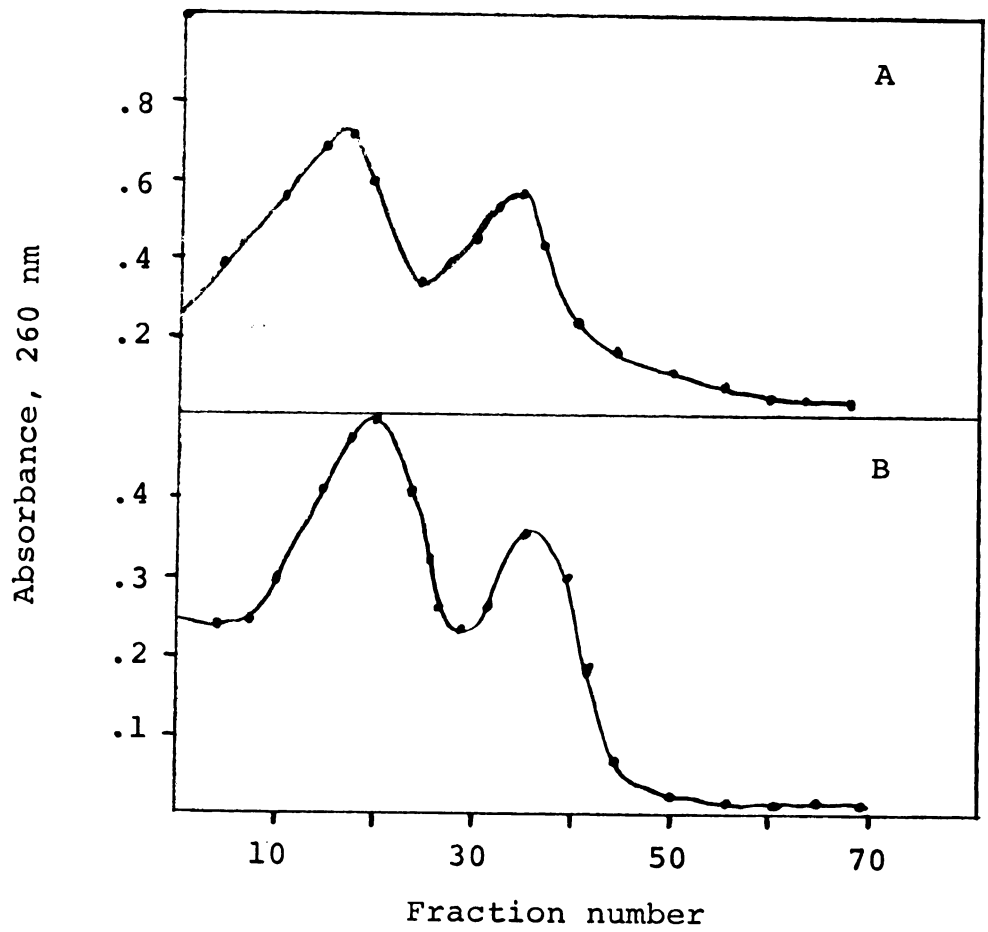


Fig. 1.--Separation of high molecular weight RNA species by sucrose density gradient contrifugation.

Sedimentation profile of 2 mg of wheat embryo rRNA (A) extracted with phenol-phosphate, and cauliflower rRNA (B) extracted with phenol-solution A. Purified RNA was centrifuged at 53,600 x g for 17 hours on 4-20% linear sucrose gradients, bottoms of the centrifuge tubes were punctured and seven drop fractions collected. Fractions were diluted with 1.5 ml of the appropriate gradient buffer (see Materials and Methods), and the absorbance at 260 nm determined.

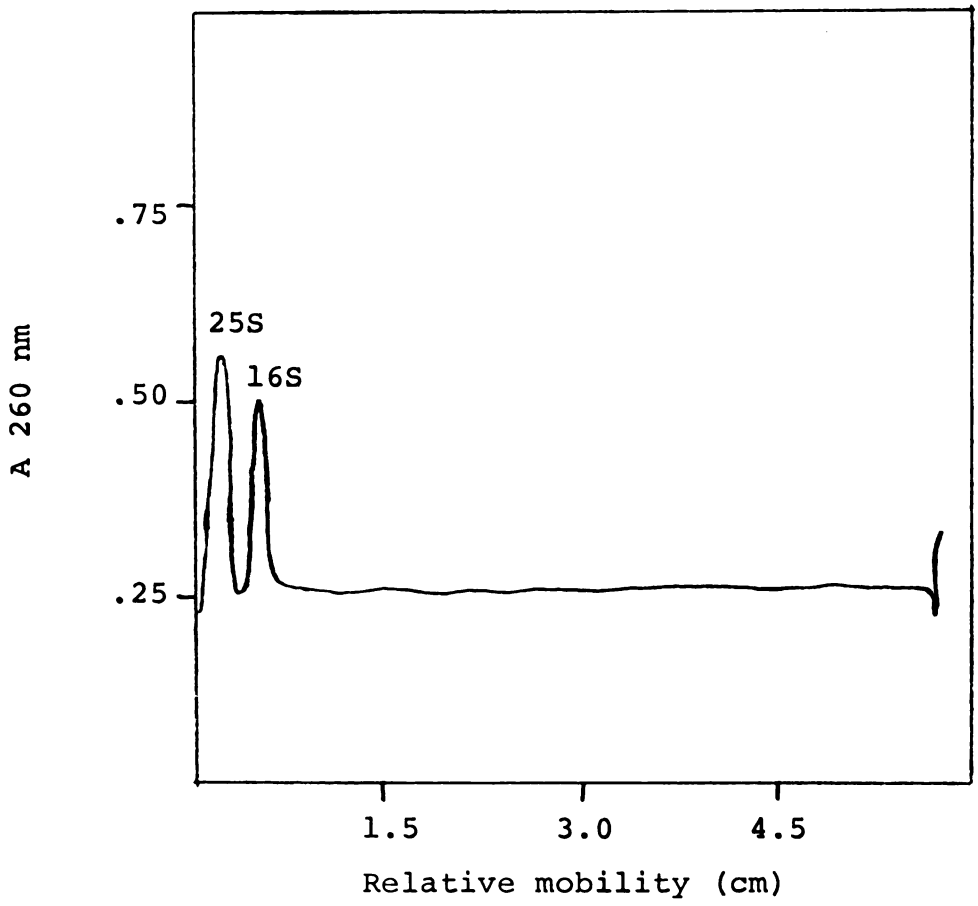


Fig. 2.--Electrophoresis of rRNA on 4.6% polyacrylamide gels.

Twenty µg of RNA prepared from cauliflower florets by the phenol-solution A procedure was applied in 50 µl of buffer containing 5% sucrose. Electrophoresis was carried out at 5 C with 10V/cm applied for 3 hrs. Under these conditions 25S RNA just enters the gel.

## Isolation of Nucleotide Phosphotransferase

The isolation of nucleotide phosphotransferase was first attempted from E. coli B (Materials and Methods, p. 40). Only a trace of nucleotide phosphotransferase activity was detected. However, this was sufficient to warrant further investigation of the enzyme from another source. The enzyme isolation, with minor modifications, was repeated on E. coli W. The results of this isolation and purification are summarized in Table 5. An increase in total phosphotransferase activity, concomitant with a decrease in hydrolase activity is observed as purification progresses. Thus it appears that the hydrolase activity, when present, counteracts the effectiveness of phosphotransferase activity.

The elution pattern from DEAE cellulose (acetate) shows two peaks having hydrolase, but no transferase activity (Figure 3). A very sharp peak exhibiting transferase activity follows closely the first phosphatase peak; some residual hydrolase activity is eluted in the peak nucleotide phosphotransferase fractions. The effect of this hydrolase is counteracted by the inclusion of a

TABLE 5.--Isolation and purification of nucleotide phosphotransferase from E. coli W.

| Purification Step | Volume (ml) | Phosphatase Activity (mg Pi/ml) | Protein Total (mg) | Nucleotide Phosphotransferase | | |
|---|---|---|---|---|---|---|
| | | | | Specific Activity* | Total Activity | Purification |
| I. Crude Extract | 40 | 0.38 | 536 | 0.04 | 21.4 | |
| II. Ammonium Sulfate fraction | 60 | 0.34 | 498 | 0.08 | 39.8 | 2X |
| III. DEAE-cellulose | 80 | 0.07 | 12 | 2.95 | 35.4 | 74X |

*Specific activity in μ moles of UMP produced/mg protein/hour.

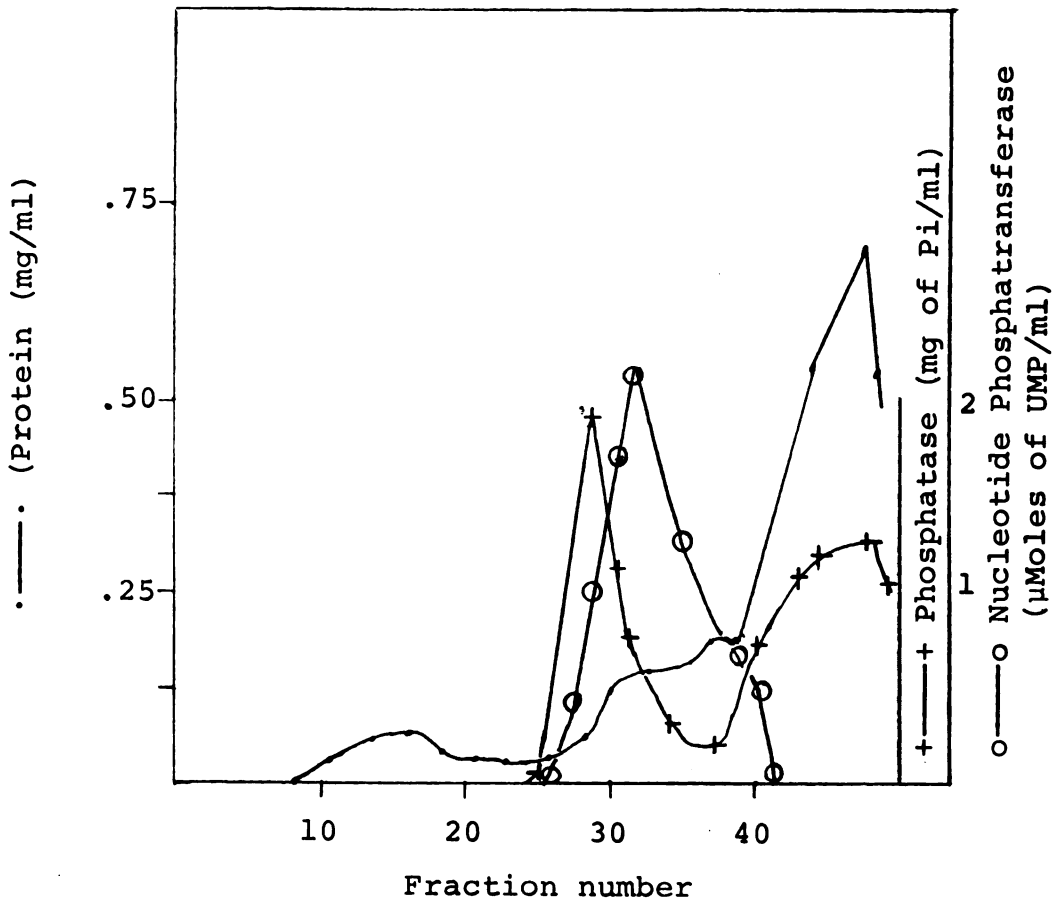


Fig. 3.--Elution of nucleotide phosphotransferase and phosphatase of E. coli W. from DEAE cellulose.

Aliquots of 10 ml fractions (Step III) enzyme eluted from a DEAE cellulose (acetate) column by a linear acetate gradient (0.005-0.3 M, pH 6), were tested for protein (·——·) conc., phosphatase (+——+) activity and nucleotide phosphotransferase (o——o) activity as described in the Materials and Methods. Maximum nucleotide phosphotransferase activity occurred at fraction 32, with a bimodal distribution of phosphatase activity, maximum activity being found at fractions 29 and 50.

large excess of phosphate donor during the synthesis experiments.

Optimal nucleotide phosphotransferase activity was determined and the percentage of nucleoside monophosphate converted to diphosphate calculated on the basis of time course studies. The samples were incubated at 37 C for the various times, samples were then spotted on Whatman no. 1 paper and developed using System A or System B. All components were separated with these systems. The spots were cut out, eluted with 0.05 N HCl and read at the appropriate wave lengths to quantitatively determine 3',5' diphosphate production (Table 6).

All of the monophosphates served as nucleotide acceptors, with a maximum of 39% conversion reached, that being the amount of UMP being converted to uridine 3',5' diphosphate using enzyme fraction II32. Incubation with enzyme fraction I A (DEAE cellulose, fractions 30-31), resulted in approximately 32% UDP yield at eight hours, and for practical purposes maximum conversion of UMP had been reached at 12 hours. The purine 5' monophosphates both produced good yields; however 24 hours was required for maximum 3',5' diphosphate production. The incubation time optimal for conversion of the respective nucleoside

TABLE 6.--Time course of phosphotransferase activity.

| 5' Nucleotide Acceptor | Nucleotide conc. µmoles/ml | Enzyme Fraction | Time incub. hrs. | Nucleoside 3',5' di-P | |
|---|---|---|---|---|---|
| | | | | amount µmoles/ml | % Yield |
| Uridine mono-P | 12.21 | IA | 2 | 1.64 | 13.4 |
| | 10.19 | | 8 | 3.23 | 31.75 |
| | 9.65 | | 12 | 3.21 | 33.25 |
| | 11.0 | | 17 | 3.3 | 30.0 |
| | 15.63 | | 24 | 5.35 | 34.25 |
| Adenosine mono-P | 21.93 | IA | 2 | 1.43 | 6.6 |
| | 20.28 | | 8 | 3.08 | 15.2 |
| | 18.8 | | 12 | 4.1 | 21.8 |
| | 22.7 | | 17 | 6.04 | 26.6 |
| | 26.27 | | 24 | 8.22 | 31.35 |
| Cytidine mono-P | 11.93 | IA | 2 | 1.68 | 14.1 |
| | 10.29 | | 8 | 2.4 | 23.3 |
| | 10.46 | | 12 | 2.68 | 25.6 |
| | 14.02 | | 17 | 3.67 | 26.2 |
| | 11.40 | | 24 | 2.89 | 25.4 |
| Guanosine mono-P | 23.94 | IA | 2 | 2.09 | 8.74 |
| | 18.22 | | 8 | 3.02 | 16.6 |
| | 21.01 | | 12 | 4.06 | 19.35 |
| | 23.11 | | 17 | 4.6 | 19.92 |
| | 24.48 | | 24 | 5.58 | 22.8 |
| Uridine mono-P | 11.92 | II32 | 2 | 1.3 | 10.9 |
| | 9.33 | | 8 | 1.71 | 18.35 |
| | 9.95 | | 12 | 3.53 | 35.4 |
| | 10.17 | | 17 | 3.53 | 34.8 |
| | 10.65 | | 24 | 4.16 | 39.15 |

monophosphates to nucleoside 3'(2')5'-diphosphates was used in the large scale preparation of the nucleoside diphosphates.

## Large Scale Preparation of Nucleoside 3',5'-Diphosphate Standards Using Nucleotide Phosphotransferase

Samples containing 0.1 ml of p-nitrophenylphosphate (200 mM/ml), and 0.1 ml of the respective nucleoside 5' monophosphates (40 mM/ml) each in 0.2 M sodium acetate pH 5.0, were incubated with 0.2 ml of the phosphotransferase for the optimal time periods as determined above. Upon completion of incubation the mixture was streaked on Whatman 3MM filter paper and then developed in system B. The component migrating to the nucleoside diphosphate position was cut out and eluted prior to further purification by passage through a G-10 Sephadex column (1.5 x 46 cm). Subsequent observation of the respective synthetic products on paper electrophoresis, by chromatography in system A and system B confirmed their purity by comparison of their migration with standards as presented in Table 7 and Table 8. No attempt was made to differentiate between

TABLE 7.--Chromatographic* migration of standard nucleosides and nucleotides in three solvent systems.

| Compound | System A | | | System B | | System C** |
|---|---|---|---|---|---|---|
| | hrs. | cm | $R_f$ | hrs. | cm | $R_f$ |
| Cytidine 3',5' di-P | | | .07 | 30 | 6.4 | .80 |
| Guanosine 3',5' di-P | | | .10 | 30 | 5.7 | .51 |
| Adensoine 3',5' di-P | | | .17 | 30 | 3.1 | .44 |
| Uridine 3',5' di-P | | | .29 | 30 | 6.6 | .73 |
| Cytidine 5' mono-P | | | .12 | | | |
| Guanosine 5' mono-P | | | .16 | 23 | 8.7 | .44* |
| Adenosine 5' mono-P | | | .24 | 23 | 14.4 | .31 |
| Uridine 5' mono-P | | | .42 | 23 | 12.2 | .69 |
| Guanosine 3' mono-P | | | | 23 | 10.9 | |
| Cytidine | | | .21 | 23 | 31.2 | .67*** |
| Guanosine | 18 | 20.6** | | 23 | 21.4 | .39*** |
| Adensoine | 18 | 28.1** | | 23 | 33.1 | .20*** |
| Uridine | 18 | 37.0** | | 23 | 29.1 | .64*** |

*Unless otherwise indicated, Whatman no. 1 paper was used.
**Designates use of Whatman 3MM paper.
***Designates use of Whatman no. 40 paper.

TABLE 8.--Paper electrophoretic separation of nucleosides and nucleotides.

| Compound | Distance of migration in cm |
|---|---|
| Cytidine 3',5' di-P | 12.1* |
| Adensoine 3',5' di-P | 12.9 |
| Guanosine 3',5' di-P | 16.3 |
| Uridine 3',5' di-P | 18.6 |
| Cytidine 5' mono-P | 4.4 |
| Adenosine 5' mono-P | 6.2 |
| Guanosine 5' mono-P | 9.8 |
| Uridine 5' mono-P | 12.2 |
| Guanosine 3' mono-P | 9.8** |
| Uridine 5' mono-P | 9.9 |
| Cytidine | -10.3 |
| Adenosine | -7.6 |
| Guanosine | -6.7 |
| Uridine | |

*Designates 9.5 hours at 7 ma for all materials above the dashed line.

**Designates 3.5 hours at 35 ma for all materials listed below the dashed line.

the formation of 2',5' and 3',5' nucleotide isomers, although the presence of both was detected in system C. For simplicity, however, in this thesis the products will be referred to as the nucleoside 3',5' diphosphates.

## Resistance of Nucleoside 3',5' Diphosphate Standards to Sodium Meta-Periodate Oxidation

The previous experiments established that a phosphate group had been transferred to the nucleoside 5' monophosphate acceptors. The position of this phosphate group, however, remained uncertain. The proposed phosphate transfer to the 3'(2') position would result in a product (nucleoside 3'(2')5' diphosphate), resistant to sodium m-periodate oxidation. Nucleoside 5' diphosphate, on the other hand, would be oxidized to a hydrated dialdehyde with concurrent alteration of electrophoretic or paper chromatographic migration properties.

The results of incubation with sodium m-periodate (Table 9) demonstrate that the nucleoside diphosphate product is resistant to oxidation while the nucleoside compound possessing the 5' phosphate is oxidized.

TABLE 9.--Effect of Periodate Treatment on the Electrophoretic and Chromatographic Properties of Nucleoside Phosphates.

| Compound | Treatment | Distance of Migration (cm) | |
|---|---|---|---|
| | | Electrophoresis | Paper Chromatograph* |
| Adenosine 3',5' diphosphate | none | 13.8 | 10.3 |
| Adenosine 3',5' diphosphate | periodate | 13.7 | 10.3 |
| Adenosine 5' phosphate | none | 9.1 | 18.5 |
| Adenosine 5' phosphate | periodate | 8.5 | 13.5 |

*System A was used for determining the migration by paper chromatography. Electrophoresis was performed at 40 ma for 3.5 hours.

## Fractionation of Nucleosides and Congeners by DEAE Cellulose (Formate) Ion Exchange, Using Dilute Salt

The four nucleosides (286.8 O.D. units), nucleoside monophosphates (175.6 O.D. units), and the nucleoside 3',5'

diphosphates (10.46 O.D. units), were applied to a DEAE cellulose (formate) 2.5 x 26 cm column, in 90 ml of 0.025 M Tris-formate buffer, pH 7.8. Elution of the nucleosides was effected with 0.025 M Tris-formate buffer, pH 7.8. The nucleoside monophosphates were eluted with 525 ml of 0.085 M Tris-formate (pH 7.8), made 7M with respect to urea in order to minimize non-ionic interactions. The nucleoside 3',5' diphosphates were eluted with 175 ml of 1 M ammonium formate, pH 9.2. An elution profile is presented in Figure 4. Paper chromatography of the respective peaks indicated that a clear separation of nucleosides, nucleoside monophosphates, and nucleoside diphosphates had been effected.

The percent recovery of nucleoside 3',5' diphosphates was determined following a series of desalting and chromatographic procedures (Adsorption to charcoal, washing, chromatography in system A, elution from paper and reading at 260 nm) to determine if any preferential loss of specific nucleoside 3',5' diphosphates occurred. These results are presented in Table 10.

The results from Table 10, analyzed by Model 1 Analysis of Variance, at the 5% level, indicated no

Fig. 4.--Fractionation of nucleosides and nucleotides by DEAE cellulose (formate) ion exchange, using batch elution with formate salts.

Nucleosides, nucleoside monophosphates and nucleoside diphosphates were eluted from a 2.5 x 26 column with Tris-formate pH 7.8 at concentrations of 0.025 M and 0.085 M (7M urea), and ammonium formate 1M, at pH 9.2, respectively. The first peak (approximately 175 ml) represents nucleoside material, the second major peak at approximately 700 ml represents the nucleoside monophosphates, and the third peak at approximately 1050 ml, represents the nucleoside diphosphates. (3.5 ml fractions were taken.)

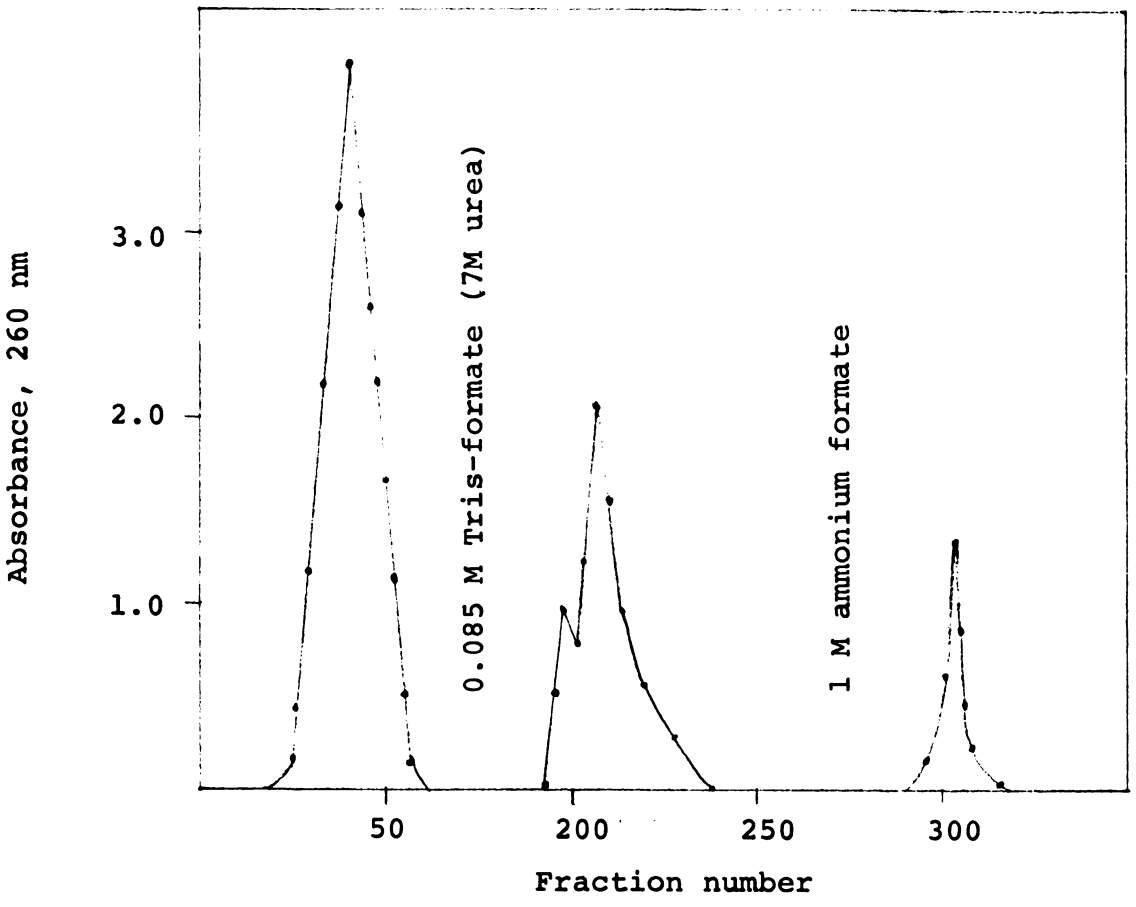
Absorbance, 260 nm
3.0
2.0
1.0
0.085 M Tris-formate (7M urea)
1 M ammonium formate
50
200
250
300
Fraction number

Figure 4

TABLE 10.--Recovery of nucleoside 3',5' diphosphate standards following desalting and paper chromatography.

| Compound* | Sample I percent recovery | Sample II percent recovery | Average recovery |
|---|---|---|---|
| Adenosine 3',5' diphosphate | 81.5 | 84.0 | 82.8 |
| Guanosine 3',5' diphosphate | 77.5 | 53.5 | 65.5 |
| Uridine 3',5' diphosphate | 92.0 | 85.5 | 88.8 |
| Cytidine 3',5' diphosphate | 74.5 | 81.5 | 78.0 |
| Average % recovery | 81.4 | 76.1 | 78.7 |

*The percent recovery values are based on an initial value of 0.75 O.D. units.

statistically significant preferential loss of nucleoside 3',5' diphosphates. This analysis is summarized in Appendix A. An *a priori* test of the percent recovery of the purines and pyrimidines also indicated no significance at the 5% level. However, an *a posteriori* test (LSR) (between the lowest (65.5) and highest (88.8) average recoveries obtained), did yield a value close to significance at the 5% level.

## Fractionation of Nucleosides and Congeners by DEAE Cellulose ($Cl^-$) Ion Exchange Using a Linear Salt Gradient

Separations on DEAE cellulose were also carried out using a 1 x 30 cm column, prepared and packed as described in the Materials and Methods. The column was equilibrated with 7M urea buffered with 0.02 M sodium acetate (pH 5.4), before applying an aqueous solution of the four respective nucleosides (138 O.D. units), nucleoside monophosphates (87 O.D. units) and nucleoside 3',5' diphosphates (29 O.D. units), in a total volume of 40 ml. Elution was effected with a linear (0.0-0.3 M NaCl gradient containing 7M urea in 0.02 M sodium acetate, pH 5.4. The elution profile is presented in Figure 5. The nucleosides were eluted in fractions 1-16, the nucleoside monophosphates in fractions 31-46, and the nucleoside 3',5' diphosphate standards in fractions 69-90.

## Separation of Nucleosides, Nucleoside Monophosphates, and Nucleoside 3',5' Diphosphates by Sephadex G-15 Gel Filtration

Gel filtration studies were carried out using Sephadex G-15 columns (2.5 x 45 cm) equilibrated with

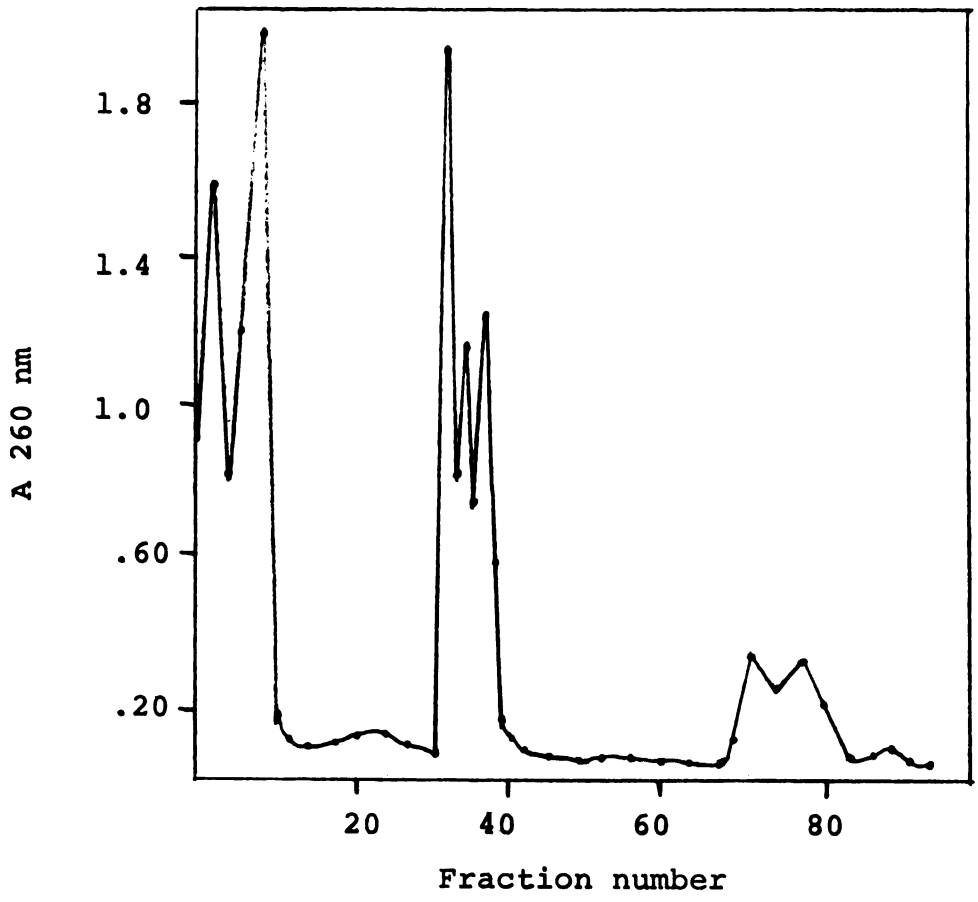


Fig. 5.--Elution pattern of standards from a DEAE cellulose ($Cl^-$) test column.

A solution of the four nucleosides, nucleoside 5' monophosphates, and nucleoside 3',5' diphosphates was applied to the column (1 x 30 cm) and subsequently eluted with a linear NaCl gradient (0.0-0.3M) in 0.02M sodium acetate containing 7M urea. The nucleosides were eluted in fractions 1-16, the nucleoside monophosphates in fractions 31-46, and the nucleoside 3',5' diphosphates in fractions 69-90. The respective initial NaCl concentrations resulting in elution of the fractions were: 0.0, 0.035, and 0.08M.

0.025 M phosphate buffer (pH 7.0). The void volume was determined with Blue Dextran 2000 to be 67.5 ml. Standards of a nucleoside 3',5' diphosphate (adenosine diphosphate), a nucleoside monophosphate (guanosine monophosphate), and a nucleoside (adenosine) were applied in a volume of five ml and eluted at 103 ml, 146 ml, and 288 ml, respectively, as seen in Figure 6a. The elution volume divided by the void volume yields a Ve/Vo value, characteristic for each substance. Values of Ve/Vo for each of the above were: ADP, 1.53; GMP, 2.08; and adenosine, 4.26.

Test columns were run to determine the elution volume expected for "trinucleotides" and "dinucleotides"; the elution profile is presented in Figure 6b. Dinucleotides and trinucleotides are those materials eluted from DEAE cellulose at 0.17 M Tris-formate pH 7.8, and at 1 M ammonium formate pH 9.2, excluding the nucleoside diphosphates. The trinucleotides were eluted at the void volume, 67.5 ml, yielding a Ve/Vo value of 1.0, while the dinucleotides were eluted at 130 ml (Ve/Vo = 1.93). Guanosine 5' monophosphate used as a marker, was eluted with 146 ml of eluant for a Ve/Vo value of 2.08.

Fig. 6.--Separation of nucleosides and nucleotides by Sephadex G-15 gel filtration.

6a. Samples layered on Sephadex 2.5 x 45 cm column were identified by characteristic elution volumes. Blue Dextran 2000 was used as a marker to determine the void volume (peak 1) of the column. Adenosine 3',5' di-P (peak 2) was eluted at fraction 28. Guanosine 5' mono-P (peak 3) was eluted at fraction 40, and adenosine (peak 4), at fraction 78.

6b. "Trinucleotides," peak a, were eluted at the void volume, fraction 18. The dinucleotides (peak b) were eluted at fraction 32. Guanosine 5' mono-P, used as a marker, was eluted at fraction 40 (peak c).

6c. The fractions of RNA hydrolysate eluted from DEAE cellulose (formate) with 1M ammonium formate were subsequently applied to a G-15 Sephadex column. Peak I, at the void volume, represents the "trinucleotides." Peak II represents the nucleoside diphosphates.

Note: Profiles represent continuous monitoring of effluent with an LKB Uvicord II at 254 nm. Fractions collected were 3.7 ml. Elution was effected in each case with 0.025 M phosphate buffer pH 7.0.

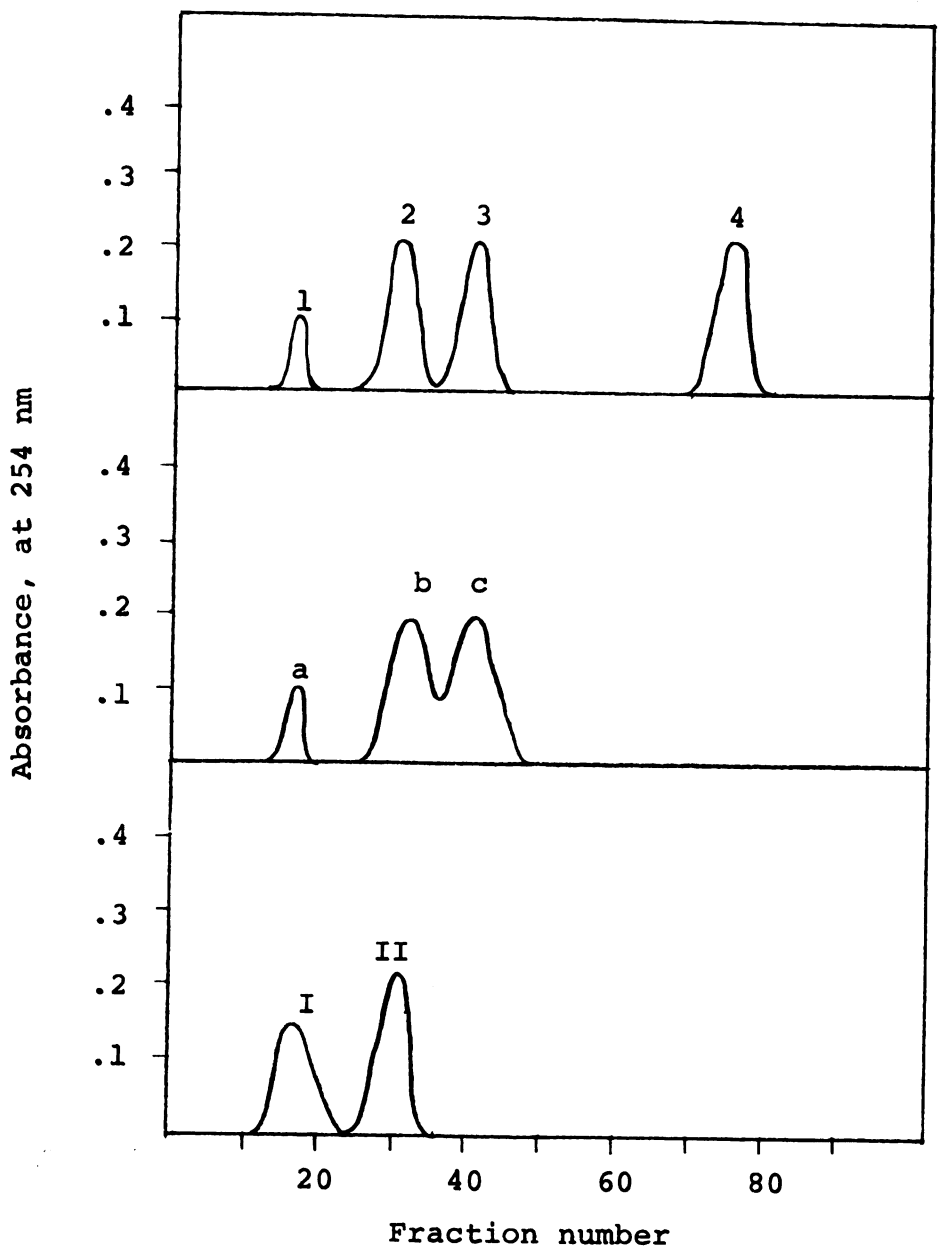
Absorbance, at 254 nm
.4
.3
.2
.1
1
2
3
4
a
b
c
I
II
20
40
60
80
Fraction number

Figure 6

Preliminary separation of rRNA hydrolysate into its respective components was accomplished by column chromatography on DEAE cellulose (formate) at pH 7.8. This separation, however, resulted in the elution of nucleoside 3',5'-diphosphates and trinucleotides in the same fractions. Since the size difference between the latter is great, Sephadex gel filtration appeared to be a useful means of separation.

The DEAE cellulose eluate containing nucleoside 3',5' diphosphates was layered on Sephadex in a 15 ml volume followed by five ml of buffer before beginning the elution with 0.025 M phosphate buffer (pH 7.0). The flow rate was maintained at about 30 ml per hour with the eluate continuously monitored with an LKB Uvicord II having a flow cell. Fractions of 4.3 ml were collected; fractions 16-20 are termed Peak I and fractions 25-32 are termed Peak II. Peak I was eluted at the void volume and Peak II at 110 ml; the elution profile is presented in Figure 6c.

The respective peaks were reduced in volume and absorbed to 30 mg of acid washed Norit A before washing each sample 14 times, and the nucleotide material eluted with ethanol as described in Materials and Methods. The

samples were dried under reduced pressure before spotting on Whatman no. 1 paper and developing in system A. The results of separating nucleoside 3',5' diphosphates by gel-filtration are presented as preparation IV in Table 14.

## Ribosomal RNA 5' Termini

### 5' Termini of Wheat Embryo rRNA

Wheat embryo rRNA (preparation IV) was extracted by the phenol-phosphate method. Sucrose density gradient analysis revealed that the RNA was free of DNA and low molecular weight RNA (Figure 1).

The neutralized alkaline hydrolysate of rRNA extracted from wheat embryo was applied to a column of DEAE cellulose ($Cl^-$), and the hydrolysate components eluted sequentially by a linear NaCl gradient. The nucleosides were subsequently separated by paper chromatography (System A). The combined amount (0.19 μ moles) of adenosine 3',5' diphosphate released from the 5' ends.

Three preparations of wheat embryo rRNA extracted as above were fractionated after alkaline hydrolysis by batch elution from DEAE cellulose (formate) with increasing

concentrations of Tris-formate, rather than with a linear NaCl gradient. The predominant 5' terminus detected in each of these preparations was also adenosine 3',5' diphosphate. The amount of RNA utilized varied from 443-632 mg. Adenosine 3',5' diphosphate was the only terminus detected in the smaller samples. In the largest sample, adenosine 3',5' diphosphate was found (0.38 μ moles), as well as traces of cytidine 3',5' diphosphate (0.03 μ moles), and guanosine 3',5' diphosphate (0.005 μ moles).

Four different methods were used to determine the identity of the nucleoside 3',5' diphosphates. These were paper chromatography in systems A and C, electrophoresis, and determination of absorption spectra. Each method indicated the 5' end group to be predominantly adenosine 3',5' diphosphate. The absorption spectrum (220-300 nm) of the wheat sample rRNA 5' terminus and of the adenosine 3',5' diphosphate standard are shown in Figure 7. A close correspondence at both the maximum and minimum wavelengths of absorbance was observed.

The absorption spectra of samples eluted from paper chromatograms developed in system A were taken without prior desalting. This practice yielded generally expected spectra without incurring loss due to additional

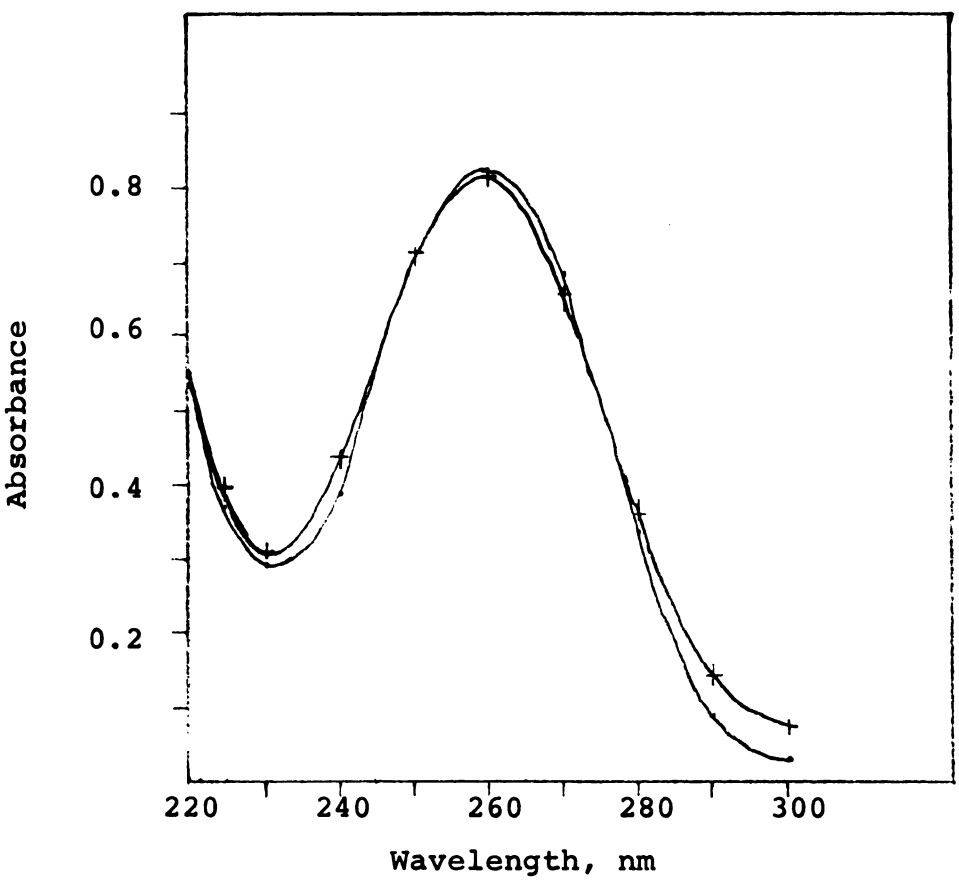


Fig. 7.--Absorption spectra (220-300 nm) for standard adenosine 3',5' diphosphate (·——·) and for material eluted from wheat rRNA hydrolysate (+——+). Maximal absorbance for both the adenosine 3',5' diphosphate standard and for the sample occurs in the region of 260 nm.

desalting procedures. Nucleotides chromatographed in system A were eluted and subsequently developed in system C, also without prior desalting. In systems A and C, co-migration of the sample with adenosine 3',5' diphosphate standard was observed (Table 11). Samples to be subjected to paper electrophoresis were desalted however. Co-migration of the sample and ADP was also observed in the paper electrophoretic system.

The amounts of guanosine 3',5' diphosphate and cytidine 3',5' diphosphate released were insufficient to perform all of the above tests. Good correspondence was observed between these samples and their respective standards in system C, paper electrophoresis, and in absorption spectra profiles (Table 11). Traces of guanosine 3',5' diphosphate and cytidine 3',5' diphosphate were observed only from wheat embryo supplied by Quaker Oats. It is interesting to note that in addition to the predominant ADP, only a trace of CDP and no GDP were released from 494 mg of Quaker rRNA, whereas 632 mg of Quaker rRNA revealed traces of both CDP and GDP, the CDP being present in a greater amount.

TABLE 11.--Identification of nucleoside 3',5' diphosphates resulting from alkaline hydrolysis of wheat embryo rRNA.

| System of Identification | Nucleoside 3',5' Diphosphates | | | Hydrolysate Component | | |
|---|---|---|---|---|---|---|
| | ADP | GDP | CDP | 1 | 2 | 3 |
| 1. Migration in System A (cm) | 12.8 | 6.6 | 5.0 | 12.8 | | |
| 2. Migration in System C (cm) | 19.4 | 23.3 | | 19.4 | 23.7 | 29.1 |
| 3. Migration in Electrophoresis (cm) | 13.6 | 16.3 | 12.5 | 13.6 | 16.7 | 12.5 |
| 4. Absorption Spectrum at pH 7.0 | | | | | | |
| max. absorbance | 259 | 253 | 270 | 260 | 255 | 267 |
| min. absorbance | 227 | 223 | 247 | 228 | 225 | 245 |
| Identity of the Nucleoside 3',5' di-P | | | | ADP | GDP | CDP |
| Yield μ moles | | | | 0.38 | 0.005 | 0.03 |
| The theoretical yield using average chain length of 2250, is 0.44 μ moles | | | | | | |

## 5' Termini of Cauliflower rRNA

Ribosomal RNA isolated by direct extraction of cauliflower florets with phenol and solution A was freed of contaminating DNA and low molecular weight RNA by successive washes with 1 M NaCl.

The cauliflower rRNA hydrolysate was fractionated by ion exchange chromatography on DEAE cellulose ($Cl^-$) with a linear (0.0-0.3 M) NaCl gradient. The elution profile is seen in Figure 8. Replicate columns were run with 500 mg of cauliflower ribosomal RNA. Nucleosides detected from the 3' end were adenosine and uridine; a trace of cytidine was also detected. A component of the hydrolysate was found to migrate adjacent to the guanosine standard in system A. However, the absorption spectrum of this spot did not coincide with those of guanosine.

Adensoine 3',5' diphosphate was the only 5' end group detected in these samples; the average amount from two 500 mg samples of rRNA was 0.198 μ moles. The data leading to the identification of ADP as the 5' terminus of cauliflower rRNA is presented in Table 12. It can be seen again, that the relative amounts of the 3' and 5' termini compare favorably (Table 13).

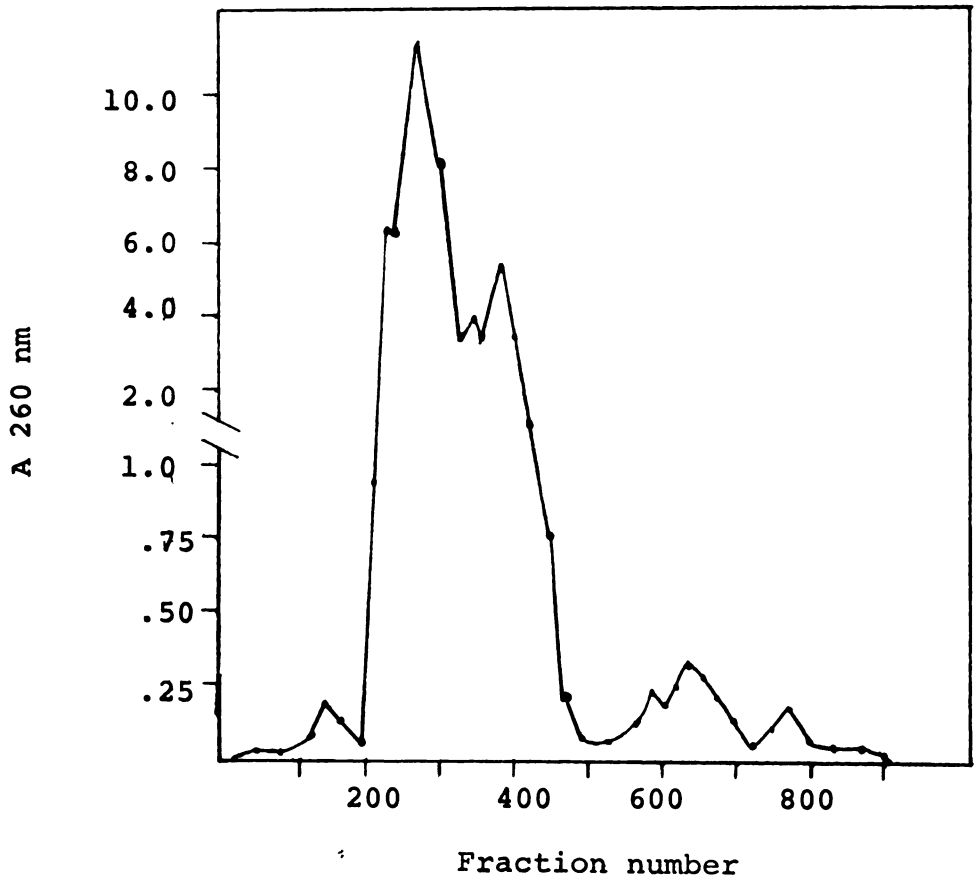


Fig. 8.--Elution pattern of neutralized RNA hydrolysate from DEAE cellulose ($Cl^-$) effected by a linear NaCl gradient.

A neutralized solution from the hydrolysis of 500 mg of RNA was applied to the column (8 x 17.5 cm) and subsequently eluted with a linear NaCl gradient (0.0-0.3 M). Peak fractions were desalted and chromatographed for identification.

TABLE 12.--Identification of nucleoside 3',5' diphosphates resulting from cauliflower floret rRNA by comparison the properties with those of adenosine 3',5' diphosphate.

| System of Identification | Nucleoside 3',5' di-P (ADP) | Hydrolysate Component |
|---|---|---|
| 1. Migration in System A (cm) | 7.0 | 6.9 |
| 2. Migration in System C (cm) | 19.3 | 19.1 |
| 3. Migration in Electrophoresis rerun (cm) | 12.7<br>13.1 | 13.1<br>13.1 |
| 4. Absorption spectrum at pH 7.0 | | |
| max. absorbance | 259 | 259 |
| min. absorbance | 227 | 230 |
| Identity of the Nucleoside 3',5' di-P | | ADP |
| Yield | | 0.17 μ moles |
| Theoretical yield | | 0.33 μ moles |

TABLE 13.--Comparison of the quantitative recover of 3' nucleoside termini and 5' terminal 3',5' nucleoside diphosphate.

| RNA Source | Preparation | 3' Terminus | 5' Terminus |
|---|---|---|---|
| Wheat Embryo | IV | 0.19 μ moles | 0.17 μ moles |
| Cauliflower Florets | I & II (ave.) | 0.17 μ moles | 0.19 μ moles |

A summary of the data on the 5' termini of plants investigated is presented in Table 14. The only 5' terminal group observed for unfractionated Randall wheat embryo rRNA and cauliflower floret rRNA was ADP. In Quaker wheat embryo rRNA the 5' terminus was predominantly ADP; traces of CDP and GDP were also observed. The data may be interpreted to mean that ADP forms the 5' end of both the 25S and 16S RNA species.

TABLE 14.--Summary of data on the 5' termini of ribosomal RNA.

| Source of RNA | Type of Fractionation* | Prep. | mg | Nucleoside 3',5' di-P detected | | | |
|---|---|---|---|---|---|---|---|
| | | | | ADP | GDP | CDP | UDP |
| Wheat Embryo | | | | | | | |
| Randall | A | I | 443 | ++ | - | - | - |
| Randall | A | II | 470 | ++ | - | - | - |
| Randall | C | III | 619 | ++ | - | - | - |
| Quaker | B | IV | 494 | ++ | - | tr# | - |
| Quaker | A | V | 632 | ++ | tr | tr | - |
| Cauliflower floret | B | VI | 500 | ++ | - | - | - |
| Cauliflower floret | B | VII | 500 | ++ | - | - | - |

*A: DEAE cellulose (formate); bulk elution by salt cuts
B: DEAE cellulose ($Cl^-$) elution by a linear (0.0-0.3 M NaCl) gradient
C: G-15 Sephadex Gel Filtration

#tr: represents trace amounts observed

# DISCUSSION

## RNA Isolation Procedures

The 5' termini of 25S and 16S rRNA comprise approximately one out of 2250 nucleotides. It was therefore critical that isolation procedures provided large quantities of non-degraded RNA of high purity. A comparison of absorbancy at 260 and 280 nm gave an indication of the amount of protein present. There was no noticeable breakdown in RNA samples stored for as long as two years, at 4 C. Two direct experimental checks for molecular integrity, sucrose density gradient centrifugation and polyacrylamide gel electrophoresis, were employed. In addition, a check for procedural consistency was made. Comparisons of replicate extractions of RNA from given plant sources indicated that consistent quantitative yields were achieved. Sucrose density gradient centrifugation and acrylamide gel electrophoresis provided stringent checks on the intactness and purity of the RNA. The presence of anomolous bands or peaks was taken to indicate either the presence of

contaminating nucleic acids or breakdown of high molecular weight RNA. Ribosomal RNA preparations satisfactory for use in end group analysis, i.e., lacking contaminating nucleic acids, gave no evidence of molecular breakdown or the presence of DNA or low molecular weight RNA. The area encompassed by the profile of the 25S peak was approximately twice that of the 16S peak.

Extraction of RNA by each of the three methods used, gave a high quality product free of DNA and sRNA. The phenol-solution A method, when used on fleshy plant parts, yielded a clean product which could be resolved as expected by sucrose density gradient centrifugation. DNA and low molecular weight RNA also released by this method were cleanly separated from the high molecular weight RNA by washes with 1 M NaCl. Tissues having high carbohydrate content, such as wheat embryo, were extracted by the phenol-phosphate method. Degradation of the 25S RNA species was observed under conditions of prolonged (50 minutes) shaking. When the suspension of tissue from which the RNA was to be extracted was shaken at high speed for only 20 minutes at room temperature, before cooling on ice, a high quality RNA product free of DNA was obtained. The NDS-phenol-cresol method used for extraction of

germinated tissue gave a high quality RNA product, without releasing DNA. This method seems particularly well suited for handling low molecular weight RNA. However, it is time-consuming and much more costly to run than the two methods previously discussed.

## Alkaline Hydrolysis of rRNA

RNA molecules cleaved by this method yield a 5' terminal nucleoside 3',5' diphosphate, intermediate nucleoside 3' monophosphates and a nucleoside at the 3' terminus. Hydrolysis begins with the formation of a cyclic 2',3' nucleoside monophosphate intermediate, by the cleavage of the phosphodiester chain linkage adjacent to the 5' position. Alkaline hydrolysis depends, therefore, on the presence of a free hydroxyl at the 2' position of the ribose moiety. This characteristic endows the process with a specificity which distinguishes between RNA and DNA or nucleotides in which the 2' hydrosyl group has undergone substitution, e.g., by a methyl group.

Some serious complications can occur, however, during alkaline hydrolysis. In the presence of high

concentrations of alkali and heat, cleavage of the phosphate linkage occurs, with the formation of inorganic phosphate and the nucleoside (78). Less severe conditions of hydrolysis (1M KOH) may result in a transfer of phosphate from one group to another (79). Prevention of such transfer is critical when identification of the termini is based on the degree of phosphorylation. Several reports in the literature (30,37) may well be anomolous because of a failure to consider the possible effects of 1 M base on phosphate transfer. For example, studies on wheat germ using 1.0 M alkali reported that all of the four nucleosides are present at the 3' terminus. These results are in contrast to data indicating a preferential termination at that position by adenosine and uridine (32,61,80-82).

## Nucleotide Phosphotransferase

Phosphorylation of nucleotides by enzymatic low energy phosphate transfer provided the means for large scale synthesis of nucleoside 3',5' diphosphate standards. These standards are necessary for the identification of the 5' terminal groups of rRNA released by alkaline hydrolysis but were unavailable at the beginning of this study.

The nucleotide phosphotransferase fraction extracted from E. coli contained a phosphatase. In contrast to reports which suggested that a single enzyme from other sources may have both hydrolase and transferase activities (77,83), the methods used in this study resulted in the detection of distinct peaks of activity for phosphatase and for nucleotide phosphotransferase. The degree of enzyme purification was such that hydrolase activity was never completely removed, however. Addition of excess phosphate substrate was sufficient to counteract the residual hydrolase.

Resistance to oxidation by sodium m-periodate indicated that phosphorylation had occurred at the 3'(2') position. Synthesis of nucleoside 3',5' diphosphates by this technique proved to be both inexpensive and efficient.

## Separation and Identification of Ribosomal RNA Terminal Groups

The techniques utilized for the separation of the rRNA hydrolysate components were based on differences of charge or degree of ionization of the components at a given pH. For example, the nucleosides, nucleoside

monophosphates, dinucleotides, trinucleotides, and nucleoside 3',5' diphosphates at pH 7.8 have net charges of 0, -2, -3, -4, and -4 respectively (37). These charges, as indicated in the Literature Review are based primarily on the primary and secondary phosphate groups. Chromatographic and electrophoretic separations also exploit the degree of ionization of the amino groups of adenine, guanine, and cytosine as well as the enolic groups of guanine, cytosine, and uracil.

Fractionation of rRNA hydrolysate by gel filtration is based on separation of substances by molecular weight and particle size. Gel filtration (Sephadex G-15) was used to check separation achieved by ion exchange methods. It is noteworthy that the results of the two methods concurred (Table 13). Disadvantages of the gel filtration method include (a) a low column load capacity; e.g., only 8-10 mg of RNA hydrolysate out of a total sample of 600 mg, may be applied at one time, (b) the resultant large number of sample transfers may result in a decreased recovery.

Implicit in most of the considerations of the 5' termini is the assumption that the end group released by alkaline hydrolysis is very likely a nucleoside 3',5'

diphosphate. The possibility also existed that the terminal group might be a triphosphonucleotide (pppNp), a dinucleotide triphosphate (pNpNp), or a diphosphonucleotide (ppNp). In each of these cases, the product of hydrolysis would be eluted from a DEAE cellulose column some time after the nucleoside 3',5' diphosphates. These latter fractions were examined for rapidly migrating components in system C and electrophoresis, with negative results. While this does not completely exclude the existence of these groups, the possibility seems remote.

Experimental losses during fractionation of the RNA hydrolysate occurred due to the numerous transfer and desalting steps. It may be important to note, however, that no significant preferential losses affecting the percentage yield of nucleoside 3',5' diphosphates were observed. A factor quite significant in monitoring the recovery of the nucleoside 3',5' diphosphates was the quantitative comparison with the nucleoside fraction. The amount of nucleoside diphosphate recovered from the rRNA 5' terminus was equivalent to the amount of nucleosides recovered from the 3' terminus (Table 12).

## The Termini of Ribosomal RNA

Unfractionated cauliflower ribosomal RNA has been reported to have predominantly adenosine and uridine at the 3' terminus (80-82). In the present study, these same bases were found as the predominant groups present. In addition, material was found which co-migrated with the guanosine standard. However, the absorption spectrum for this material did not correspond to that of the guanosine standard. Adenosine and uridine have previously been reported as the predominant 3' terminus for E. coli (61), L cells (38), and mammalian reticulocytes (83).

In the studies reported here, adenosine 3',5' diphosphate was the only 5' terminal group obtained following alkaline hydrolysis of unfractionated (25S and 16S) cauliflower rRNA. This was demonstrated by its co-migration with authentic standards in two paper chromatographic solvent systems, paper electrophoresis, Dowex-1 ion exchange chromatography, and absorption spectra at 220-300 nm. Similarly, adenosine 3',5' diphosphate was also found at the predominant end group obtained from two different sources of wheat embryo rRNA. Traces of guanosine 3',5' diphosphate and cytidine 3',5' diphosphate

were also found when large quantities of Quaker wheat embryo rRNA was hydrolyzed. The relative yield of nucleoside 3',5' diphosphate compared favorably with the total amount of nucleosides obtained from the 3' end.

In conclusion, the 5' termini of ribosomal ribonucleic acids appear to be adenosine 3',5' diphosphate in both higher plant species tested. Purines are commonly the 5' bases initiating RNA molecules synthesized *in vitro* with *E. coli* DNA-dependent RNA polymerase. Purines were also reported as the 5' termini for RNA isolated from several bacteriophages and some plant viruses. Likewise, both the 16S and 23S rRNA species from *E. coli* were reported to contain purines at the 5' terminus.

While the presence of a purine base at the 5' terminus of rRNA may be a tool useful in the study of RNA initiation, it should be mentioned that the phenomenon is not universal. Pyrimidines have also been reported at the 5' terminus of *Bacillus* rRNA and both the 16S and 23S rRNA species of L cells.

## Future Applications

The observation that adenosine 3',5' diphosphate is the 5' end group of both plant species studied suggests that a mechanism common to both may control the initiation of synthesis or specific enzymatic cleavage of these RNA molecules. As indicated earlier, *in vitro* RNA synthesis is initiated by a purine at the 5' terminus. Transfer RNA synthesis also begins with a purine base. It appears therefore that in these cases, the RNA terminus is complementary to a pyrimidine on the DNA chain. One may speculate then that knowledge of the 5' terminus of RNA may aid our understanding of the process controlling initiation of RNA.

One immediate application of these results may be to study RNA-protein interactions within the ribosome. Data on ribosomal proteins have very recently been published which in conjunction with both 5' and 3' RNA terminal group analysis may be used to determine whether specific rRNA termini are related to the activity of ribosomes. These experiments would involve isolation and fractionation of ribosomes to separate and purify the ribosomal proteins and the rRNA. Selective blockage of

a specific chain terminus of rRNA could then be followed by reassembly of the ribosomal proteins and nucleic acids to form intact ribosomes. The latter could then be tested for ability to translate from a synthetic messenger RNA to produce specific amino acid sequences.

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## LITERATURE CITED


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# APPENDIX

## APPENDIX A

### Model 1 Analysis of Variance, Comparison of Means and Groups of Means of Nucleoside Diphosphate % Recovery

| | ADP | GDP | UDP | CDP | (a) |
|---|---|---|---|---|---|
| (n) observation 1 | 81.5 | 77.5 | 92.0 | 74.5 | 325.5 |
| 2 | 84.0 | 53.5 | 85.5 | 81.5 | 304.5 |
| | 165.5 | 131.0 | 177.5 | 156.0 | |

Anova

1. $\sum_{a}^{n}\sum Y = 630.0$ Grand Total

2. $\sum_{a}^{n}\sum Y^2 = 50,534$ Sum of squared observations

3. $\frac{\sum_{a}^{n}\sum(Y)^2}{m} = 50,312$ Sum of squared groups/n

4. $\frac{\sum_{a}^{n}\sum(Y)^2}{an} = 49,612$ Correction term

5. $\sum_{a}^{n}\sum Y^2 - CT = 50,534 - 49,612 = 922$ Sums of Squares (Total)

$$6.\quad \sum_{an}\sum Y^2 - \frac{\left(\sum_{an}\sum Y\right)^2}{an} = \begin{array}{r} 50,312 \\ \underline{49,612} \\ 700 \end{array}$$

Sums of squares (Groups)

$$7.\quad \left[\sum_{an}\sum Y^2 - CT\right] - \left[\sum_{an}\sum Y^2 - \frac{\left(\sum_{an}\sum Y\right)^2}{an}\right] = \begin{array}{r} 922 \\ \underline{700} \\ 222 \end{array}$$

Anova Table

| Source of variation | | df | SS | MS | Fs |
|---|---|---|---|---|---|
| $\bar{Y}-\bar{\bar{Y}}$ | Between groups among treatments | 3 | 700 | 233 | $\frac{\text{MS groups}}{\text{MS within}} = 4.19$ |
| $Y-\bar{Y}$ | Within groups (error, replicates) | 4 | 222 | 55.5 | ns |
| $Y-\bar{\bar{Y}}$ | | 7 | 922 | | |

$F.05(3,4) = 6.59$ $F.01(3,4) = 16.7$ $F.001(3,4) = 56.2$

## APPENDIX B

### List of Abbreviations and Symbols

| | |
|---|---|
| rRNA | Ribosomal ribonucleic acid |
| mRNA | Messenger ribonucleic acid |
| tRNA | Transfer ribonucleic acid |
| *E. coli* | *Escherichia coli* |
| (S) | Svedberg units |
| pNp | Nucleoside 3'5' diphosphate |
| pNpN | Dinucleotide phosphate |
| NpmeN | Methylated dinucleotide |
| pNpNpNpN | Oligonucleotide resulting from nucleosides linked by phosphodiester bonds |
| NDS | Naphthalene 1,5 disulfonate |
| DEAE | Diethylaminoethyl |
| Tris | Tris (hydroxymethyl) aminomethane |
| EDTA | Ethylenediaminetetraacetic acid |
| O.D. | Optical density |
| NMP | Nucleoside monophosphate |