THE 5' -LINKED TERMINI OF PLANT RIBOSOMAL RIBONUCLEIC ACIDS

Thesis for the Degree of Ph. D.

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JOHN McDONELL HALLOIN

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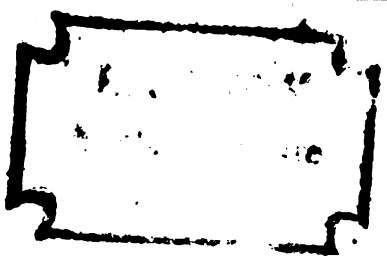
THESIS



This is to certify that the

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JOHN McDONELL HALLOIN

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Major professor

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ABSTRACT

THE 5'-LINKED TERMINI OF PLANT
RIBOSOMAL RIBONUCLEIC ACIDS

by John McDonell Halloin

This thesis results from an investigation of the 5'-linked termini of plant ribosomal ribonucleic acids (r-RNA). The objectives were to determine (a) the identity of 5'-linked termini, (b) if plant r-RNA's are preferentially terminated by a particular nucleoside or nucleosides and (c) if the same termini occur on r-RNA from several plant species. RNA was obtained from microsomal pellets of cauliflower, cabbage and mushroom (Agaricus campestris) by extraction and deproteinization with phenol-sodium dodecyl sulfate. It was hydrolyzed with 0.3 N KOH, and the hydrolysate was neutralized with a sulfonic acid resin, Dowex 50-H^+. Nucleosides released from 5'-linked chain terminal positions by KOH hydrolysis were separated and identified by paper chromatograph and spectrophotometry.

Adenosine and uridine were the only nucleosides obtained upon alkaline hydrolysis of unfractionated (18 S + 28 S) cauliflower r-RNA, and adenosine was approximately twice as abundant as uridine. Similarily, only adenosine

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and uridine were isolated from hydrolysates of unfractionated r-RNA from cabbage and mushrooms, however, in both of these cases uridine was approximately twice as abundant as adenosine. Studies on the individual subunits of cauliflower r-RNA, after separation on sucrose density gradients, revealed that both adenosine and uridine terminate 28 S r-RNA. Phosphorylation of the nucleoside fraction from 18 S r-RNA with ^{32}P-polypohosphate revealed that uridine is present as a 5'-linked terminus on this RNA: cytidine was not detected. This method is applicable only for the detection of pyrimidine nucleosides, as purine nucleosides are not phosphorylated by polyphosphate. Other studies in our laboratory showed that adenosine and uridine are also the only nucleosides isolated from alkaline hydrolysates of parsnip and spinach r-RNA.

The results presented above suggested that all plant r-RNA's may terminate preferentially in adenosine and uridine, however, other laboratories reported that all four ribonucleosides were isolated from alkaline hydrolysates of wheat germ r-RNA. A study was made, therefore, to determine the 5'-linked termini of wheat germ r-RNA, using the techniques employed in our laboratory. RNA was isolated from wheat germ by direct phenol extraction of raw wheat

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germ by direct phenol extraction of raw wheat germ. Adenosine and uridine, plus a small amount of cytidine (5 percent of the total nucleoside) were the only nucleosides isolated from preparations of wheat germ r-RNA which had been shown to be free of contamination by DNA and s-RNA. Another preparation of wheat germ r-RNA, contaminated with both DNA and s-RNA, yielded all four nucleosides upon alkaline hydrolysis. All four nucleosides were also isolated, following hydrolysis with 1 M NaOH and neutralization with 3 N HCl (the procedure used in other laboratories), from still another wheat germ r-RNA preparation. Since the latter results were similar to those reported by others, dephosphorylation of nucleosides during neutralization with HCl was the suspected cause of the appearance of all four nucleosides in their studies.

The preferential termination with adenosine and uridine of all r-RNA's studied suggested that addition of termini subsequent to RNA chain synthesis might occur with r-RNA by a method similar to that known for s-RNA. A series of experiments was done using cauliflower r-RNA labeled with 2-^{14}C-uridine in the presence and absence of actinomycin D, which has been shown to inhibit DNA-dependent RNA synthesis,

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to determine if end-group addition occurred. These experiments were based on the assumption that if 5'-linked termini are added after r-RNA chain synthesis, the ratio of terminal to non-terminal ^{14}C-uridine will be higher with r-RNA chains labeled under conditions of actinomycin D-inhibited DNA-dependent RNA synthesis than with r-RNA chains labeled in the absence of such inhibition. This would be expected, because terminal addition would occur at a proportionately higher rate under conditions of inhibition than in the absence of inhibition. The same ratio of ^{14}C-nucleoside: ^{14}C-nucleotide was obtained upon alkaline hydrolysis of cauliflower r-RNA labeled under both inhibited and uninhibited conditions, suggesting that addition of termini subsequent to ribosomal chain synthesis is not responsible for the preferential termination of cauliflower r-RNA.

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THE 5'-LINKED TERMINI OF PLANT RIBOSOMAL RIBONUCLEIC ACIDS

by

John McDonell Halloin

A THESIS

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INTRODUCTION

The termination of soluble ribonucleic acid (s-RNA) (5, 26, 27, 29, 32, 46, 78, 103, 104) and tobacco mosaic virus RNA (TMV-RNA) (79, 88, 89, 96) with specific nucleotides is well established. Information on the termini of ribosomal RNA (r-RNA), with the exception of wheat germ r-RNA (45, 46, 49, 80), was not available prior to the start of these investigations; and although the role of ribosomes in protein synthesis is well established, little is know of either the primary structure or function of r-RNA.

Evidence existed that the base ratios of r-RNA's from different species were different, as were those of the r-RNA subunits from a single species (14, 66, 67). It has been established that there is a multiplicity of DNA sites for the synthesis of r-RNA (2, 101, 102), and that the subunits are synthesized on different sites (2, 102). Based on these facts, an investigation was undertaken to determine the identity of plant r-RNA termini. It was conceived that such an investigation might lead to a more intimate knowledge

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of the role of the RNA, if any, in protein synthesis, or possibly contribute to knowledge concerning the synthesis of r-RNA. For example, preferential termination of all r-RNA with a single nucleoside could be indicative either that this nucleoside participates somehow in binding of the r-RNA to ribosomal protein, or that the nucleoside could be necessary for biological activity. Finally, since the 5'-linked nucleoside is apparently the last one attached to a growing chain of RNA, such information could contribute to knowledge of the mode of biosynthesis of r-RNA.

An abstract of the first phase of this investigation, dealing with the nature of 5'-linked termini of plant r-RNA's, has been published (69), and these results together with the results of others (36, 43, 47, 53, 59, 62), showed that the 5'-linked end of r-RNA chains terminate preferentially in adenosine and uridine. Because our results were quite different from the results obtained with wheat germ r-RNA by Lane and co-workers (45, 46, 80) and by Lee and Gilham (49), and because their isolation and alkaline hydrolysis procedures differed from the methods usually employed in this thesis, we reinvestigated the 5'-linked termini of wheat germ r-RNA using several isolation procedures and two different procedures of alkaline hydrolysis.

The existence of enzymes which preferentially add the sequence p-cytidine-p-cytidine-p-adenosine (CCA) to the 5'-linked termini of s-RNA's (11, 12, 35, 26, 27, 55), and enzymes from rat liver which add homopolymer chains to the 5'-linked termini of snythetic and natural polyribonucleotides, including r-RNA (40, 41, 65); together with the observation that growth conditions could influence the proportion of 5'-linked terminal uridine in r-RNA from Escherichia coli (60), suggested to us that such an enzyme might be involved in the addition of uridine to the 5'-linked termini of plant r-RNA's. Accordingly, ^{14}C-labeled uracil was incorporated into RNA by cauliflower hypocotyls, in the presence and absence of actinomycin D. It was hoped that by inhibiting DNA-dependent r-RNA synthesis with actinomycin D, it would be possible to detect any addition of uridine to the 5'-linked terminal group.

LITERATURE REVIEW

The Primary Structure of Ribosomal RNA

The structure of ribosomes and the ribonucleic acids associated with them have been reviewed by Ebel (20), Peterman (66), Spirin (81, 82) and Ts'o (92). Ribosomes are subcellular particles which have sedimentation coefficients between 70 and 80 S, and are composed of 40 to 60 percent protein and 40 to 60 percent RNA. Lowering the magnesium ion concentration in the surrounding medium causes a dissociation of the particles into two unequal subunits; one with a sedimentation coefficient of 30 to 40 S, and the other with a sedimentation coefficient of 50 to 60 S. The smaller subunits contain RNA molecules with sedimentation coefficients varying from 13 to 19 S, which corresponds to molecular weights of 5 to 7 x 10^5, or 1400 to 2000 nucleotides; whereas RNA molecules isolated from the larger subunits have sedimentation coefficients of 23 to 34 S and molecular weights of 1.0 to 1.5 x 10^6, corresponding to 3000 to 4500 nucleotides.

End group studies on wheat germ r-RNA by Lane and co-workers (45, 46, 80) and by Lee and Gilham (49) showed one 5'-linked end group for each 1200 1500 nucleotides. McIlreavy and Midgley (53) found one 5'-linked terminus for each 1200 to 1500 nucleotides in the larger r-RNA subunit from E. coli. Midgley (56, 58) found that two smaller RNA molecules corresponding in size to the smaller r-RNA subunit, were produced when the larger subunit of E. coli r-RNA was incubated at pH 9, in the absence of magnesium ion or in the presence of ethylenediamine tetraacetic acid (EDTA). These results suggested that the larger molecules may be discontinuous, being composed of two RNA chains similar to the smaller subunit. Beck et al. (3), have demonstrated, however, that when yeast r-RNA is isolated and incubated in the presence of polyvinyl sulfate, which inhibits ribonuclease, no dissociation of the large subunit occurs. This indicates that in the absence of enzymatic or chemical hydrolysis, the larger subunit is a continuous polynucleotide chain.

The existence of a difference in base ratios between the large and small subunits (14, 66, 67) is evidence that the larger subunit is not a dimer of smaller subunits. Further evidence are the results of Yankofsky and Spiegelman

(102), who found through hybridization studies that the two subunits of Bacillus megaterium r-RNA are non-competitive in their binding to DNA. They concluded that the two subunits are synthesized on different genetic loci.

A third r-DNA subunit associated with the larger ribosomal subunit has recently been isolated from E. coli (9, 17, 22, 77), KB cells (22) and Blastocladiella emersonii (17). This r-RNA is 100 to 105 nucleotides in length and has a sedimentation coefficient of 5 S. It differs from s-RNA in that it lacks methylated bases and amino acid accepting activity.

The generalized structure of RNA may be represented as a continuous polynucleotide chain, in which the ribonucleosides adenosine (A), guanosine (G), cytidine (C) and uridine (U) are joined together by phosphodiester linkages between the 5' position of one nucleoside and the 3' position of the nucleoside adjacent to it. The nucleoside in the 3'-linked terminal position contains a 5'-lined phosphomonoester and a hydroxyl group in the 2' position, whereas the 5'-linked terminal nucleoside contains hydroxyl groups in both the 2' and 3' positions. Non-terminal nucleosides contain hydroxyl groups only in the 2' position. This is illustrated in Figure 1A. Alkaline hydrolysis of various

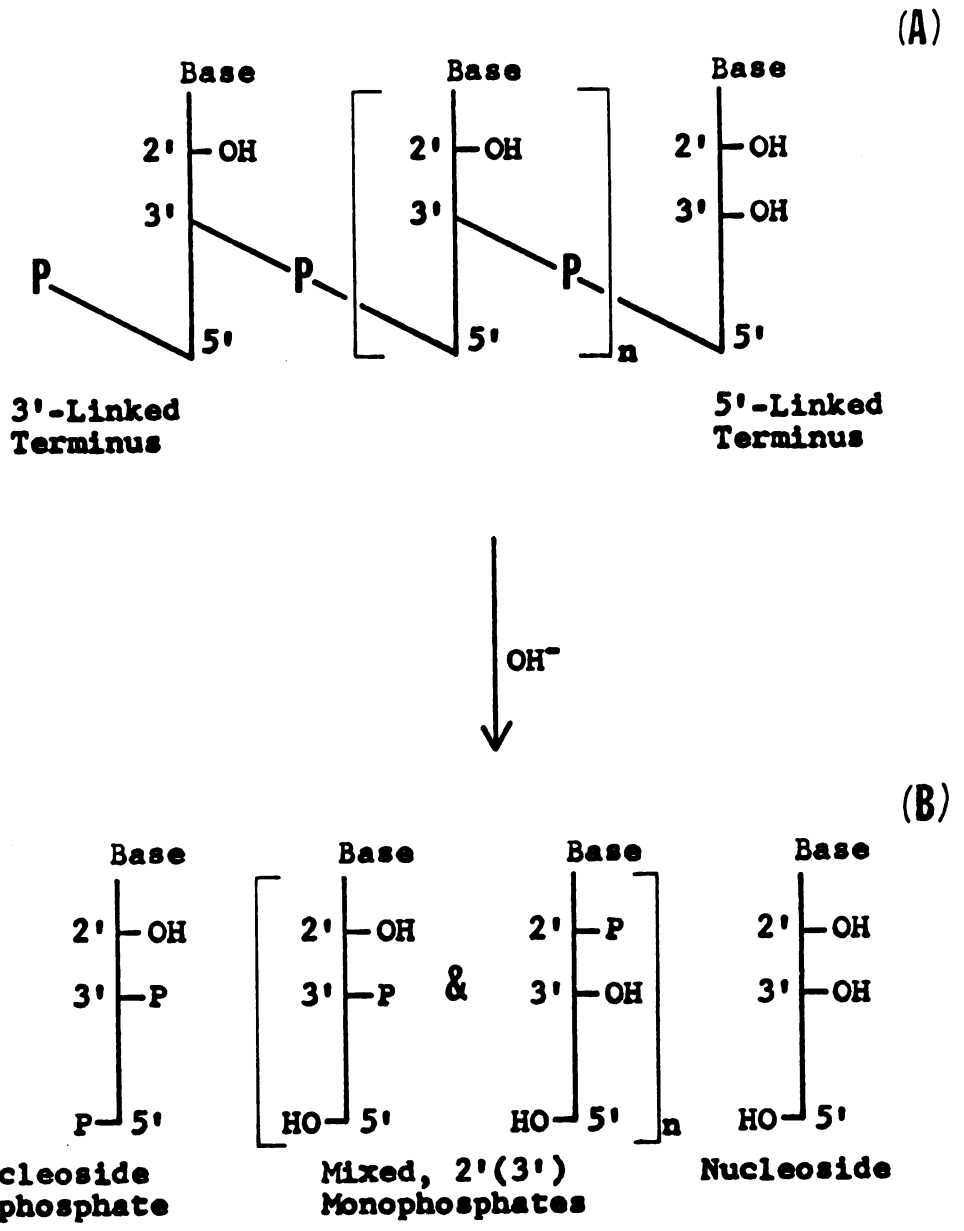

Figure 1: (A) Stylized representation of an RNA molecule, adapted from Sugiyama and Fraenkel-Conrat (88). (B) Products of alkaline hydrolysis of RNA: nucleoside-2'(3'),5'-diphosphate from 3'-linked terminus, mixed 2'(3')-monophosphates from internal positions and nucleoside from 5'-linked terminus.

RNA's has confirmed this structure (see later discussion of alkaline hydrolysis in Literature Review), as both nucleosides and nucleoside-2'(3'),5'-diphosphates were obtained upon alkaline hydrolysis of r-RNA from E. coli (9, 62, 77) L. cells (47), and wheat germ (45, 80), as well as s-RNA from E. coli, yeast (5), rat liver (29), rabbit liver (78) and wheat germ (46). The only exception to this structure, observed to date, is TMV-RNA. Sugiyama and Fraenkel-Conrat (88) observed that no phosphate was released when TMV-RNA was incubated with E. coli alkaline phosphatase, indicating that this RNA molecule is not phosphorylated at either terminus. This was substantiated by the observation that nucleosides are released both by alkaline hydrolysis (88), and venom phosphodiesterase hydrolysis (89) of TMV-RNA. These hydrolytic procedures release nucleosides from non-terminally phosphorylated 5'-linked and 3'-linked termini respectively.

The rest of this review is divided into three parts. The first part deals with methods of detecting RNA termini, the second with the nature of RNA termini and the third with the enzymatic addition of terminal groups to RNA.

Methods of Detecting RNA Termini

RajBhandary and Stuart (70) have recently reviewed methods used to detect RNA termini. Most of these methods depend upon the existence of polynucleotides of the type illustrated in Figure 1A, and many of the methods are used in conjunction with each other.

Enzymatic Methods

1. *Escherichia coli* alkaline phosphatase

Literature dealing with *E. coli* alkaline phosphatase has been reviewed by Hummel and Kalnitsky (33). The enzyme is synthesized adaptively when *E. coli* cells are grown on phosphate-deficient media. Heppel *et al.* (28) found that the purified enzyme acts nonspecifically, only on phosphate monoesters and is therefore a useful tool for the removal of terminal phosphates from nucleic acid chains, thereby rendering the termini accessible to certain reagents used for detecting terminal groups. Sugiyama and Fraenkel-Conrat (88) used this enzyme on ^{32}P-labeled TMV-RNA, and determined that neither terminus is phosphorylated in this RNA.

2. Snake venom phosphodiesterase

The action of phosphodiesterase from the venom of *Crolatus sp.* (rattlesnake) and other snakes has been reviewed

by Laskowski (48). This enzyme acts primarily as an exonuclease, starting at the end of the chain bearing a free 3' hydroxyl group, and sequentially liberating 5' nucleoside monophosphates (73). If RNA is incubated first with E. coli alkaline phosphatase, and then with venom phosphodiesterase, the 3'-linked terminus will be released as a nucleoside, while all other chain components will be released as monophosphates (89). Another use of the enzyme is for the sequential degradation of RNA, with subsequent identification of released nucleotides, to determine 5'-linked termini (23, 79). Singer and Fraenkel-Conrat (79) recommended that this enzyme be used at 0° C for sequential degradation. Disadvantages to this procedure, however, are that values for number of cleavages are only average values and do not represent identical degradation of all chains (79, 84), and also, even purified preparations of the enzyme exhibit limited amounts of endonuclease activity (73, 79). This internal cleavage appears to occur preferentially at sequences of the type -pUpA$\overset{\downarrow}{p}$X-, which could lead to disproportionately high values for terminal A and U (79). This enzyme was utilized by Sugiyama and Fraenkel-Conrat (89) to identify the 3'-linked terminus of TMV-RNA.

3. Spleen phosphodiesterase

The action of spleen phosphodiesterase has been studies by Razzell and Khorana (74). This enzyme acts in a manner opposite to that of venom phosphodiesterase, as it hydrolyzes nucleic acids by sequential attack from the end bearing a free 5' hydroxyl group and releases nucleoside 3' monophosphates. The 3'-linked terminus may be freed of its 5'-linked phosphate by the action of E. coli alkaline phosphatase. A 5'-linked terminus with a free 3' hydroxyl group is released as a nucleoside, which may be separated from the nucleotides. No endonuclease activity is detected in purified preparations of this enzyme.

4. Pancreatic ribonuclease

The mechanism of action of pancreatic ribonuclease has been reviewed by Witzel (99) and by Hummel and Kalnitsky (33). This enzyme cleaves phosphodiester linkages of the type pyrimidine-p-X, giving as products pyrimidine-3'-monophosphates and oligonucleotides of the type -X-p-pyrimidine-p. Nucleosides are released from the 5'-linked terminus, if the penultimate base is a pyrimidine. If the penultimate base is a purine, the oligonucleotide will contain 2' and 3' hydroxyls on the 5'-linked terminus. Hydrolysis by this enzyme involves the formation of a cyclic

pyrimidine-2'(3')-phosphodiester, which then gives rise, preferentially, to the pyrimidine 3'-phosphomonoester. Hunt (36) has utilized this technique in conjunction with isonicotinic acid hydrazide-labeling to determine the 5'-linked terminal and penultimate nucleosides in rabbit reticulocyte r-RNA.

5. Polynucleotide kinase

Polynucleotide kinase has been isolated from cell-free extracts of E. coli infected with bacteriophage T_2 (63) or T_4 (75). This enzyme catalyzes the transfer of phosphate from adenosine triphosphate (ATP) to the free 5' hydroxyl position of a 3'-linked terminal nucleoside of either RNA or DNA. Selectivity of the enzyme is limited, however, as it will also transfer phosphate, at a reduced rate, to the 5' position of adenosine-2(3')-monophosphate, but not to adenosine or cytidine (75). This enzyme has been used to transfer ^{32}P from γ-^{32}P-ATP to RNA in attempts to identify 3'-linked nucleosides (86, 90). RNA in these studies was first incubated with E. coli alkaline phosphatase to remove the 5'-linked terminal phosphate moieties.

6. Nucleoside phosphotransferase

Brawerman and Chargaff (7) isolated an enzyme from malt diastase which catalyzes the transfer of phosphate from

phenyl phosphate to nucleosides. Tunis and Chargaff (93) have isolated and partially purified a similar enzyme from carrot. The enzyme from carrot leaf tissue has been further purified by Becker (4), who studied its ability to transfer phosphate from ^{32}P-p-nitrophenyl phosphate to the 5' position of nucleosides. The enzyme can be used to study nucleosides released from the termini of RNA by the nucleolytic enzymes which have been discussed, or by alkaline hydrolysis. Purified enzyme preparations posess phosphomonoesterase activity, which remains associated with the enzyme through all purification steps which have been studied, making precise quantitative estimates of chain terminal groups difficult. Becker (4) used this technique to phosphorylate nucleosides isolated from alkaline hydrolysates of cauliflower r-RNA.

Chemical Methods

1. Alkaline hydrolysis

The mechanism of alkaline hydrolysis of RNA has been reviewed by Witzel (99). As is shown in Figure 1B, hydrolysis releases mixed 2'(3')-monophosphates from non-terminal positions on the RNA chain, nucleosides-2'(3'),5-diphosphates from the 3'-linked terminus and nucleosides from the 5'-linked terminus. As in the case of pancreatic ribonuclease, hydrolysis proceeds via the formation of a cyclic 2',3'—

nucleoside phosphodiester intermediate (51, 99), however, in alkali this diester is cleaved non-preferentially, to give mixed nucleoside-2'(3')-monophosphates. Alkaline hydrolysis is thus dependent upon the presence of a free hydroxyl in the 2' position, and is therefore ineffective on DNA or on dinucleotides in which the 3'-linked member is substituted in the 2' position. Hudson et al. (32) have shown that alkali-stable dinucleotides in wheat germ s-RNA have the general structure NxpNp, where Nx is the 2'-O-methyl derivative of a nucleoside.

Whitfeld (96) and Lee and Gilham (49) have discussed a side reaction which can occur in alkaline hydrolysis, and which can give misleading results in analyses of 5'-linked termini. This reaction involves the transfer of a phosphate from the 2' or 3' position of a nucleotide, to the 2' or 3' position of a nucleoside, and thereby converting the original nucleotide to a nucleoside. The reaction, which apparently proceeds by the formation of a phosphodiester linkage between the two nucleoside moieties, is slow, due to the low concentration of nucleosides in alkaline hydrolysates, and is apparently of importance only in long-term hydrolyses. Cohn (16) has reported that use of excess alkali may cause further hydrolysis of nucleotides.

2. Periodiate oxidation

Whitfeld and Markham (98) discovered that ribonucleosides react with sodium meta periodate, giving a dialdehyde oxidation product. The reaction involves cleavage of the C-C bond between the 2' and 3'-hydroxylated positions of the ribosyl moiety, with concomitant formation of aldehyde groups at these positions. This reaction is the first step in each of the next five methods.

A. Amine catalyzed cleavage of dialdehydes

The dialdehydes produced by periodate oxidation of dinucleoside phosphates were shown to react with glycine, to yield free bases and nucleoside-3'-monophosphates (8, 98). Brown, et al. (8) suggested that this method might be used for the sequential degradation of RNA. Ogur and Small (64) found that lysine was more effective than glycine, and it now appears that any primary amine is effective in catalyzing this reaction. Among the other amines which have been used are cyclohexylamine (103), phenylhydrazine (38) and aniline (84). This method may be used on intact RNA chains and on nucleosides and 5' nucleotides produced by hydrolysis. Neu and Heppel (61) have described a modification of the amine catalyzed cleavage reported by Whitfeld and Markham (98), which involves the use of higher temperature (45° C, rather

than room temperature) and excess periodate, thereby facilitating recovery of the purine of pyrimidine base in a free state.

Amine catalyzed cleavage of the dialdehydes involves the formation of Schiff's bases (38, 39, 61, 103), which are stable at pH 9, but which break down at pH 7-8 to yield free base plus an aglycone (38, 39).

Periodate oxidation and amine catalyzed cleavage have been used to characterize the 5'-linked termini of a variety of nucleic acids (69, 84, 97, 103). Whitfeld (97) has demonstrated the use of this technique, in conjunction with E. coli alkaline phosphatase, to determine the 5'-linked terminal sequence of TMV-RNA. The terminal nucleoside was determined by subjecting the TMV-RNA to periodate oxidation followed by amine catalyzed cleavage. The terminal base released was subsequently identified, and the TMV-RNA was dephosphorylated with E. coli alkaline phosphatase, and the procedure repeated, to identify the penultimate base.

B. Formation of bisphenylhydrazones

Khym and Cohn (38) have described conditions for the formation of bisphenylhydrazones by the reaction of dialdehydes, formed by periodate oxidation, with phenylhydrazine. A serious disadvantage to this procedure, however, is the

observation that excess phenylhydrazine causes a dissociation of the derivatives to bases and two- and three-carbon fragments. This disadvantage probably accounts for the fact that this method has not been used in end group assays.

C. Formation of trialcohols

Another reaction described by Khym and Cohn (38) is the formation of trialcohols by the reduction of the dialdehyde oxidation products with sodium borohydride. This method, employing tritium-labeled sodium borohydride, has been used to characterize nucleosides produced by alkaline hydrolysis of cauliflower r-RNA (69).

D. Formation of semicarbozones and thiosemicarbazones

Methods analagous to the previous two, in that they involve formation of derivatives of dialdehydes, have been described by Steinschneider and Fraenkel-Conrat (83) and by Dulbecco and Smith (19) for the formation of semicarbazones and thiosemicarbazones, respectively. These methods involve the reaction of either ^{14}C-semicarbazide or ^{35}S-thiosemicarbazide with dialdehydes, with subsequent formation of either semicarbazones. Like the previous method, these allow the detection of small quantities of nucleoside by means of radioactivity. Steinschneider and Fraenkel-Conrat (84) have used ^{14}C-semicarbozones to determine the rate of release of

terminal bases, by amine catalyzed cleavage following periodate oxidation of TMV-RNA.

E. Formation of isonicotinyl hydrazones

Hunt (34) has described the synthesis of isonicotinyl hydrazone derivatives of the dialdehyde oxidation products described previously. These derivatives are formed by the reaction of the dialdehydes with isonicotinic acid hydrazide, and, in contrast with the amine Schiff bases which are stable at pH 9 but not at pH 7-8, these derivatives are stable at low pH, but are destroyed at pH 7 or above.

Periodate oxidation and formation of isonicotinyl hydrazones, followed by hydrolysis of RNA with pancreatic ribonuclease, has been used by Hunt (36) to determine the 5'-linked terminal sequences in rabbit reticulocyte r-RNA. In these studies he used ^{3}H-labeled isonicotinic acid hydrazide. Midgley (57, 58) has used this technique in attempts to determine chain length in E. coli r-RNA, and McIlreavy and Midgley (53, 59) have used it to determine the 5'-linked chain termini in E. coli r-RNA.

3. Acetylation

A method which has not been used on RNA, which should be applicable for detecting and quantifying small amounts of nucleoside isolated from RNA hydrolysates, has been developed

by Stuart and Khorana (85). They found that ^{3}H-acetic anhydride reacts specifically with terminal hydroxyls of DNA. Jackson et al. (37) used this method to determine the 5'-linked chain termini of DNA associated with crystalline yeast cytochrome b_2. This method would not be applicable for use with intact RNA chains, as acetic anhydride would react with free 2' hydroxyls of the ribonucleotides.

4. Phosphorylation with polyphosphate

Weiss et al. (95) have described a method for the synthesis of cytidine monophosphate from cytidine and polyphosphate. This method, when used with ^{32}P-polyphosphate, allows the detection of small quantities of nucleoside. A similar synthesis has been described by Hall and Khorana (24), for the synthesis of pyrimidine nucleoside-2'(3'),5'-diphosphates, and appreciable quantities of monophosphates are formed as by-products of the reaction. This method is only suitable for the phosphorylation of pyrimidine phosphates, as purines are not phosphorylated. Waehneldt and Fox (94) used the polyphosphate method to synthesize both 2'(3') and 5' monophosphates of all four ribonucleosides, but yields in their experiments were low, being in the range of 10 to 15 percent.

5. Phosphorylation with cyanoethyl phosphate

The use of cyanoethyl phosphate and dicyclohexylcarbodiimide (DCC) for the synthesis of mono- and diphosphates from nucleosides has been described by Tener (91). He found that cyanoethyl phosphate reacts with alcohols, such as free hydroxyl groups on the ribosyl moiety of nucleosides, yielding a phosphodiester of the cyanoethyl group and the alcohol. DCC acts as a coupling agent in the reaction. The cyanoethyl moiety is then removed by mild alkaline hydrolysis, yielding nucleotides. Tener (91) used nucleosides which had 2' and 3' hydroxyls blocked with isopropylidine, to synthesize 5' nucleotides. If this blocking is not done, phosphorylation of nucleosides by this method gives mixed products of all possible monophosphates and diphosphates. This method has been used in the synthesis of many of the ribo- and deoxyribonucleosides (21, 91) and in the characterization of nucleosides released upon alkaline hydrolysis of cauliflower r-RNA subunits (68).

6. Formation of methyl phosphate derivatives

Another method, related to the previous one, employs condensation reactions to add a ^{14}C label to the 3'-linked terminus of nucleic acids. This reaction, described by RajBhandary et al. (71), involves the reaction of the

5'-terminal phosphate with ^{14}C-methyl phosphomorpholidate. Methanol-^{14}C is reacted with cyanoethyl phosphate in the presence of DCC. The methyl phosphate produced by alkaline hydrolysis of this product is then reacted with morpholine, in the presence of DCC, to give methyl phosphormorpholidate. This product reacts specifically with a phosphomonoester to form a pyrophosphate linked methyl phosphate derivative.

This method has been used to determine the 3'-linked termini of yeast s-RNA (71) and of the DNA associated with yeast cytochrome b_2 (37). This method should also be applicable for the introduction of a ^{32}P label to the 3'-linked chain terminus, as well as a ^{14}C label, since both a phosphate and a methyl group are added in the reaction.

7. Formation of phosphoroanilidate derivatives

Ralph et al. (72) have described the synthesis of ^{14}C-phosphoroanilidate derivatives of 5' nucleotides. The reaction involves the condensation of phosphomonoesters with aniline, in the presence of diisopropylcarbodiimide to form a phosphodiester. This method has been used to attach a ^{14}C label to the 5' phosphate of the 3'-linked terminal nucleotide in yeast s-RNA, and could have general application for the detection of this terminus in other nucleic acids.

The Termini of Ribonucleic Acids

Viral RNA

Lee and Gilham (49) found all four ribonucleosides (A, G, C and U) in alkaline hydrolysates of both f_2 and MS 2 bacteriophage RNA, but concluded that adenosine was the 5'-linked terminus of both, since this nucleoside composed more than 75 percent of the product in both cases. These results, for MS 2 bacteriophage, were confirmed by Sugiyama (87), using dyrolysis with both alkali and pancreatic ribonuclease.

The termini of TMV-RNA have been investigated by Fraenkel-Conrat and co-workers (79, 84, 88, 89) and by Whitfeld (96, 97). Their results, obtained by alkaline hydrolysis (88, 89, 96), hydrolysis with venom phosphodiesterase (79) and pancreatic ribonuclease (96) and by periodate oxidation followed by amine cleavage (84, 97) showed adenosine to be the major nucleoside component of the 5'-linked terminus. Steinschneider and Fraenkel-Conrat (84) observed that elimination of the terminal nucleoside by periodate oxidation and amine cleavage resulted in a 95 percent loss of infectivity of the RNA. Activity was not restored by removal of the 3' terminal phosphate by E. coli alkaline phosphatase. These findings were in conflict with

results obtained by Singer and Fraenkel-Conrat (79), which showed only a 10 percent loss of infectivity with an average of one cleavage per RNA chain with venom phosphodiesterase. Steinschneider and Fraenkel-Conrat (84) concluded that values for cleavage of RNA chains with phosphodiesterase actually represent average numbers of cleavages per chain, rather than actual cleavage of every chain.

Sugiyama and Fraenkel-Conrat (88, 89) studied the 3'-linked terminus of TMV-RNA by the use of E. coli alkaline phosphatase and extensive digestion with venom phosphodiesterase. Using E. coli alkaline phosphatase (88) they determined that, unlike other RNA's, TMV-RNA contains no 5'-linked phosphate at its 3'-linked terminus. Studies with venom phosphodiesterase (89) revealed that about 50 percent of the nucleoside released was adenosine. They concluded that adenosine probably represented the 3'-linked terminus, thereby indicating that TMV-RNA has adenosine at both termini.

Thus, in summary, it may be said that the 5'-linked end group of all the viruses studied is adenosine. Results discussed for the termini of viral RNA's are summarized in Table 1.

Table 1. Summary of data on the termini of viral ribonucleic acid.

Source	Terminus	Mole Percent				Ref.
		A	G	C	U	
f_2	5'-linked	76.5	4.2	15.1	4.2	49
MS 2	5'-linked	79.0	4.2	12.6	4.2	49
		78.4	8.1	5.4	8.1	87
TMV	5'-linked	65.4	9.9	7.4	17.3	79
		91.6	2.8	0.9	4.6	84
		72	8	4	16	88
		68.2	12.1	12.1	7.6	89
		79.7	3.7	7.6	9.0	96
		64.1	12.0	10.9	13.1	97
TMV	3'-linked	48.8	17.6	16.8	16.8	89

Soluble RNA

All s-RNA's studied to date have been found to terminate preferentially in either cytidine or adenosine at the 5'-linked end. Zillig, et al. (104) found that adenosine was the only nucleoside produced by alkaline hydrolysis of E. coli s-RNA, whereas McIlreavy and Midgley (53) found both adenosine and cytidine as termini when samples of E. coli s-RNA were labeled with ^{3}H-isonicotinic acid hydrazide. Eighty percent adenosine and 20 percent cytidine were found in alkaline hydrolysates of yeast s-RNA by Bell, et al. (5), whereas Yu and Zamecnik (103) found about 30 percent adenosine, 60 percent cytidine and 10 percent uridine, using both alkaline hydrolysis and periodate oxidation followed by amine cleavage. Hecht and Zamecnik (26) and Herbert and Canellakis (29) found mostly adenosine and cytidine in alkaline hydrolysates of rat liver s-RNA; similarily, Singer and Cantoni (78) found mostly adenosine and cytidine (at least 50 percent adenosine) upon alkaline hydrolysis of rabbit liver s-RNA. Studies on wheat germ s-RNA by Lane and Allen (46) and by Hudson, et al. (32) revealed that adenosine and cytidine comprised most of the nucleoside function released by alkaline hydrolysis.

Heidelberger, et al. (27) found that an enzyme system from rat liver would incorporate adenosine at the 5'-linked terminus of s-RNA, and that the penultimate nucleoside was cytidine. Canellakis (11) found that this enzyme system would only add adenosine to a 5'-linked terminal cytidine residue, and Hecht, et al. (26) reported that the enzyme was specific for s-RNA, and that it sequentially added cytidine and adenosine. The enzyme had a very low activity for the incorporation of uridine (18, 26). Herbert and Canellakis (30) observed that when s-RNA is degraded by approximately 10 percent with venom diesterase, its ability to accept cytidine or uridine in the rat liver enzyme system is increased, whereas its ability to accept adenosine is decreased. The presence of all four ribonucleoside triphosphates in the reaction mixture increases incorporation of adenosine and cytidine, and decreases incorporation of uridine. Among the uridine-containing termini added sequentially in a similar system studied by Klemperer and Canellakis (42) were: -pU, -pUpA, -pUpC and -pCpU.

The requirement for sequential addition of a -pCpCpA terminus for acylation of s-RNA with amino acids, was discovered by Hecht, et al. (25), through a study on the kinetics of nucleotide incorporation with the rat liver enzyme system.

They found that two cytidine residues were incorporated for each adenosine, and that adenosine was not added until after cytidine had been incorporated. Acylation would not occur until a terminal adenosine was present. The CCA terminus was lost when s-RNA was incubated in the presence of pyrophosphate. They concluded that the CCA terminus is necessary for the incorporation of nearly all amino acids, because all of the 14 amino acids studied showed increased acylation with addition of the CCA terminus, and were non-competitive with each other. Merits (55), studying incorporation of ^{32}P-labeled nucleosides into s-RNA, found that in the presence of actinomycin D, most of the radioactivity was in the CCA terminus, indicating that incorporation was due to end group addition, and that this process was independent of DNA-dependent RNA synthesis. Rosset and Monier (76) found that s-RNA from exponentially growing yeast cells had mostly adenosine as its 5'-linked terminus, whereas cells in a stationary growth state had both adenosine and cytidine at this terminus. They concluded that there was a slow turnover of terminal adenosine in exponentially growing yeast cells, but not in those in a stationary state of growth, resulting in the observation of cytidine in the latter cells, due to enzymatic hydrolysis of terminal adenosine.

Guanosine has been shown to be the 3'-linked terminus of s-RNA. Zillig, et al. (104) found pGp as the only diphosphate produced upon alkaline hydrolysis of E. coli s-RNA, whereas Bell, et al. (5) and McLaughlin and Ingram (54) found that it comprised approximately 80 percent of the diphosphate produced upon alkaline hydrolysis of yeast s-RNA. Studies on rat liver s-RNA and rabbit liver s-RNA by Herbert and Canellakis (29) and Singer and Cantoni (78), respectively, showed that pGp was the main diphosphate produced by alkaline hydrolysis of these s-RNA's. Similar results were obtained by Hudson, et al. (32) in their study on s-RNA from wheat germ. Ralph, et al. (72) formed ^{14}C-phosphoroanilidate derivatives of yeast s-RNA, and found that most of the label was bound to quanosine; and RajBhandary, et al. (71) found that when ^{14}C-methl phosphate derivatives of yeast s-RNA were formed, 75 percent of the label was associated with guanosine.

Tables 2 and 3 summarize data on the termini of s-RNA.

Ribosomal

Studies on the termini of r-RNA's have been reviewed by Ebel (20).

1. 3'-linked termini

Studies done by Sugiura and Takanami (86), on the 3-'linked termini of r-RNA's of yeast (Saccharomyces cerevisiae)

Table 2. Summary of data on the 5'-linked termini of soluble ribonucleic acids.

Source of RNA	Mole Percent				
	A	G	C	U	Ref.
E. coli	100	n.d.	n.d.	n.d.	104
	51	-	38	n.d.	53
Yeast	80	n.d.	20	n.d.	5
	28.6	n.d.	61.4	10.0	103
Rat liver	+	n.d.	+	n.d.	26
	98.5	0.5	0.5	0.5	27
	67	14	7	12	29
Rabbit liver	>50	tr.	+	tr.	78
Wheat germ	77.3	1.8	17.0	3.9	32
	75.5	6.8	12.0	5.7	46

\+ detected, but quantitative data not available

\- no information given

tr. trace

n.d. not detected

Table 3. Summary of data on the 3'-linked termini of soluble ribonucleic acids

Source of RNA	Mole Percent				Ref.
	A	G	C	U	
E. coli	n.d.	100	n.d.	n.d.	104
Yeast	7	78	5	10	5
	4.1	75.5	5.1	10.1	71
	16.6	83.4	n.d.	tr.	54
	+	+	n.d.	+	72
Rat liver	n.d.	100	n.d.	n.d.	29
Rabbit liver	-	>80	-	-	78
Wheat germ	3.8	89.5	1.0	5.7	32

\+ Detected, but quantitative data not available

\- no information given

tr. trace

n.d. not detected

and four species of bacteria: Bacillus cereus, B. subtilis, B. stearothermophilus and Saracina lutea, using γ-^{32}P-ATP and polynucleotide kinase, revealed that all r-RNA's studied terminated preferentially in uridine except the heavy subunit of S. lutea, which had mostly adenosine at the 3'-linked terminus. These results are summarized in Table 4.

Nichols and Lane (62), using alkaline hydrolysis, and Takanami (90), using plynucleotide kinase, -^{32}P-ATP and alkaline hydrolysis, determined that E. coli r-RNA has mostly adenosine on the 3'linked terminus of 16 S r-RNA, and mostly guanosine on 23 S r-RNA. The 5 S r-RNA from E. coli (9, 77) has been shown, through alkaline hydrolysis, to have uridine at the 3'-linked terminus.

Alkaline hydrolysis of r-RNA from L cells revealed that this r-RNA has mostly uridine at the 3'-linked terminus of the 16 S subunit, and cytidine at the 3'-linked terminus of the 28 S subunit. Unfractionated wheat germ r-RNA has been shown by alkaline hydrolysis to have no preferential termination at the 3-linked end, as all four nucleoside diphosphates were present in hydrolysates (45, 80).

Data on the 3'-linked termini of r-RNA from E. coli, L cells and wheat germ are summarized in Table 5.

The observations that TMV-RNA, s-RNA and E. coli r-RNA

Table 4. Summary of data on the 3'-linked termini of ribosomal ribonucleic acids. I. The termini of bacterial and yeast ribosomal RNA as reported by Sugiura and Takanami (86).

Source of RNA	Size of RNA	Mole Percent			
		A	G	C	U
B. cereus	Light	13.2	1.6	4.4	85.0
	Heavy	9.6	2.5	3.0	81.0
B. subtilis	Light	22.0	2.2	9.8	66.0
	Heavy	23.0	3.4	2.7	71.0
B. stearothermophilus	Light	10.7	1.1	2.2	85.7
	Heavy	21.8	3.5	3.8	70.9
S. lutea	Light	10.8	2.5	3.1	83.6
	Heavy	82.0	2.8	2.4	12.7
S. Cerevisiae	Light	11.1	4.0	6.0	84.5
	Heavy	31.6	9.6	1.9	57.0

Table 5. Summary of data on the 3'-linked termini of ribosomal ribunucleic acids. II. The termini of ribosomal RNA from *E. coli*, L. cells and wheat germ.

Source of RNA	Size of RNA	Mole Percent A	G	C	U	Ref.
E. coli	16 S	74.5	7.0	8.7	9.7	90
		41	25	10	23	62
	23 S	16.5	68.2	6.8	8.4	90
		20	59	9	12	62
L. cells	16 S	5	24	11	60	47
	28 S	9	28	52	11	47
Wheat germ	18 S + 28 S	9.4	39.6	23.9	27.0	45
		9.1	31.8	15.9	43.2	80

all have purines at their 3-linked termini may have some relationship to the observation of Maitra and Hurwitz (50), that products of DNA-dependent RNA polymerase from E. coli are preferentially initiated by purines. The results of Sugiura and Takanami (86), however, suggest that this is not a universal phenomenon.

2. 5'-linked termini

Lane (43), using alkaline hydrolysis, found that the 16 S + 23 S r-RNA of E. coli had mostly adenosine and uridine at the 5'-linked terminus. These results were confirmed by Midgley and McIlreavy (59) and by McIlreavy and Midgley (53), both my means of alkaline hydrolysis, and by experiments involving isonicotinyl hydrazones. These latter experiments involved periodate oxidation of 5'-linked r-RNA chain termini and labeling with ^{3}H-isonicotinic acid hydrazide, followed by hydrolysis of the isonicotinyl-RNA with either pancreatic ribonuclease or ribonuclease T_1. Studies on the 5'-linked termini of individual subunits of E. coli r-RNA by Nichols and Lane (62), using alkaline hydrolysis, and by McIlreavy and Midgley (53) using ^{3}H-isonicotinic acid hydrazide derivatives, revealed that the 16 S subunit terminates primarily as adenosine, and the 23 S subunit primarily as uridine.

Rabbit reticulocyte r-RNA was studied by Hunt (35,

36) via formation of isonicotinyl hydrazones and enzymatic hydrolysis. He found that there were essentially two types of 5'-linked termini in 30 S r-RNA, one being -pyrimidine-p-U and the other -G-p-U. The 17 S r-RNA terminates preferentially as -pyrimidine-p-A. Lane and Tamaoki (47) used alkaline hydrolysis to determine that r-RNA from L cells has mostly adenosine at the 5'-linked terminus of the 16 S subunit, and uridine at that terminus of the 28 S subunit.

Hadjiolov, et al. (23) have reported that upon sequential degradation of rat liver r-RNA with venom phosphodiesterase, the contents of adenosine and uridine in early hydrolysates (30 percent hydrolysis) are higher than are those obtained upon complete hydrolysis. Nucleotides isolated following 30 percent hydrolysis of 18 S r-RNA were composed of 20.8 percent adenosine monophosphate and 24.5 percent uridine monophosphate, and those from 28 S r-RNA were composed of 19.4 percent adenosine monophosphate and 20.9 percent uridine monophosphate. Complete hydrolysis with alkali, however, gave products from 18 S r-RNA containing 12.1 percent adenosine monophosphate and 22.3 percent uridine monophosphate; and from 28 S r-RNA containing 14.9 percent adenosine monophosphate and 20.9 percent uridine monophosphate. It was suggested that the segments of the chains

near the 5'-linked termini may be higher in adenosine and uridine than in quanosine and cytidine. These results are subject to question, however, because of the observations that venom phosphodiesterase has some endonuclease activity (73, 79), and that this gives preferential cleavage at sequences of the type -pUpA↓pX- (79).

Alkaline hydrolysis of unfractionated wheat germ r-RNA has revealed that there is no preferential termination at the 5'-linked end of this r-RNA. Because of this finding, and that reported previously for the 3'-linked terminus, wheat germ r-RNA provides the only exception observed to the preferential termination of RNA's. The possible significance of this will be discussed in greater detail later.

Table 6 summarizes data on the 5'-linked termini of r-RNA's.

Midgley and McIlreavy (60) also found that when E. coli cells were grown on "casamino acids" or broth, rather than on glucose or succinate, the percent of terminal uridine increased on both subunits. A summary of their results is presented in Table 7.

Alkaline hydrolysis of the 5 S r-RNA from E. coli (9, 77) revealed that this subunit has uridine at its 5'-linked terminus.

Table 6. Summary of data on the 5'-linked termini of ribosomal ribonucleic acids.

Source of RNA	Size of RNA	Mole Percent				Ref.
		A	G	C	U	
E. coli	16 S + 23 S	47.4	9.2	13.2	30.3	43
		43	-	-	38	53
		43	11	8	38	59
	16 S	71	-	-	16	53
		60	10	10	19	62
	23 S	26	-	-	48	53
		19	6	4	70	62
Rabbit reticulocyte	17 S	+	-	-	-	36
	30 S	-	-	-	+	36
L. cells	16 S	75	6	4	13	47
	28 S	12	24	6	58	47
Wheat germ	18 S + 28 S	21.4	31.0	27.4	20.2	46
		17.9	30.8	25.6	25.6	45
		19.8	23.6	31.1	25.4	49
		20.5	30.8	24.4	24.4	80

\+ detected, but quantitative data not available

\- no information given

Table 7. Summary of data on the effect of nutrients on the nature of the 5'-linked termini of E. coli ribosomal RNA, as reported by Midgley and McIlreavy (60).

Growth Medium	RNA Fraction	Mole Percent of Terminus		
		A	U	Other
Glucose and salts	16 S + 23 S	43	36	21
	16 S	70	19	11
	23 S	28	48	24
Succinate	16 S + 23 S	44	40	16
	16 S	68	20	12
	23 S	23	53	24
Broth	16 S + 23 S	23	69	8
	16 S	30	59	11
	23 S	16	75	11
Casamino acids and salts	16 S + 23 S	20	74	6
	16 S	37	53	10
	23 S	13	78	9

Enzymatic Addition of Termini

The action and importance of enzymes which add the terminus -CCA to the 5'-linked ends of s-RNA have been discussed in the section on s-RNA termini.

An enzyme which incorporates uridine derived from uridine triphosphate into the 5'-linked termini positions of RNA of an unspecified type, has been isolated from Ehrlich Ascites carcinoma cells by Burdon and Smellie (10). Action of this enzyme was unaffected by the addition of triphosphates of adenosine, guanosine and cytidine, but was surprisingly stimulated by the addition of a mixture of amino acids. Another enzyme isolated in the same study gave incorporation of uridine into both 5'-linked terminal and non-terminal positions, and was stimulated by the addition of a mixture of nucleoside triphosphates.

Klemperer (40, 41) isolated an enzyme from the pH 5, 105,000 g supernatant fraction of rat liver homogenate which incorporated adenosine monophosphate or uridine monophosphate, derived from the corresponding triphosphates, as homopolymer chains on the 5'-linked end of natural or synthetic polyribonucleotides. Although this enzyme would add to the termini of s-RNA, it differed from previously studied enzymes in that it was not specific for s-RNA, and would also

work on rat liver r-RNA and synthetic polyribonucleotides. Klemperer found that incorporation of uridine monophosphate was dependent upon the presence of magnesium ions, and incorporation of adenosine monophosphate was dependent upon the presence of manganese ions.

Recently, Page, et al. (65) reported that a similar enzyme from rat liver microsomes incorporates all four ribonucleotides into homopolymer chains on the 5'-linked termini of r-RNA, and that such incorporation is dependent upon the presence of manganese ions. That all four nucleotide triphosphates show competitive inhibition indicates involvement of the same enzymatic sites for incorporation of all of them. When manganese ions are replaced by magnesium ions in the system, only uridine monophosphate is incorporated into the terminal chains.

Enzymes which add terminal groups may be involved in the findings of Midgley and McIlreavy (60) summarized in Table 6, which showed an increased ratio of 5'-linked terminal uridine to terminal adenosine in cells grown on broth or casamino acids, as compared to those grown on glucose or succinate. These authors concluded, however, that the increase in terminal uridine was due to a different response of genetic elements controlling the synthesis of r-RNA, in

different environments. They reported that the synthesis of "A-terminal" r-RNA was constant in all systems, whereas the synthesis of "U-terminal" r-RNA was stimulated in broth or casamino acids. Taken on the basis of molecules per cell, the quantity of "A-terminal" r-RNA was constant under all conditions, whereas the quantity of "U-terminal" r-RNA was increased by broth or casamino acids.

MATERIALS AND METHODS

Experimental Materials

Plant tissues used in these studies, cauliflower (Brassica oleracea, L. var. botrytis, L.), cabbage (B. oleracea, L. var. capitata), mushroom (Agaricus campestris, L. ex Fr.) and wheat germ (Triticum aestivum, L.), were purchased from local commercial sources; cauliflower seeds (Snowball) were purchased from Joseph Harris Company, Rochester, New York. Gills and the outer surface of mushroom caps were removed prior to use, as were leaves and heavy stem sections of cauliflower, while cabbage heads were shredded.

Rexyn 201 (Cl^--quartenary amine resin) was purchased from Fisher Scientific Company, Detroit, Michigan; Dowex 50 (H^+-sulfonic acid resin) was purchased from Sigma Chemical Company, St. Louis, Missouri. Both resins were washed successively with portions of acetone, 50 percent ethanol and 20 percent concentrated hydrochloric acid, followed by extensive washing with distilled water, prior to use. Carrier-free

^{32}P-phosphoric acid was purchased from New England Nuclear Boston, Massachusetts, and 2-^{14}C-uracil was purchased from Schwarz BioResearch, Inc., Orangeburg, New York. Nucleoside phosphotransferase from carrot leaf tissue and ^{32}P-p-nitrophenyl phosphate were gifts of Veryl E. Becker; Actinomycin D was a gift of Merck Sharp and Dohme, Research Laboratories, Rahway, New Jersey. Other chemicals were reagent grade and are available at most chemical supply houses.

Preparation and Hydrolysis of RNA

Preparation of Ribosomal RNA by Direct Extraction

1. Extraction with phenol and sodium dodecyl sulfate

Plant tissues were homogenized for two to three minutes at 80 percent line voltage in a Waring Blendor, with two volumes each (w/v) water-saturated phenol and a solution consisting of 0.001 M magnesium chloride, five percent (w/v) sodium dodecyl sulfate, 0.28 M lithium sulfate and 0.1 M sodium acetate, pH 5.0. The latter solution, which will subsequently be referred to as solution A, was adapted from McCarthy and Hoyer (52).

The homogenate was then centrifuged for 10 minutes, at 10,000 g and the upper aqueous phase was removed. It was added to an equal volume of phenol and deproteinization was repeated. Following centrifugation, the RNA was precipitated

from the aqueous phase with an equal volume of cold absolute ethanol and the resulting RNA precipitate was collected by centrifugation. The pellet was then homogenized in cold 3 M sodium acetate, pH 6.0, and the insoluble material was collected by centrifugation. Unless otherwise noted, all r-RNA was washed two times with 3 M sodium acetate to remove s-RNA. This procedure was modified by increasing the solution A: plant tissue ratio to 4:1 for the extraction of r-RNA from wheat germ.

Phenol used in the deproteinization steps was Baker Analyzed Reagent, since use of other brands often resulted in a discolored, pink or gray, product. Click and Hackett (15) reported that use of phenol other than Baker Analyzed Reagent resulted in low yields and poor resoltuion of r-RNA.

Other solutions used for extraction were modified from those described by Click and Hackett (15). These consisted of equal volumes of water-saturated phenol prepared by dissolving phenol in distilled water containing 0.001 M ethylenediamine tetraacetic acid (EDTA), and a solution containing one percent (w/v) sodium dodecyl sulfate, 0.01 M EDTA, pH 9.5. This latter solution will be referred to as solution B. The extraction procedure was the same as the one described previously.

2. Extraction with phenol and phosphate buffer

The methods of Singh and Lane (80) were used to prepare one batch of wheat germ r-RNA. Wheat germ was placed in 6.5 volumes (w/v) 0.05 M phosphate buffer, pH 7.0, containing 6.5 volumes of water-saturated phenol and shaken vigorously at room temperature for 20 minutes. The suspension was then centrifuged at 10,000 g for 10 minutes and the aqueous phase was siphoned off and made 1 M in sodium chloride. This solution was allowed to stand for 24 hours, at 0° C. The insoluble RNA was collected by centrifugation, and subsequently washed by homogenization successively in 100 ml portions of absolute ethanol and diethyl ether. The RNA was then resuspended in phosphate buffer and again collected by making the solution 1 M in sodium chloride.

Preparation of Cytoplasmic RNA by Differential Centrifugation and Phenol Extraction

The following methods of preparing cytoplasmic RNA were developed as a part of this research.

1. Ribosomal RNA

Equal quantities (w/v) of chilled plant tissue and a solution of 0.01 M magnesium chloride, 0.01 M calcium chloride, 0.25 M sucrose and 0.05 M Tris, pH 7.8; were homogenized in a Waring Blendor at 50 percent line voltage for two minutes.

The resulting slurry was filtered through two layers of cheesecloth, and the filtrate reused as homogenizing medium for three to four successive batches of plant tissue. The homogenizing medium was chilled in ice water before each homogenization, so that the temperature never exceeded 8° C. The last filtrate from the cheesecloth was filtered through four layers of facial tissue, and the resulting filtrate was centrifuged at 15,000 g for 15 minutes, to remove nuclei, mitochondria and cell wall material. The 15,000 g supernatant solution was spun for 30 minutes at 105,000 g, with a number 40 rotor in a Spinco Model L. ultracentrifuge. Microsomal pellets were then suspended in solution A, combined with an equal volume of water-saturated phenol, and homogenized in a Tenbroeck all-glass homogenizer. RNA was extracted by the procedure described previously for preparation of RNA by direct extraction with phenol and solution A.

2. Soluble RNA

An equal volume of absolute ethanol was added to the 105,000 g supernatant solution (postmicrosomal fraction), and the precipitate was collected by centrifugation. Soluble RNA was prepared from this pellet by the same procedure described for the preparation of r-RNA by direct extraction with phenol and solution A, except that the phenol deproteinization step

was done four times, and the material soluble in 3 M sodium acetate was collected, as s-RNA, by ethanol precipitation.

Because of suspected contamination with DNA, wheat germ r-RNA preparations were checked for DNA content with the diphenylamine test described by Ashwell (1). This test involved the addition of 1 ml of sample (RNA solution) to 2 ml of a solution formed by the addition of 1 g of diphenylamine and 2.75 ml of concentrated sulfuric acid, to 100 ml of glacial acetic acid. The mixture was heated for 10 minutes in a boiling water bath, and following cooling, the absorbance was read at 595 and 650 mμ.

All RNA used in these studies was of high optical purity (OD 260/280 = 1.95 - 2.15), and quantitative estimations of RNA were based on the assumption that 1 mg of RNA in 1 ml would have an OD of 24 at 260 mμ.

Sucrose Density Gradient Centrifugation

Linear sucrose gradients (4-20 percent, w/v) were prepared in either 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7 (saline citrate), or 0.05 M sodium chloride, 0.05 M sodium acetate, 0.0001 M magnesium chloride, pH 5.3 (saline acetate). Ordinarily, from 12 to 50 OD units (260 mμ) of RNA per bucket were centrifuged for routine examination of sedimentation profiles, whereas 80 to 100 OD

units per bucket were centrifuged in runs for preparation of r-RNA subunits. Gradients were centrifuged at 0^{o} C for 15 - 19 hours at 23,000 rpm with a Spinco 25.1 rotor. RNA subunits were recycled without precipitation, after dialysis at 2^{o} C overnight against the buffer used to prepare the gradients. Centrifuge tubes were pierced through the bottom and the gradients collected in 5-drop fractions which were then diluted with 3 ml of the gradient buffer. unless otherwise noted. Sedimentation profiles were obtained routinely for all r-RNA preparations.

Alkaline Hydrolysis

Unless otherwise noted, RNA was hydrolyzed for 18-24 hours, at 37^{o} C, in 0.3 M potassium hydroxide. Hydrolysis was stopped by adjusting the solution to pH 7, in the cold, with Dowex 50 H^{+} resin.

Chromatography and Electrophoresis

Anion-exchange Chromatography

Nucleosides were collected from neutralized alkaline hydrolysates as described by Cohn (16), by passing them through a column of Rexyn 201-Cl^{-}, quartenary amine resin. Material which came off the column during loading, during a water wash and before 0.003 N hydrochloric acid began to elute

cytidylic acid, was taken as the nucleoside fraction. As is illustrated in Figure 2, cytidylic acid is eluted as a sharp peak with 0.003 N hydrochloric acid, and fractions preceeding this peak may be considered devoid of nucleotides.

The nucleoside fraction was then neutralized with dilute sodium hydroxide, evaporated to dryness under reduced pressure, and the residue extracted with boiling pyridine. Following filtration, to remove undissolved material, the pyridine was evaporated under reduced pressure. Successive small quantities of concentrated ammonium hydroxide were added to the residue and evaporated to remove last traces of pyridine. The nucleoside residue was then dissolved in 0.05 N hydrochloric acid and applied to paper for chromatography.

The nucleotide fraction from cauliflower hypocotyl r-RNA was eluted with 1 N hydrochloric acid.

Paper Chromatography

All paper chromatography was descending, on Whatman No. 1 paper, with chromatograms developed in the following solvent systems: (A) 2-propanol, one percent ammonium sulfate, 2:1, paper impregnated with one percent ammonium sulfate; (B) 75 percent ethanol, paper impregnated with 10 percent-saturated ammonium sulfate; (C) 2-propanol, concentrated hydrochloric acid, water, 65:15:20; (D) water-saturated

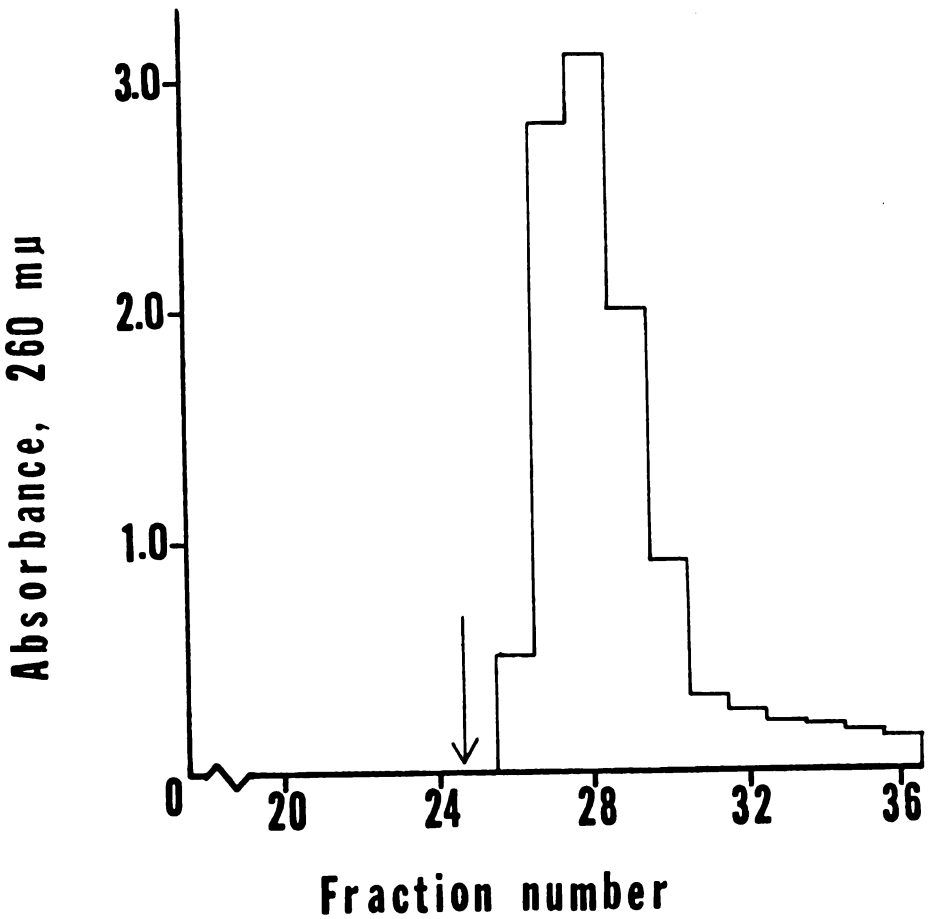

Figure 2: Pattern of elution of cytidylic acid from Rexyn 201, Cl^-. A neutralized alkaline hydrolysate of 450 mg of RNA was loaded on to a 2 X 10 cm column of Rexyn 201, Cl^-, rinsed with distilled water and eluted with 0.003 N HCl. Seventy ml fractions were collected, and all material which was passed through the column during loading, the distilled water rinse and to the point indicated by the arrow, was saved as the nucleoside fraction. Cytidylic acid was identified by its absorption spectrum and by chromatography in system B.

butanol, in an ammonia-saturated atmosphere; (E) isopropanol, concentrated ammonium hydroxide, 0.1 M boric acid, 7:1:2; (F) t-butanol, 88 percent formic acid, water, 70:15:15; and (G) 0.1 M sodium phosphate, pH 6.8 (1000 ml), ammonium sulfate (600 g), n-propoanol (20 ml). Systems A, B, C and D have been described by Hall and Khorana (24), Lane (44), Wyatt (100), and Hotchkiss (31), respectively, whereas systems E, F and G are listed in the 1967 catalog from Schwartz Bio-Research Inc., Orangeburg, New York.

Following paper chromatography, spots were detected with ultraviolet light and were eluted from the paper with 0.1 N hydrochloric acid. Often areas adjacent to standards were eluted, whether ultraviolet light absorption was detected or not. Identification of nucleosides was based on chromatographic mobilities and on absorption spectra at both acidic and alkaline pH. Quantitative determinations were based on ΔOD (260 mμ - 280 mμ) at acidic pH (pH 1).

Samples containing undesired inorganic salt, such as those eluted from papers developed in systems A and B, were absorbed on Norit A charcoal (0.02 - 0.1 g), in the presence of 0.05 N hydrochloric acid, prior to further chromatographic purification. After washing the Norit A 2- 20 times with distilled water, the samples were eluted with two 10 ml

portions of 80 percent ethanol containing one percent concentrated ammonium hydroxide, followed by one 10 ml portion of 50 percent ethanal containing one percent concentrated ammonium hydroxide. Norit A was removed from the solutions, at each step, by centrifugation, and the combined alcoholic solutions were evaporated to dryness under reduced pressure.

Paper Electrophoresis

Paper electrophoretic separation of nucleotides was achieved in a Beckman RD-2 electrophoresis unit, using two different systems. The first system, as described by Bell, et al. (5) employed 0.05 M citrate buffer, pH 5, with a potential of 300 volts. The second system, described by Chandra and Varner (13), and modified by Becker (4), contained 0.01 M EDTA (tetrasodium salt) and 0.34 ml of pyridine in 600 ml of water. The pH was adjusted to 3.5 with acetic acid. A potential of 400 volts was applied across the paper to achieve separation.

Radioisotope Counting

Radioactive samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, using a solution consisting of 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene and 5 g of 2,5-diphenyloxazole per liter of toluene.

Chromatograms and electrophoretograms were cut in strips which were counted directly in the toluene solution, whereas ^{14}C-labeled plant tissues were dried in counting bials at 95° C, prior to addition of the solution. Aqueous samples of ^{14}C-labeled nucleoside and nucleotides, from cauliflower hypocotyl r-RNA, were pipetted into counting vials and evaporated to dryness at 95° C, prior to counting. Cauliflower hypocotyl r-RNA from sucrose density gradients was prepared for counting according to a method modified from Bollum (6). Fractions from sucrose gradients were spotted on filter paper strips, air dried and then rinsed successively, twice in five percent tricholoracetic acid, once in 75 percent ethanol, twice in absolute ethanol and once in diethyl ether. Air dried strips were then placed in vials for counting.

Phosphorylation of Nucleosides

Use of Nucleoside Phosphotransferase

Nucleosides, purified from wheat germ r-RNA hydrolysates, were incubated for six hours, at 37° C, with 0.1 ml of 0.1 M sodium acetate, pH 5.1, 50 µl of ^{32}P-p-nitrophenylphosphate (15.8 mg/ml, activity = 10,000 cpm/µg) and 0.1 ml of a nucleoside phosphotransferase preparation. The reaction was stopped by spotting the material on

chromatography paper and evaporating to dryness.

Use of ^{32}P-polyphosphate

The techniques of Weiss, et al. (95) were used for the phosphorylation of nucleosides derived from 18 S cauliflower r-RNA. Nucleosides were evaporated to dryness and were reacted with 40 µl of 0.1 M ^{32}P-phosphoric acid (specific activity = 0.75 mc/µ mole) and approximately 5 mg of phosphorus pentoxide for two hours in an 80° C incubator. The reaction was stopped by the addition of 4 ml of water, and the mixture was placed in a boiling water bath for 45 minutes. The sample was then diluted 10 ml, centrifuged and the residue discarded, after which the dissolved products were adsorbed onto Norit A and washed 20 times to remove traces of radioactivity due to unreacted ^{32}P. Following elution from Norit A, the products were co-chromatographed with nucleotide standards.

Incorporation of 2-^{14}C-Uracil by Cauliflower Hypocotyls

Cauliflower seeds were germinated three to four days at room temperature, prior to excision of hypocotyls. The hypocotyls were stored in cold, one percent sucrose until ready for use. Experiments on incorporation into the ethanol-insoluble fraction were done with seven to ten hypocotyls per

tube, whereas experiments involving RNA extraction were done with 100-200 hypocotyls per tube.

The incubation medium consisted of one percent sucrose, 50 μg/ml of streptomycin sulfate and 10^{-2} M potassium phosphate, pH 6.8. This medium was also used to make up solutions of actinomycin D and 2-^{14}C-uracil. Hypocotyls were preincubated for 2 hours, in either the incubation medium or incubation medium and actinomycin D, prior to the addition of 2-^{14}C-uracil.

Incorporation was stopped, in studies on ethanol-soluble incorporation, by the addition of five volumes of ethanol to each tube, followed by five minutes of boiling. Hypocotyls were then washed by boiling five successive times in 5 ml portions of 75 percent ethanol, containing 0.2 mg/ml of unlabeled uracil. Following two successive washings in boiling absolute ethanol, the hypocotyls were dried at 95° C and their radioactivity determined.

Synthesis of ^{14}C-labeled RNA was stopped by direct extraction of RNA from the hypocotyls with phenol and solution B. Hypocotyls were removed from the incubation medium and placed in a mixture of 15 ml of water-saturated phenol, 12 ml of solution B, 1 ml of a solution containing 0.2 mg/ml of unlabeled uracil and 5 mg of unlabeled cauliflower cytoplasmic

r-RNA. The mixture was homogenized in a Tenbroeck all-glass homogenizer, and the r-RNA was extracted and purified as described previously.

RESULTS

RNA Isolation Procedures

Cytoplasmic Ribosomal RNA

Cytoplasmic r-RNA was isolated from the microsomal fraction obtained by differential centrifugation of homogenates of cabbage, cauliflower and mushrooms. The microsomal fraction was extracted with phenol and solution A. Because it was felt that contamination with r-RNA from cellular organelles might give misleading results, and because large quantities of r-RNA (300-500 mg) were needed for detection of terminal residues by means of chromatography and spectrophotometric analysis, a method was needed which would provide relatively high yields of cytoplasmic r-RNA. This was achieved through repeated reuse of the homogenizing medium (tris:sucrose, pH 7.8) for the homogenization of plant tissue. That no noticeable degradation of the r-RNA or preferential loss of the large subunit resulted may be accounted for by the relatively high concentration of magnesium ion (0.01 M) in the homogenizing medium. This method was found unsuitable

for the preparation of wheat germ r-RNA, because the sucrose homogenizing medium became so viscous that the appropriate subcellular fractions failed to sediment out at either the 15,000 g or 105,000 g steps. This high viscosity was apparently due to the presence of dissolved polysaccharide and DNA. Attempts to isolate r-RNA from giant ragweed (Ambrosia trifida, L), goldenrod (Solidago sp.) and burdock (Arctium minus, [Hill], Bernh.) were abandoned, because a black material, associated with the first ethanol precipitate, could not be separated from the RNA.

In a typical preparation, three to four kg of cauliflower yielded 250 mg of r-RNA. However yields varied greatly between batches of cauliflower. Yields of r-RNA were lower from cabbage and higher from mushrooms, than those obtained from cauliflower. Good resolution of r-RNA subunits on sucrose density gradients was achieved with these preparations, as is illustrated in Figure 3. Yields of s-RNA were found to be 20 mg per six to eight kg of plant tissue.

Total Ribosomal RNA

Subunits of wheat germ r-RNA isolated by direct extraction with phenol and solution A were not resolved on sucrose density gradient as well as the r-RNA's extracted from the microsomal fractions, as described above. In preparation of

this RNA, difficulty was experienced in sedimenting the insoluble material, following each of the first two homogenizations in 3 M sodium acetate, due to the high viscosity of the homogenate. Wheat germ RNA preparations were found to be contaminated with DNA, which was eliminated only after repeated homogenization in 3 M sodium acetate. Pollard (68), however, experienced no such difficulties, and achieved good resolution of subunits upon sucrose density gradient centrifugation, with r-RNA prepared from cauliflower by this method.

None of the problems of viscosity or DNA contamination encountered with the previous system were found when wheat germ r-RNA was isolated by direct extraction with phenol and solution B. This material gave less satisfactory resolution with sucrose density gradient centrifugation (Figure 7B), however, and the profile was skewed toward the heavy side of the 28 S subunit.

Isolation of wheat germ r-RNA by direct extraction with phenol and phosphate buffer, pH 7.0, gave a product which was free of DNA and whose subunits could be resolved well upon sucrose density gradient centrifugation, as is illustrated in Figure 10.

The 5'-Linked Termini of Cytoplasmic RNA

Ribosomal RNA

1. Unfractionated r-RNA

Cytoplasmic r-RNA was prepared from cauliflower, cabbage and mushrooms, by means of differential centrifugation, followed by extraction of microsomal pellets with phenol and solution A. A sedimentation profile of caulflower r-RNA is shown in Figure 3. This figure shows the usual r-RNA subunits, having a distribution of 67 percent 28 S and 33 percent 18 S, as calculated from areas under the peaks; as well as an absence of material in the s-RNA area (4-5 S). Further evidence of the absence of s-RNA will be presented in the section dealing with 5'-linked termini of s-RNA. Similar profiles were obtained with r-RNA's from cabbage and mushrooms.

The nucleoside fraction isolated after hydrolysis of 550 mg of unfractionated cauliflower r-RNA was chromatographed in system, B, and was found to contain 0.33 μ moles of adenosine and 0.19 μ moles of uridine. In four repetitions of this experiment, adenosine was consistently found to predominate over uridine. No cytidine or guanosine was detected in any of these preparations, although in some experiments spots were detected adjacent to the quanosine

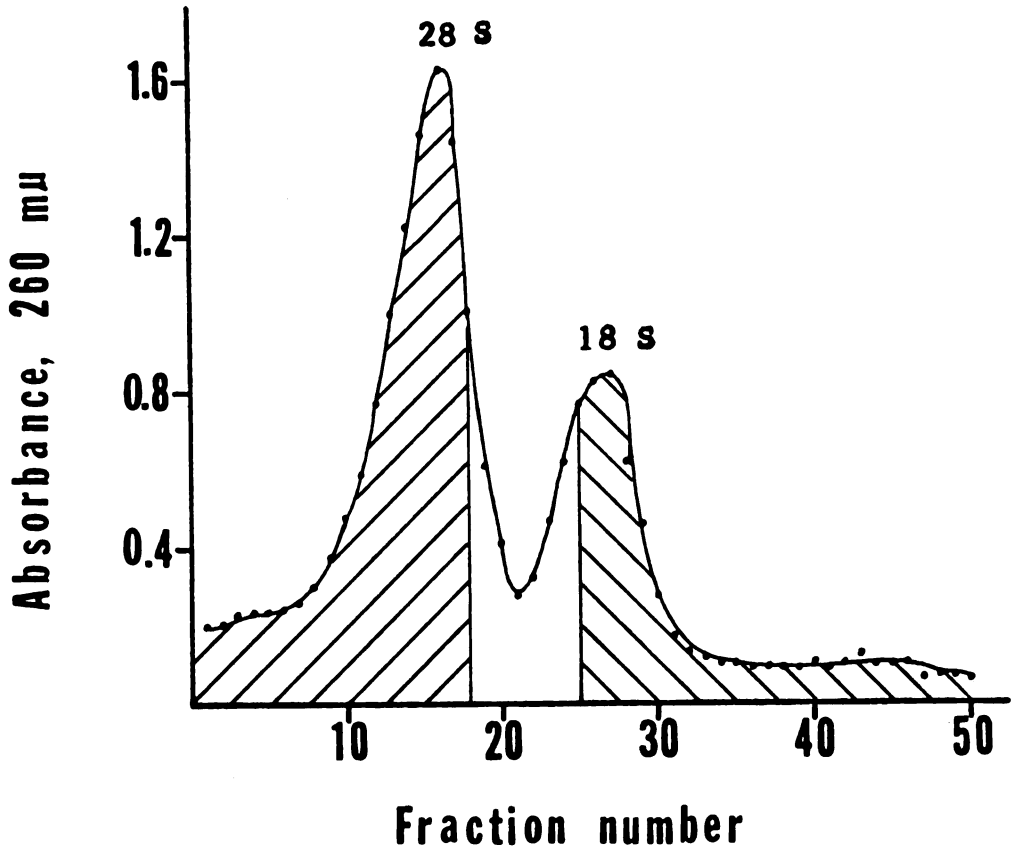

Figure 3: Sedimentation profile of a typical preparation of cauliflower cytoplasmic r-RNA. Hatched areas represent the portions of the gradient saved for studies on the termini of the individual subunits. RNA (24 OD units) was placed on a linear sucrose gradient (4-20 Percent, in saline citrate buffer) and was centrifuged for 16.5 hours.

standard on chromatograms developed in system B. Absorption spectra of these spots, however, differed from those of guanosine, and upon rechromatography in acidic solvents, they were resolved into several spots, with mobilities differing from quanosine.

When mixtures of 50 to 100 μg each of authentic nucleosides were subjected to these procedures, recoveries of from 50 to 90 percent were realized, no preferential losses being noted.

In three experiments on cabbage r-RNA only adenosine and uridine were detected in the nucleoside fractions. However, these results differed from the results obtained with cauliflower r-RNA, in that uridine was more abundant than adenosine. In a typical experiment, hydrolysis of 455 mg of 4-RNA gave 0.091 μ moles of adenosine and 0.318 μ moles of uridine. Similarily, 464 mg of mushroom r-RNA yielded 0.308 μ moles of adenosine and 0.691 μ moles of uridine. Again, no cytidine or quanosine was detected.

The yield of nucleoside did not exceed the theoretical yield, based on equal molar ratios of 18 S and 28 S RNA, in any of the experiments on unfractionated cauliflower or cabbage cytoplasmic r-RNA, but was twice the expected yield with mushroom cytoplasmic r-RNA. The reason for

higher than expected yields of nucleosides from mushroom r-RNA is not known, but may have been due to ribonuclease activity, although no such enzymatic hydrolysis was evident in sedimentation profiles of this RNA. Data comparing expected and actual yields of nucleosides are presented in Table 12.

Results of typical experiments with unfractionated cytoplasmic r-RNA are summarized in Table 8. Values are expressed as percent of total nucleoside detected.

Table 8. Nucleosides resulting from the hydrolysis of unfractionated, cytoplasmic r-RNA in typical experiments.

Source of RNA	Mole Percent of Nucleosides			
	A	G	C	U
Cauliflower	63	n.d.	n.d.	37
Cabbage	22	n.d.	n.d.	78
Mushroom	31	n.d.	n.d.	69

n.d. not detected

Cauliflower r-RNA subunits, represented by the hatched areas of Figure 3, were collected in attempts to determine if any preferential termination with either adenosine or uridine could be found on individual subunits.

Sedimentation profiles obtained by recycling of the individual subunits are presented in Figure 4.

2. 28 S RNA

Eighty-nine mg of 28 S cauliflower RNA were collected from sucrose gradients by ethanol precipitation, over a period of one month. The nucleoside fraction obtained from the alkaline hydrolysate of this RNA was chromatographed in system B, and the individual nucleoside areas located by means of an ultraviolet light. Spectrophotometric analysis showed that the sample contained 0.037 μ moles of adenosine and 0.064 μ moles of uridine. No other nucleosides were detected.

3. 18 S RNA

The nucleosides isolated from an alkaline hydrolsate of 29 mg of 18 S cauliflower RNA were phosphorylated with ^{32}P-polyphosphate, in order to facilitate detection of pyrimidine nucleosides. As previously mentioned, this method is unsatisfactory for phosphorylation of purine nucleosides. Preliminary experiments indicated that 0.5 μg samples of pyrimidine nucleosides could be phosphorylated by this technique and could be easily detected. Products included 2'(3')- and 5'-monophosphates and 2'(3'), 5'-diphosphates. Half of this sample was chromatographed successively in systems B and

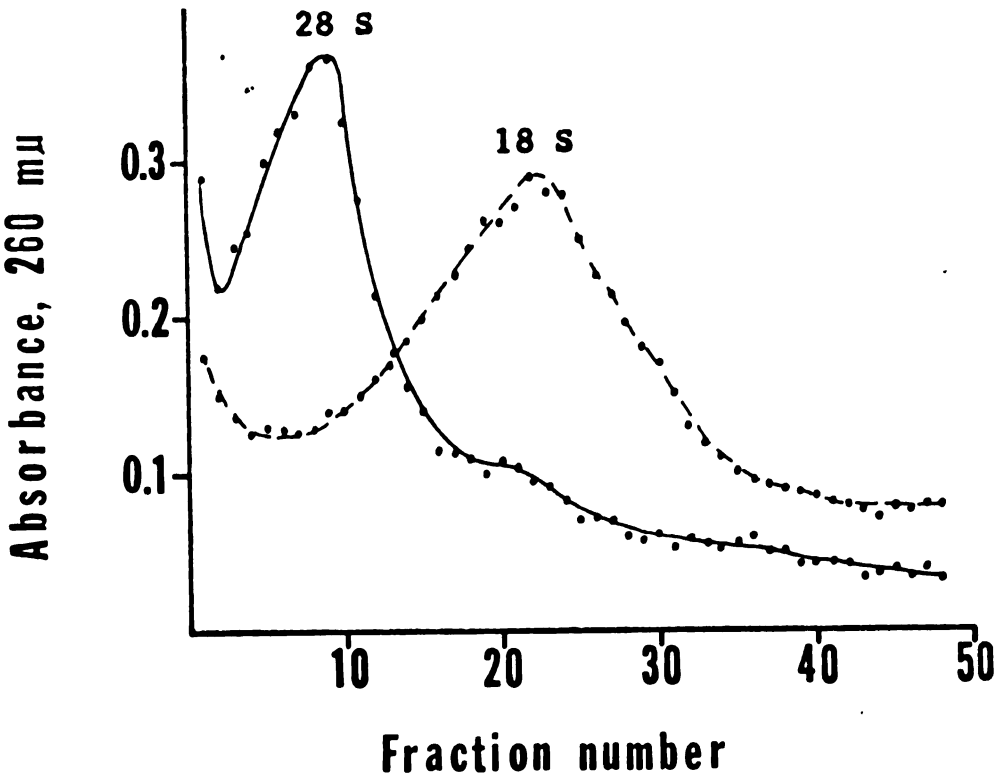

Figure 4: Sedimentation profiles of individual cauliflower r-RNA subunits. Subunits from hatched areas of Figure 3 were centrifuged simultaneously for 15 hours in separate linear sucrose gradients, using saline acetate buffer. Four OD units of 18 S r-RNA and six OD units of 28 S r-RNA were used. Fractions were diluted with one ml of saline acetate buffer.

E, and was found to contain 2693 cpm in uridine-5'-monophosphate and 6175 cpm in uridine -2'(3')-monophosphate. The distribution of these products upon rechromatography in system A is shown in Figure 5, and the distribution of the 2'(3')-monophosphate upon electrophoresis in 0.05 M citrate buffer, pH 5, is illustrated in Figure 6. The other half of the phosphorylated sample, following successive chromatography with cytidine monophosphate standards in systems B, C and A gave only 15 cpm above background.

Pollard (68) reacted the periodate-oxidized nucleoside fraction of cauliflower 18 S RNA with tritiated sodium borohydride, and was able to isolate both adenine and uridine trialcohols.

These results constitute good evidence that nolecules of both the 18 S and 28 S subunits of cauliflower r-RNA may terminate as either adenosine or uridine.

Soluble RNA

The nucleoside fraction derived from alkaline hydrolysis of 20 mg of cauliflower s-RNA was found following chromatography with system B, to contain 0.021 μ moles of adenosine, 0.034 μ moles of guanosine, 0.076 μ moles of cytidine and 0.045 μ moles of uridine. These results demonstrate that all four nucleosides may be produced by

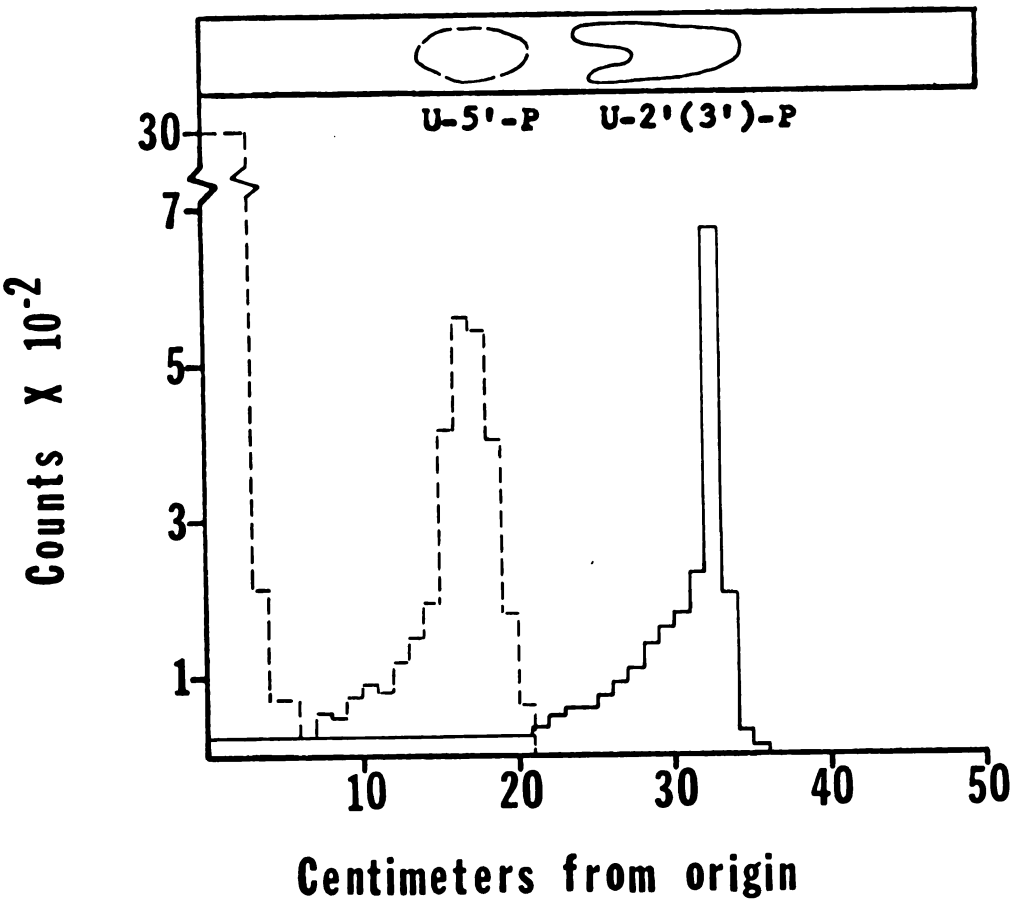

Figure 5: Radioactivity of chromatogram, from system A, of uridine monophosphates resulting from phosphorylation of cauliflower 18 S r-RNA nucleoside fraction with ^{32}P-polyphosphate. The isomers were chromatographed successively on Whatman No. 1 paper in systems B and E, and then chromatographed in adjacent lanes in system A. The drawing at the top represents the ultraviolet-absorbing areas of unlabeled standards with which the samples were co-chromatographed. Counts per 10 minutes are given for the 5' isomer (broken line) and counts per minute for the mixed 2'(3') isomers (solid line).

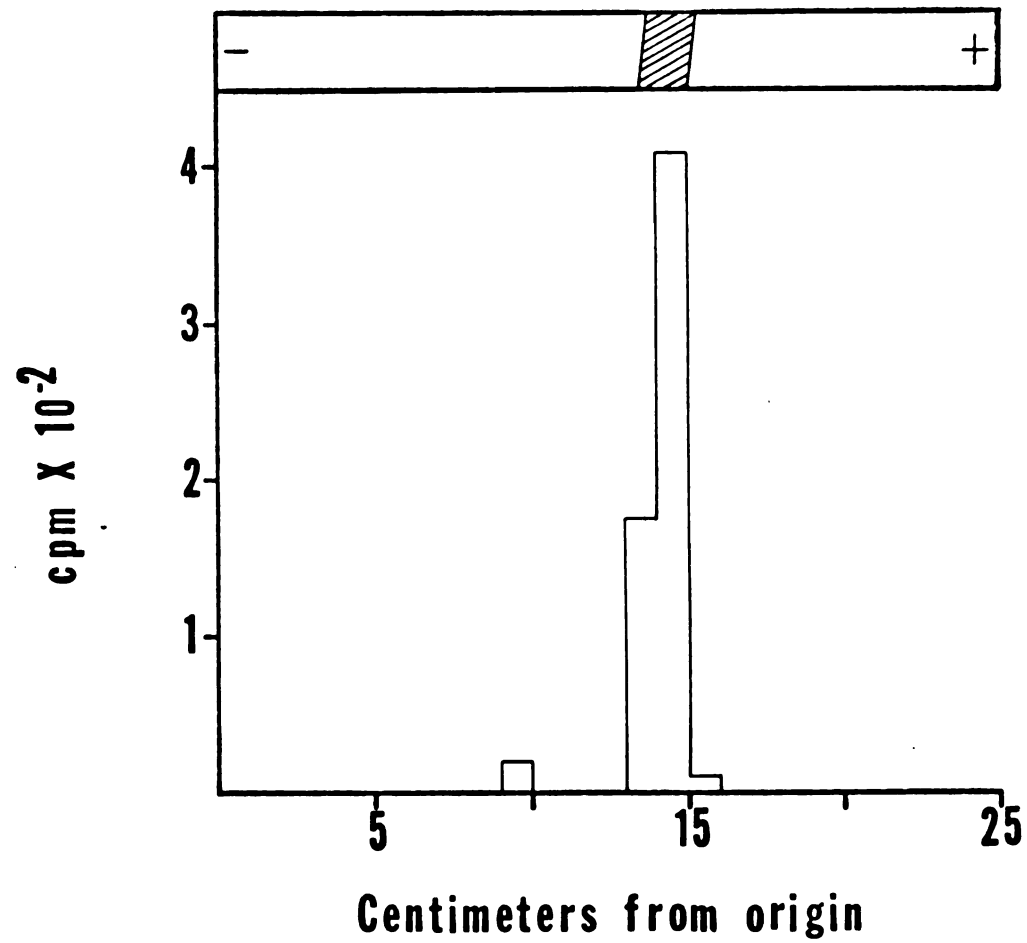

Figure 6: Radioactivity of paper electrophoretogram of uridine-2'(3')-monophosphate, resulting from phosphorylation of cauliflower 18 S r-RNA nucleoside fraction with ^{32}P-polyphosphate, and eluted from a paper chromatogram developed in system A. Electrophoresis was done for 3 hours at 300 V, in a Beckman RD-2 electrophoresis unit, using 0.05 M citrate buffer, pH 5.0, and Whatman 3 mM electrophoresis paper. The hatched area at the top represents the ultraviolet-absorbing area of unlabeled standard.

alkaline hydrolysis, thereby indicating that guanosine and cytidine were not preferentially lost in experiments on r-RNA. Although the finding of all four nucleosides in alkaline hydrolysates of s-RNA is not unique, typical results of others usually show that adenosine and cytidine are the only nucleoside products of alkaline hydrolysis of s-RNA (see Table 2). The results we obtained upon hydrolysis of 20 mg of cabbage s-RNA, showed that 0.049 μ moles of adenosine and 0.055 μ moles of cytidine were released. No quanosine or uridine were detected. These results, expressed as percent of total nucleoside fraction, are summarized in Table 9.

Table 9. Nucleosides resulting from the hydrolysis of s-RNA.

Source of s-RNA	Mole Percent of Nucleosides			
	A	G	C	U
Cauliflower	12	19	43	26
Cabbage	47	n.d.	53	n.d.

n.d. not detected

Yields of nucleosides from s-RNA were only 13 to 22 percent of those which would be expected from molecules with molecular weights of 2.5×10^4, indicating that s-RNA

preparations may have been contaminated with larger RNA species. Purity of s-RNA preparations was not varified by sucrose density gradient centrifugation. Data comparing expected and actual yields of nucleosides are presented in Table 12.

The 5'-Linked Termini of Total Wheat Germ Ribosomal RNA

The results of Lane and co-workers (45, 46, 80) and of Lee and Gilham (49), who found all four nucleosides in alkaline hydrolysates of wheat germ r-RNA is different from our finding of only adenosine and uridine in hydrolysates of cauliflower, cabbage and mushroom r-RNA. Attempts were made to explain these differences. The experimental approach followed was to identify the nucleosides resulting from alkaline hydrolysis of r-RNA (a) isolated and hydrolyzed under the conditions used in our laboratory and (b) under the conditions used by Lane and co-workers (80). In addition, special attention was given to the sedimentation profiles of the RNA since large quantities of RNA had to be employed. The contribution of nucleoside from 1 mg of s-RNA is approximately equivalent to that from 20 mg of 18 S RNA or 40 mg of 28 S RNA. Contaminating s-RNA could very easily escape notice unless sufficient amounts of RNA were applied on the gradients.

A sample of wheat germ r-RNA, which will be referred to as preparation I, was obtained by direct extraction of wheat germ with phenol and solution A, and was washed six times by homogenization in 3 M sodium acetate. A sedimentation profile of this r-RNA is presented in Figure 7A. No DNA was detected in this preparation. Only adenosine (0.240 μ moles) and uridine (0.159 μ moles) were detected in the hydrolysate of 350 mg of this preparation, following chromatography in system B.

The sedimentation profile of a second RNA preparation (preparation II) is illustrated in Figure 7B. This RNA was isolated by direct extraction with phenol and solution B, was homogenized two times with 3 M sodium acetate, and like preparation I, was found to be free of DNA. The nucleoside fraction, following alkaline hydrolysis of 600 mg of RNA, was chromatographed in system B, and was found to contain 0.425 μ moles of adenosine, 0.039 μ moles of cytidine and 0.400 μ moles of uridine. No guanosine was detected. Rechromatography of the adenosine and uridine products in system G showed that they migrated with authentic standards in that system as well.

Ten percent of the nucleoside fraction from preparation II was incubated with purified carrot leaf-nucleoside

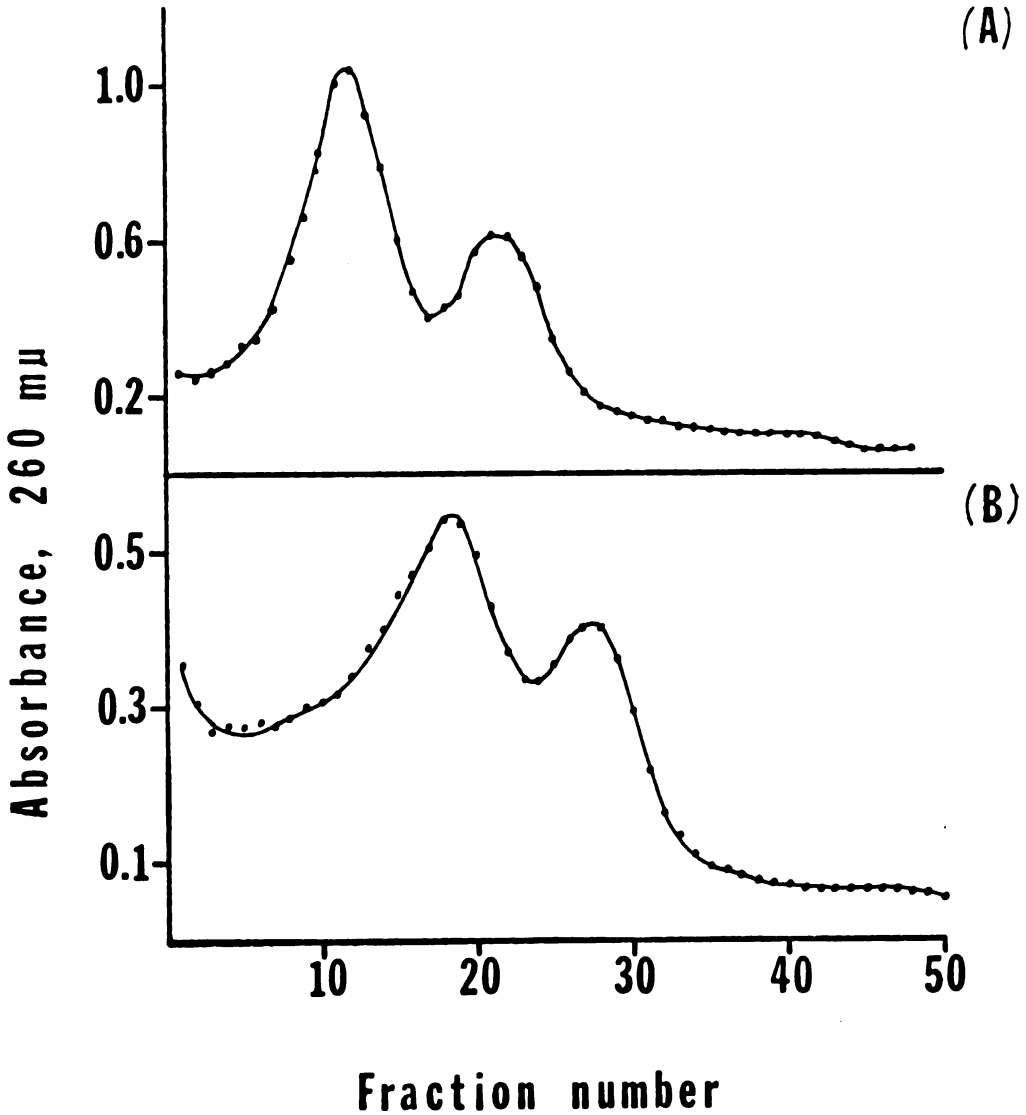

Figure 7: (A) Sedimentation profile of 48 OD units of wheat germ r-RNA isolated by direct extraction of wheat germ with phenol and solution A. The preparation was washed six times with 3 M sodium acetate, pH 6.0. Centrifugation was done for 18 hours, using saline citrate buffer. (B) Sedimentation profile of 42 OD units of wheat germ r-RNA isolated by direct extraction of wheat germ with phenol and solution B. The preparation was washed two times with 3 M sodium acetate, pH 6.0. Centrifugation was done for 16.5 hours, using saline citrate buffer.

phosphotransferase and ^{32}P-p-nitrophenyl phosphate, spotted on chromatography paper, and the chromatogram developed with system F for 37 hours. The nucleotides remained near the origin, while inorganic phosphate and p-nitrophenyl phosphate migrated more rapidly and were thus eliminated. Elution of the nucleotide area, followed by chromatography in system B, gave the distribution of radioactivity shown in Figure 8. Subsequent electrophoresis of each of the nucleotide samples individually in the pH 3.5, acetic acid:pyridine system, showed the distribution of radioactivity presented in Table 10.

Table 10. Radioactivity of nucleotides produced by the reaction of ^{32}P-p-nitrophenyl phosphate with the nucleoside fraction from wheat germ r-RNA.

Activity	Nucleotide			
	AMP	GMP	CMP	UMP
cpm	74	7	161	554
Percent	9	1	20	70

Radioactivity in the guanosine monophosphate area of the chromatogram shown in Figure 8 migrated with cytidine monophosphate in the electrophoretic separation, and was probably due to overlap of the two spots on the chromatogram. These results provide qualitative support for the

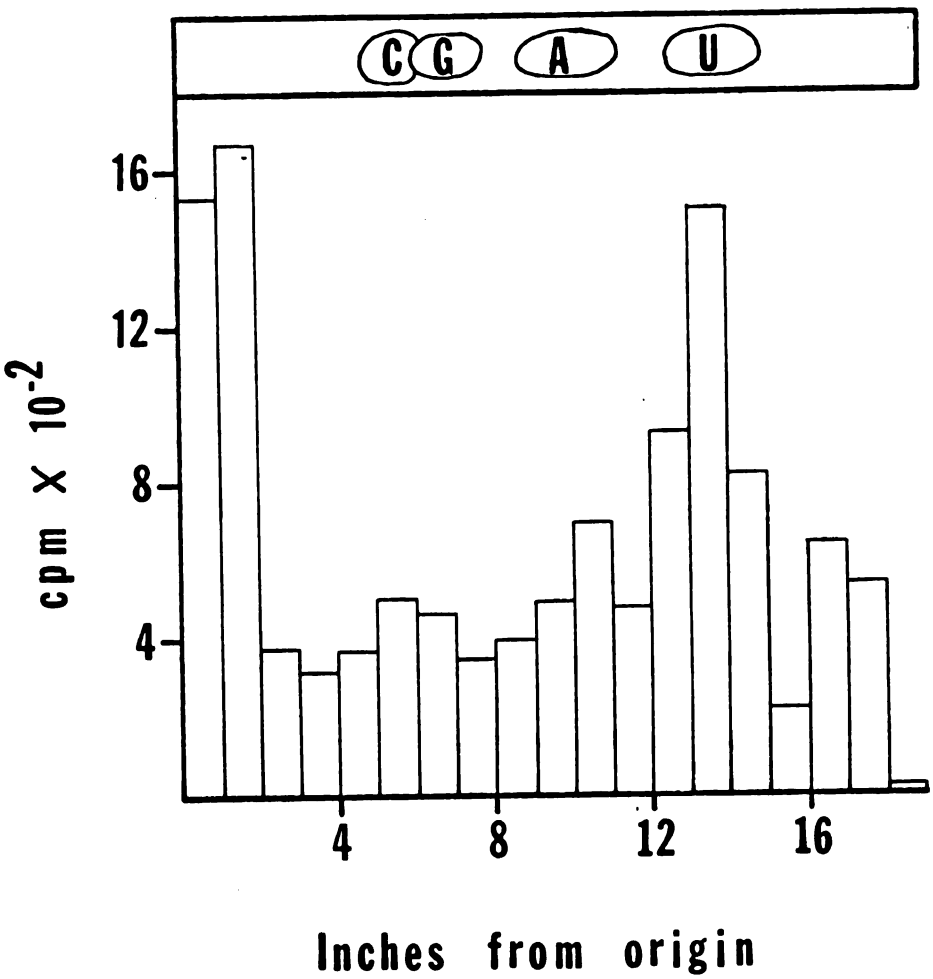

Figure 8: Radioactivity on chromatogram, developed in system B, of nucleoside monophosphates resulting from phosphorylation of wheat germ r-RNA nucleoside fraction with ^{32}P-p-nitrophenyl phosphate and nucleoside phosphotransferase. The nucleotides were chromatographed first in system F, to remove ^{32}P and ^{32}P-p-nitrophenyl phosphate. The drawing at the top represents the ultraviolet-absorbing areas of unlabeled standards with which the sample was co-chromatographed.

identity of nucleosides reported for this RNA preparation, as the monophosphates of adenosine, cytidine and uridine formed the major portion of the product. The relative amounts of radioactivity, however, are probably misleading, as this criterion showed cytidine to be twice as abundant as adenosine, whereas spectral evidence showed that adenosine was more than ten times as abundant as cytidine.

Preparation III of wheat germ r-RNA (Figure 9) was isolated by direct extraction with phenol and solution A, followed by two homogenizations in 3 M sodium acetate, instead of six homogenizations as done with preparation I. The sample was shown to contain four percent DNA, as indicated by the diphenylamine test. The hump on the light side of the 18 S RNA peak, between fractions 31 and 40, was believed to be enzymatically degraded r-RNA. Thus it is clear from a comparison of Figure 9 with Figure 7A that extensive treatment with 3 M sodium acetate is required for obtaining a good preparation of wheat germ r-RNA, when solution A has been employed for extraction of RNA.

Nucleosides produced by hydrolyzing 675 mg of RNA from preparation III were found, following chromatography in system B, to consist of 0.381 μ moles of adenosine, 0.220 μ moles of guanosine, 0.336 μ moles of cytidine and

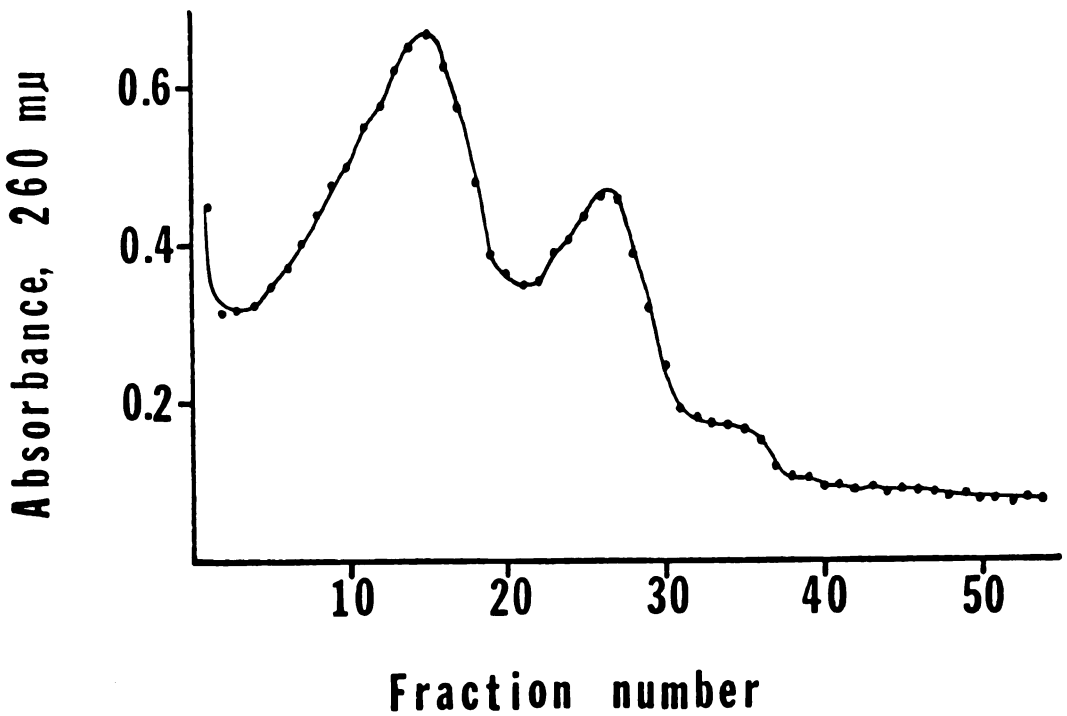

Figure 9: Sedimentation profile of 48 OD units of wheat germ r-RNA isolated by direct extraction of wheat germ with phenol and solution A. The preparation was washed two times with 3 M sodium acetate, pH 6.0. The gradient was made up in saline citrate buffer, and was centrifuged for 19 hours.

0.407 μ moles of uridine.

The occurrence of all four nucleosides in the hydrolysate of preparation III, which was shown to be contaminated with DNA and low molecular weight RNA, was in agreement with the results of Lane and co-workers (45, 46, 80) and Lee and Gilham (49), (see Table 7). This fact indicated the possibility that wheat germ r-RNA preparations used by these workers may have been contaminated in much the same manner as preparation III. Another wheat germ r-RNA preparation (preparation IV), was isolated, therefore, according to the procedures of Singh and Lane (80). This involved direct extraction of RNA with phenol and phosphate buffer pH 7.0, and precipitation of the r-RNA from 1.0 M sodium chloride. No DNA was detected in this preparation, and good resolution of the r-RNA subunits was achieved with sucrose density gradient centrifugation (Figure 10). The RNA was also hydrolyzed according to Singh and Lane (80), using 1.0 M sodium hydroxide at room temperature, rather than 0.3 M potassium hydroxide at 37° C, as was done for our other preparations. Another difference in this procedure was that the hydrolysate was neutralized with 3 N hydrochloric acid, rather than with Dowex 50-H^+. Purification and chromatography of nucleosides were the same as described for

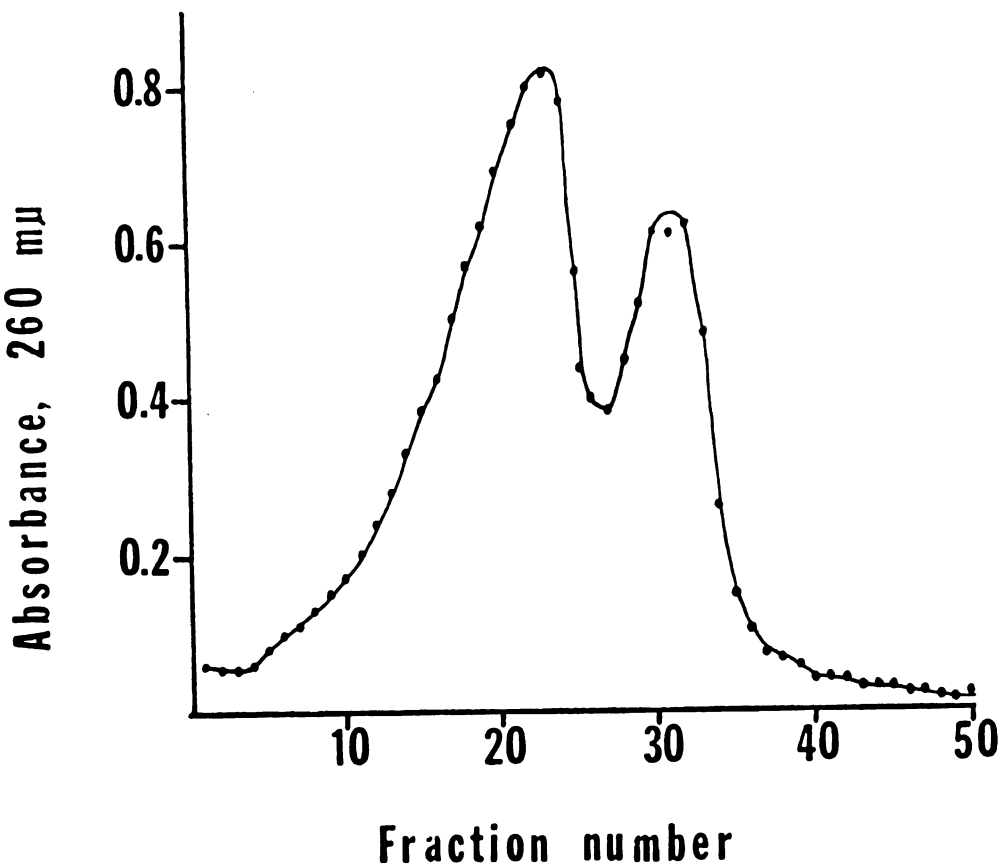

Figure 10: Sedimentation profile of wheat germ r-RNA isolated by the methods of Singh and Lane (80). The gradient was made up in saline citrate buffer. Forty-two OD units of r-RNA were centrifuged for 16 hours.

other preparations. Nucleosides from 300 mg of RNA were shown to consist of 0.054 μ moles of adenosine, 0.093 μ moles of guanosine, 0.165 μ moles of cytidine and 0.216 μ moles of uridine, thereby supporting reports of all four nucleosides in alkaline hydrolysates of wheat germ r-RNA when these methods were used.

Results on all four wheat germ r-RNA preparations are summarized and compared with those of Lane and co-workers (45, 46, 80), and Lee and Gilham (49), in Table 11.

Yields of nucleosides varied between different wheat germ r-RNA preparations, and may have influenced the nature of the product which was obtained. The yield of nucleosides from preparation I was approximately that which would be expected from nolecules with an average weight of 10^6, and only adenosine and uridine were detected. Preparation II yeidled 44 percent more nucleosides than expected, and in this case, in addition to adenosine and uridine, cytidine was detected. Yields of nucleosides from preparations III and IV were respectively 99 and 76 percent greater than expected, and in both cases, all four nucleosides were detected. These data are summarized in Table 12.

Table 11. Summary of data on the 5'-linked termini of wheat germ r-RNA.

Preparation	Mole Percent of Nucleoside			
	A	G	C	U
I	60	n.d.	n.d.	40
II	49	n.d.	5	46
III	28	17	25	30
IV	10	18	31	41
A[a]	20	31	26	23
B[b]	20	24	31	25

n.d. not detected

[a] average values from data of Lane and co-workers (45, 46, 80) presented in Table 7.

[b] data of Lee and Gilham (49).

Table 12. Yield (percent of expected) and identity of nucleosides obtained from alkaline hydrolysates of RNA preparations studies in this thesis. Only adenosine and uridine were detected in hydrolysates of cauliflower, cabbage and mushroom r-RNA, regardless of the yield. Only adenosine and uridine were isolated from wheat germ r-RNA when the yield was approximately that which was expected, whereas cytidine and guanosine were also detected when yields were greatly in excess of those exptected. Low yields obtained from s-RNA appear to be independent of the nucleosides isolated, and may be due to contamination of these preparations by larger RNA.

Source of RNA	Size of RNA	Yield of Nucleosides (Percent of Expected)	Nucleosides Detected
Cauliflower	18 S + 28 S	95	A, U
Cabbage	18 S + 28 S	90	A, U
Mushroom	18 S + 28 S	216	A, U
Cauliflower	28 S	164	A, U
Wheat germ			
Preparation I	18 S + 28 S	114	A, U
Preparation II	18 S + 28 S	144	A, U, C
Preparation III	18 S + 28 S	199	A, U, C, G
Preparation IV	18 S + 28 S	176	A, U, C, G
Cauliflower	s-RNA	22	A, U, C, G
Cabbage	s-RNA	13	A, C

Incorporation of 2-^{14}C-Uracil by Cauliflower hypocotyls

The preferential termination of plant r-RNA's with adenosine and uridine, and the existence of enzymes which add 5'-linked terminal nucleotides to RNA's other than s-RNA (10, 40, 41, 65), suggest that terminal addition, subsequent to DNA-dependent RNA synthesis, might be involved in the preferential termination of plant r-RNA. Because all of the enzymes mentioned above were found to add uridine monophosphate, and because the proportion of terminal uridine on *E. coli* r-RNA has been shown to vary with environmental conditions, experiments were done to determine if uridine is added to plant r-RNA chains subsequent to DNA-dependent RNA synthesis. The experimental approach followed was to isolate the nucleoside and nucleotide fractions from alkaline hydrolysates of caulfilower hypocotyl r-RNA labeled with 2-^{14}C-uracil, in both the presence and absence of actinomycin D. It was conceived that if 5'-linked-terminal uridine is added to the r-RNA chains after DNA-dependent chain synthesis, a higher ratio of ^{14}C-nucleoside:^{14}C-nucleotide should be obtained from RNA labeled in the presence of actinomycin D than from that labeled in its absence.

Effect of Actinomycin D Concentration

Prior to studies on the ratio of terminal to non-terminal uridine in cauliflower hypocotyl r-RNA, in the presence and absence of actinomycin D; it was necessary to determine suitable concentrations of actinomycin D and optimum incorporation times. A concentration of actinomycin D which would give approximately 90 percent inhibition of incorporation was desired, as it was reasoned that at this level of inhibition it would be possible to detect preferential addition of 5'-linked terminal uridine to existing RNA chains.

Ten hypocotyls were weighed and placed in each tube where they were preincubated for two hours in incubation media with actinomycin D concentrations of 0 to 60 μg per ml. Each concentration was run in triplicate. Incorporation was started by the addition of 0.2 μc of 2μ-^{14}C-uracil, was allowed to proceed for six hours and was stopped by boiling the reaction mixture in ethanol. Following extraction of alcohol-soluble material, the radioactivity was measured. The results of this experiment, which was the third in a series of experiments, are presented in Figure 11. An actinomycin D concentration of 60 μg per ml gave 90 percent inhibition of 2-^{14}C-uracil incorporation. In the first of

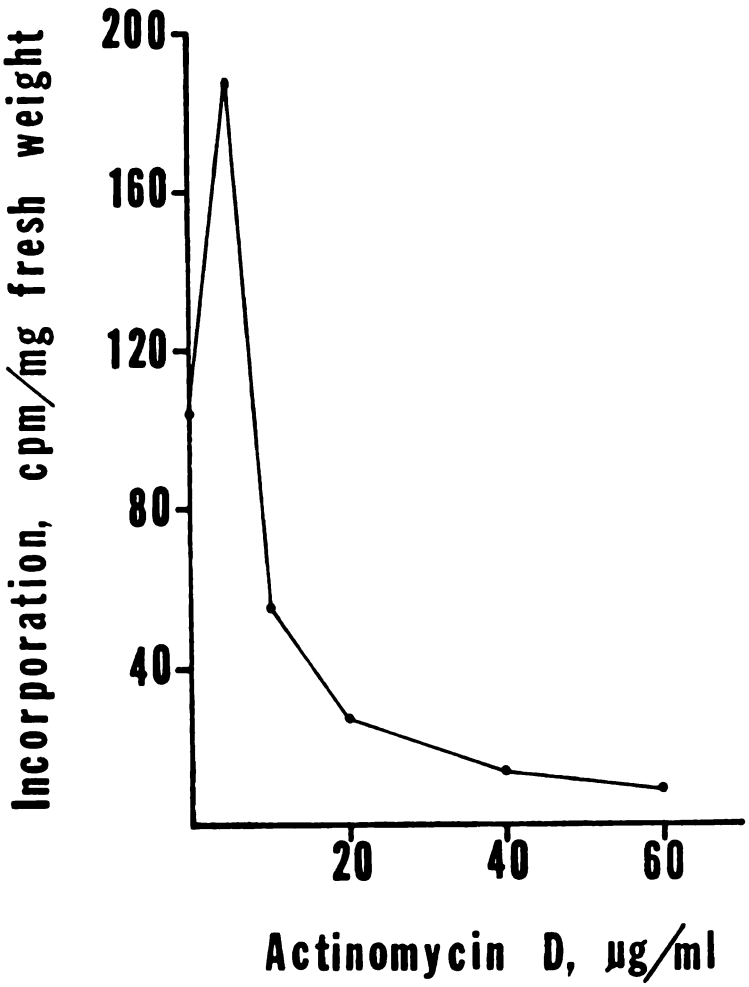

Figure 11: Incorporation of 2-^{14}C-uracil into alcohol-insoluble material by cauliflower hypocotyls with increasing concentrations of actinomycin D. Ten hypocotyls were incubated in each tube for 6 hours at room temperature following the addition of 2-^{14}C-uracil. Each point represents the mean of three replications.

these experiments no preincubation period was used, and much less inhibition resulted at the same concentrations, indicating that penetration of actinomycin D into the hypocotyl cells occurs more slowly than penetration of uracil. The second of these experiments involved the use of actinomycin D concentrations ranging from 0 to 50 μg per ml, and although results were similar to those reported for the third experiment, the desired level of inhibition (90 percent) was not achieved.

An interesting aside to these experiments, which was not pursued in greater detail but which was consistently observed, was a stimulation of uracil incorporation by actinomycin D at concentrations of 0.2 to 5 μg per ml (Figure 11).

The validity of the assumption that inhibition of incorporation of uracil into ethanol-insoluble material is equivalent to inhibition of RNA synthesis was established in another set of experiments. Preincubated hypocotyls were incubated for eight hours in the presence and absence of 60 μg per ml of actinomycin D, which had been shown (Figure 11) to give approximately 90 percent inhibition of alcohol-insoluble uracil incorporation. RNA was isolated by extraction with phenol and solution B. Results of these

experiments are presented in Table 13. Thus it is established that incorporation into the ethanol-insoluble fraction corresponds to incorporation into RNA.

Table 13. Inhibition of incorporation of 2-^{14}C-uracil into cauliflower hypocotyl r-RNA by 60 μg/ml of actinomycin D.

Experiment Number	CPM per 100 Hypocotyls		Percent Inhibition
	Control	+ Actinomycin D	
I	11,300	1,250	89
II	174,000	22,300	87

Time Course of 2-^{14}C-uracil Incorporation

An experiment was done on the time course of uracil incorporation to determine an appropriate incubation time for experiments involving incorporation of 2-^{14}C-uracil into r-RNA. An incubation time was wanted which would insure adequate incorporation of 2-^{14}C-uracil into r-RNA of actinomycin D-inhibited hypocotyls to enable detection of terminal nucleosides, but which would not be so long as to lead to a breakdown of inhibition. For these experiments, hypocotyls were treated as in the previous experiments, except that only seven hypocotyls per tube were used, and only one concentration of actinomycin D (60 μg/ml) was

compared with the control. Results are presented in Figure 12. Based on these results, an incubation time of eight hours was selected for experiments involving incorporation of 2-^{14}C-uracil into RNA, as it was felt that adequate incorporation could be obtained in that length of time, and inhibition approximated the 90 percent level which was desired.

The Ratio of Terminal to Non-terminal Uridine in Cauliflower Hypocotyl r-RNA

Incorporation of uracil into RNA was accomplished using an eight hour incubation period, with 100 hypocotyls in 3 ml of incubation medium for controls, and 200 hypocotyls in 6 ml of incubation medium for actinomycin D treatments. Control hypocotyls were supplied with 50 μc of 2-^{14}C-uracil, whereas actinomycin D treated ones were supplied with 100 μc.

RNA extracted from the hypocotyls was applied to sucrose density gradients; the resulting sedimentation profiles are shown in Figures 13 and 14. Although the added carrier RNA appears to have undergone a partial dissociation of the 28 S subunit during the extraction procedure, the ^{14}C-labeled RNA remained intact and showed good resolution into subunits on the sucrose gradient. In subsequent experiments carrier RNA was added after homogenization with

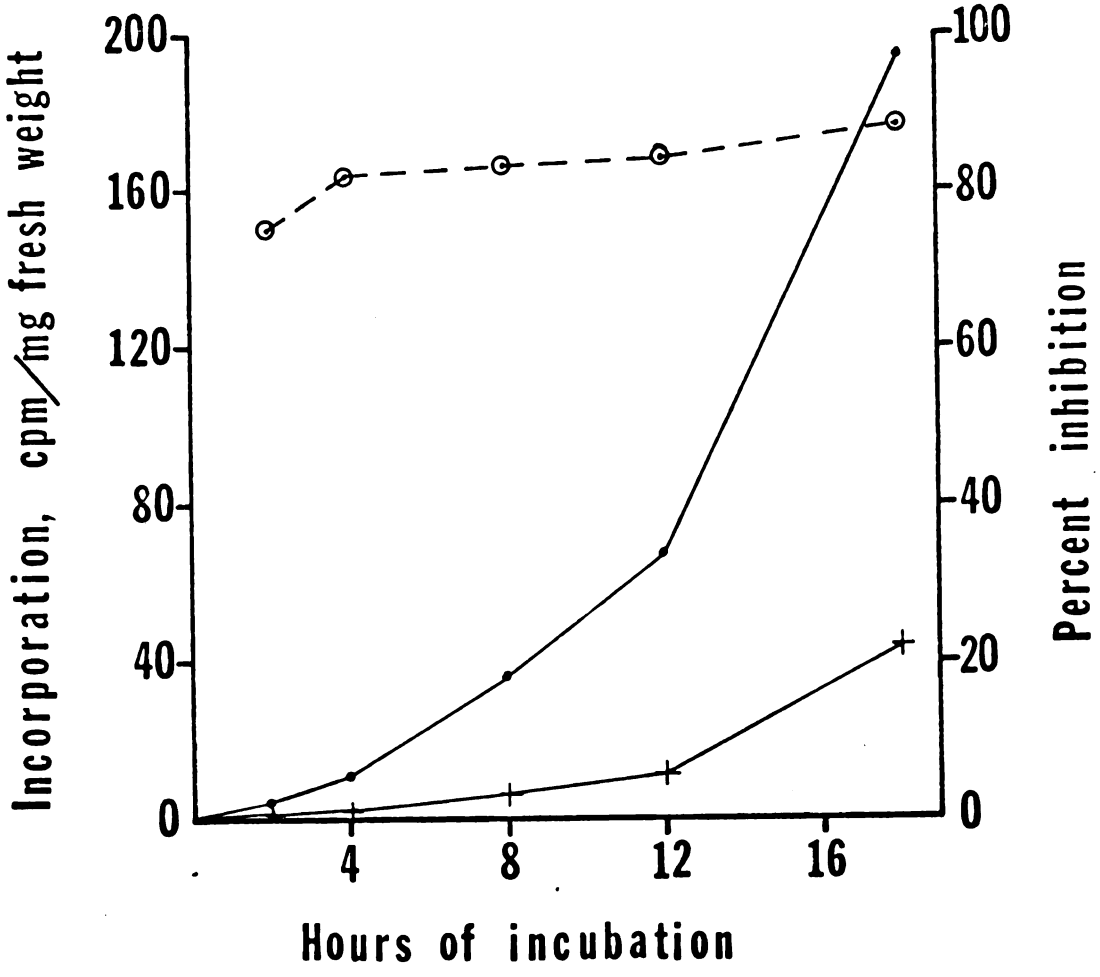

Figure 12: Incorporation of 2-^{14}C-uracil, into alcohol-insoluble material, by cauliflower hypocotyls with increasing time, in the presence and absence of 60 μg/ml of actinomycin D. Seven hypocotyls were incubated in each tube at room temperature. Each point represents the mean of three replications.

•———• Incorporation, cpm/mg fresh weight in control hypocotyls

+———+ Incorporation, cpm/mg fresh weight in actinomycin D treated hypocotyls

⊙– – –⊙ Percent inhibition

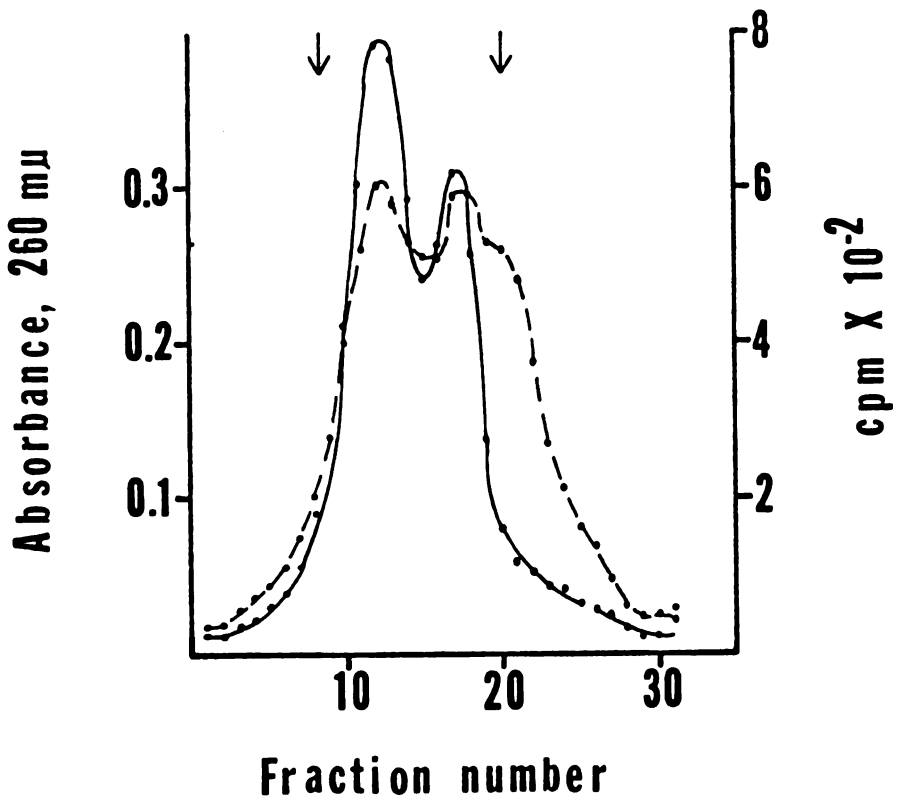

Figure 13: Sedimentation profiles of r-RNA isolated from cauliflower hypocotyls incubated for 8 hours in 2-^{14}C-uracil, and of unlabeled carrier r-RNA. The gradient, prepared in saline citrate buffer, was centrifuged for 16 hours. The fractions between the arrows at the top were collected for alkaline hydrolysis of the RNA.

•——• Radioactivity of cauliflower hypocotyl r-RNA

•– – –• Absorbance at 260 mμ of 15 OD units of added carrier, cauliflower r-RNA

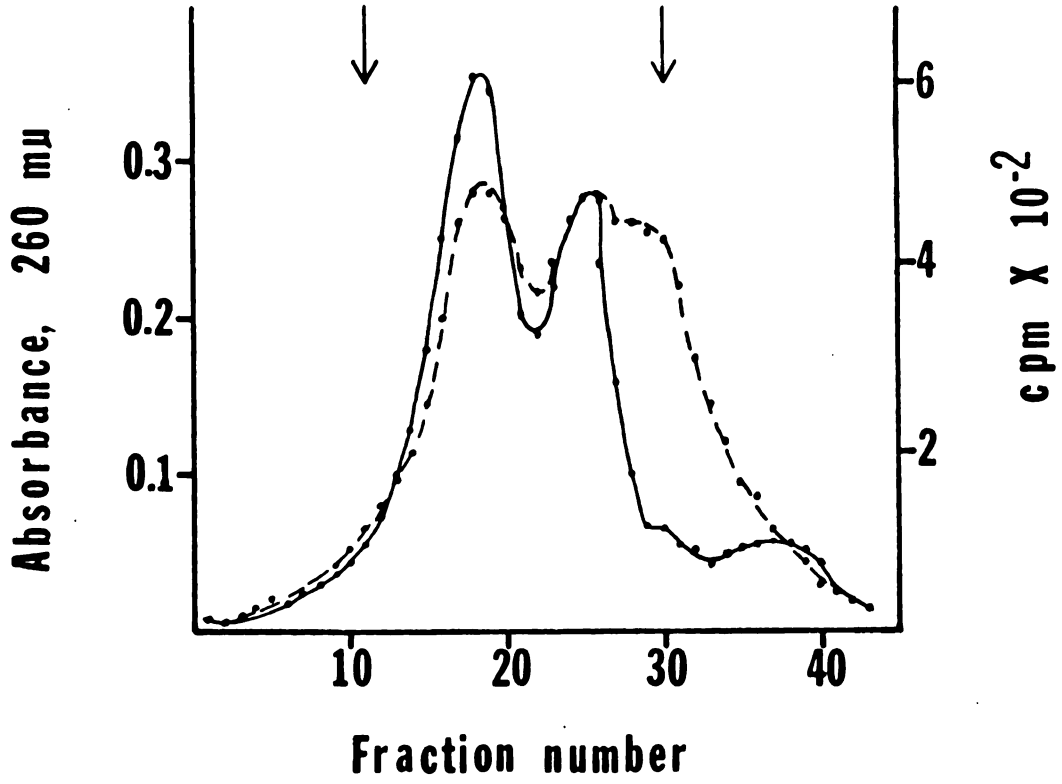

Figure 14: Sedimentation profiles of r-RNA isolated from cauliflower hypocotyls incubated for 8 hours in 2-^{14}C-uracil and 60 μg/ml of actinomycin D, and of unlabeled carrier r-RNA. The gradient, prepared in saline citrate buffer, was centrifuged for 16 hours. The fractions between the arrows at the top were collected for alkaline hydrolysis of the RNA.

•——— Radioactivity of cauliflower hypocotyl r-RNA

•– – –• Absorbance at 260 mμ of 15 OD units of added carrier, cauliflower r-RNA

solution B and phenol, and good resolution was achieved on gradients, as measured by absorbance at 260 mμ. The presence of radioactive material in the s-RNA region of Figure 14, not eliminated by homogenization in 3 M sodium acetate, made it necessary that all labeled RNA used in the remainder of these studies be subjected first to sucrose density gradient centrifugation, so that only the r-RNA was collected. Inclusion of the low molecular weight fraction could have led to disproportionately high values for terminal nucleoside in alkaline hydrolysates.

RNA was collected from sucrose gradients, hydrolyzed, and the nucleoside fraction collected as previously described; after which the nucleotides were eluted from the column with 0.1 N hydrochloric acid. These fractions were evaporated to dryness under reduced pressure and redissolved in 5 ml of 0.05 N hydrochloric acid. One ml aliquots of each fraction were pipetted into each of three counting vials, evaporated to dryness, and their radioactivity determined. Results of three repetitions of this experiment are presented in Tables 14 and 15.

The results, with a 50 percent overlap of standard deviations, indicate that no difference exists in the ratio of terminal to non-terminal uridine in cauliflower hypocotyl

Table 14. Radioactivity of nucleosides and nucleotides isolated from alkaline hydrolysates of r-RNA labeled in the presence and absence of 60 μg/ml of actinomycin D.

Experiment Number	CPM Control		CPM Actinomycin D	
	Nucleoside	Nucleotide	Nucleoside	Nucleotide
1	119	61855	90	41031
2	57	28401	12	6102
3	38	21577	16	8437

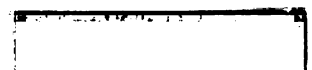

Table 15. Ratios of terminal to non-terminal uridine in r-RNA isolated from cauliflower hypocotyls incubated with 2-^{14}C-uracil in the presence and absence of 60 μg/ml of actinomycin D. Results are based on the assumption that all radioactivity isolated is in the form of uridine or uridine-2'(3')-monophosphate.

Experiment Number	Ratio in Control	Ratio in Actinomycin D Treated
1	1/520	1/456
2	1/498	1/509
3	1/568	1/529
Average and Standard Deviation	1/529 ± 29	1/498 ± 31

r-RNA labeled in the presence or absence of actinomycin D. Surprisingly, the ratio of approximately 1/515 was much higher than the ratio which was anticipated (1/1500 to 1/1600). A suitable explanation of this observation is not known.

DISCUSSION

RNA Isolation Procedures

A potentially important contribution of these studies is the development of techniques for obtaining large quantities of cytoplasmic r-RNA from plants. This may be especially significant if studies on the base sequence of r-RNA are undertaken. Since it has become apparent that organelles possess r-RNA, and since it has been possible to demonstrate that chloroplast ribosomes have distinctly different S-values from cytoplasmic ribosomes, it might be desirable to utilize cytoplasmic r-RNA as a basis for studies on base sequences, in order to minimize the pssibility of obtaining heterogeneous r-RNA, for example, r-RNA from different organelles.

By improving techniques for the separation of RNA's, it is possible that systems may be developed which will separate r-RNA's varying by very small differences in S values, or differing in some other manner. If so, then it may be possible to investigate the differences of r-RNA in

organelles, or even the structural heterogeneity of cytoplasmic r-RNA subunits.

In view of such possible future needs for large quantities of r-RNA, it might be well to briefly asses the methods used in this thesis.

Extraction of RNA from microsomal pellets from cauliflower, cabbage and mushrooms with phenol and solution A yielded a product which showed good resolution on sucrose density gradients. This method is particularily applicable to tissues such as fleshy or leafy plant parts, where low yields of RNA can be expected, and large volumes of tissue must be used. Inadequacy of the method for tissues high in nucleic acids and protein is demonstrated by the difficulties encountered in attempts to isolate r-RNA from wheat germ.

Since DNA and s-RNA were also extracted in attempts to isolate r-RNA from wheat germ with phenol and solution A, it is probably the least satisfactory of the homogenizing media used for the extraction of r-RNA from this tissue. Use of either solution B or pH 7.0 phosphate buffer gave products free of DNA or s-RNA contamination. The method of Singh and Lane (80), using phosphate buffer and phenol, gave the best results, based upon resolution of the subunits by sucrose density gradient centrifugation.

A difficulty which may be encountered in the isolation of RNA with phenol and solution B is illustrated by the apparent decrease in the amount of 28 S r-RNA and concomitant appearance of a larger, bimodal peak in the 18 S region of carrier r-RNA added during the isolation of cauliflower hypocotyl r-RNA (Figures 13 and 14). Beck, et al. (3) demonstrated that dissociation of the larger r-RNA subunit into two smaller molecules, at alkaline pH (pH 9) and in the presence of EDTA, as described by Midgley (56, 58), was due to the action of ribonuclease. Since solution B has a pH of 9.5, and contains EDTA, a dissociation of the type described by Midgley (56, 58) appears to be involved; this resulted in the sedimentation profiles illustrated in Figures 13 and 14. Apparently sufficient ribonuclease was present in the cauliflower to cause a partial hydrolysis of the carrier r-RNA added prior to homogenization with phenol. The reason that the hypocotyl r-RNA did not undergo this same dissociation may have been that this r-RNA was shielded by ribosomal proteins. Solution B was apparently not directly responsible for the dissociation, because when carrier r-RNA was added immediately following homogenization, rather than prior to it, no dissociation was apparent.

These observations on the use of different isolation

techniques demonstrate that in any attempts to isolate RNA, the methods used must be checked to determine the quality of the product obtained, and that one method is not necessarily applicable to all tissues.

The 5'-Linked Termini of Cytoplasmic r-RNA

Adenosine and uridine were the only nucleosides isolated from alkaline hydrolysates of unfractionated cytoplasmic r-RNA from cauliflower, cabbage and mushrooms, and from 28 S r-RNA from cauliflower. Uridine was identified as a 5'-linked terminal groups of the 18 S subunit. The presence of both adenosine and uridine as 5'-linked termini of both subunits of cauliflower r-RNA has been confirmed by Pollard et al. (60) using tritiated trialcohols, formed by reduction of the dialdehyde oxidation products of nucleosides. Cauliflower r-RNA isolated by direct extraction with phenol and solution A was found by Pollard (68) to also contain only adenosine and uridine as 5'-linked termini, indicating that this phenomenon is not confined to cytoplasmic r-RNA. The difference in ratios obtained with r-RNA from cabbage and cauliflower indicates that the ratios of the termini within a species is not necessarily constant.

Similar reports, of a predominance of adenosine and uridine as 5'-linked termini, have been made by others in

studies on r-RNA from E. coli (43, 53, 59, 60, 62), rabbit reticulocytes (36) and L cells (47), (see Table 7). Pollard, et al. (69) have demonstrated that adenosine and uridine are the only nucleosides isolated from alkaline hydrolysates of spinach and parsnip r-RNA. Although these results do not constitute proof of the universality of this phenomenon, they do suggest that it is wide-spread. Worthwhile extension of these findings could be achieved through the use of different techniques to confirm them, and studies on a greater diversity of organisms.

The results of Midgley and McIlreavy (60) demonstrate that the ratios of termini may be changed by altering the nutrient status of E. coli. Another line of investigation, which might beproductive, would be to determine if organisms other than E. coli exhibit this phenomenon.

The significance of these findings, a predominance of 5'-linked terminal adenosine and uridine, is unclear at this time due to a deficiency of information on the structure and function of r-RNA. It is possible that the nature of the termini of r-RNA may be related to the activity of ribosomes in protein synthesis. This possibility will be discussed in greater detail later.

The 5'-Linked Termini of Total Wheat Germ r-RNA

Results of experiments to determine the 5'-linked termini of wheat germ r-RNA indicate that this RNA terminates predominantly as adenosine or uridine. Hydrolysis of the r-RNA with 0.3 M alkali gave adenosine and uridine primarily, small amounts of cytidine and no quanosine. Qualitative confirmation of these results was achieved by phosphorylation of the nucleosides with nucleoside phosphotransferase and ^{32}-P-p-nitrophenyl phosphate. Hydrolysis with 1.0 M alkali, and neutralization with 3 N hydrochloric acid, rather than with Dowex 50-H^+, resulted in the production of all four nucleosides, confirming the results of Lane and co-workers (45, 46, 80) and of Lee and Gilham (49). This conflict of results may be due to artifacts introduced by the hydrolysis or neutralization procedures. Sugiyama and Fraenkel-Conrat (88) have reported that neutralization of alkaline hydrolysates with acid can result in dephosphorylation of nucleotides. Dephosphorylation of this type was probably responsible for the appearance of all four nucleosides in the hydrolysate of preparation IV. A reexamination of the procedures employed and use of other techniques, such as labeling with ^{3}H-isonicotinic acid hydrazide as described

by Hunt (36) are desirable. RNA isolated by the methods of Singh and Lane (80) would probably be the best to use in such studies, because of its good resolution on sucrose density gradients.

Incorporation of 2-^{14}C-Uracil by Cauliflower Hypocotyls

Experiments to determine the ratio of terminal to non-terminal uridine in r-RNA synthesized by control and actinomycin D-inhibited cauliflower hypocotyls, revealed that there was no difference in this ratio between the two treatments. These results suggest that termination of these r-RNA molecules, unlike that of s-RNA, is not due to end group addition or turn-over. Termination with specific nucleosides is probably due directly to genetic activity (transcription), as suggested by Midgley and McIlreavy (60), although the possibility also exists that termination is controlled by ribonuclease activity, subsequent to RNA synthesis.

A worthwhile extension of this work would be a determination of the ratios of terminal to non-terminal nucleosides, using highly labeled ^{14}C r-RNA.

Future Application of the Results

The consistent observation of adenosine and uridine as 5'-linked termini on all r-RNA's studied to date suggests

that some mechanism, common to all of the organisms studied, may control the termination of these RNA's. One possible explanation of this phenomenon, discussed by Midgley and McIlreavy (70), is that these termini occur at natural stopping points in the DNA which codes for r-RNA: that is to say, at adenosine and thymidine moieties on DNA. Other possible explanations would be (1) the existence of enzymes which give preferential cleavage, at adenosine or uridine, of larger RNA molecules, thereby giving rise to r-RNA, or (2) the addition of adenosine and uridine to the 5'-linked termini of r-RNA. Although the results of experiments with labeled r-RNA presented in this thesis suggest that the latter mechanism is not involved, they do not completely preclude the possibility of its existence.

The possibility that specific r-RNA termini are related to the activity of ribosomes was mentioned earlier. Although conclusive proof of such a proposal is not possible at this time, due to inadequacy both of techniques and of knowledge concerning the structure and function of r-RNA, circumstantial evidence for it could be obtained by experiments based on the experiments of Midgley and McIlreavy (60), which have already been discussed. These experiments would involve the use of ribosomes from E. coli grown both on

glucose and on "casamino acids," which are known to have different proportions of 5'-linked terminal adenosine and uridine. These ribosomes could be used in a cell-free amino acid-incorporating system to determine their relative rates of amino acid incorporation. Higher rates of amino acid incorporation for either the "high adenosine-" or "high uridine-terminal" r-RNA would provide circumstantial evidence of the essentiality of that terminus. Findings of equal rates of incorporation with both types of ribosomes would provide no evidence either for or against the proposal.

Conclusive proof of the essentiality of particular termini, such as exists for s-RNA and TMV-RNA (see Literature Review), will probably not be obtained until techniques are developed by which biologically active ribosomes can be disassembled and reassembled. In this way, the RNA molecules could be removed from ribosomes, their termini removed, such as by periodate oxidation and amine catalyzed cleavage, followed by hydrolysis with E. coli alkaline hydrolysis, and the RNA then put back into ribosomes.

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