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The Presence of $O^{2'}$-Methylpseudouridine in the 18S + 26S Ribosomal Ribonucleates of Wheat Embryo†

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ABSTRACT: $O^{2'}$-Methylpseudouridine (ψm) has been identified as a constituent of wheat embryo cytosol rRNA. The corresponding 5′-nucleotide, pψm, was recovered from snake venom hydrolysates of the 18S + 26S ribonucleates of wheat embryo. The identity of this nucleotide was established by its ultraviolet absorption spectra, chromatographic and electrophoretic properties, and conversion to ψm by treatment with alkaline phosphatase. Control experiments have eliminated the possibility that contaminating tRNA could have been the source of the pψm isolated from wheat embryo 18S + 26S rRNA. Alkaline hydrolysis of 18S + 26S rRNA released ψm in the form of an alkali-stable dinucleotide, ψm-Ap. Since the quantity of ψm-Ap in alkaline hydrolysates (0.016 mol %) was equal to the amount of pψm found in venom hydrolysates (0.017 mol %), all of the ψm in wheat embryo 18S + 26S rRNA is apparently contained in the sequence 5′ . . . ψm-A . . . 3′. Neither ψm-Cp nor ψm-Up, the two ψm-containing dinucleotides previously isolated from wheat embryo tRNA (Hudson, L., Gray, M., and Lane, B. G. (1965), *Biochemistry 4,* 2009–2016), was detected in alkaline hydrolysates of wheat embryo rRNA. In the course of this investigation, an alkali-stable dinucleotide containing unmethylated pseudouridine, Cm-ψp, was also isolated from wheat embryo 18S + 26S rRNA. From a consideration of the analytical data obtained in this study, it has been concluded that Cm-ψp and ψm-Ap can each be present in either the 18S or 26S rRNA species, but not in both, and that each sequence occurs with a frequency of not more than once per chain. These two sequences should therefore serve as specific chemical markers of one or both of the high molecular weight cytosol rRNA species of wheat embryo.

The ribosomal RNA (rRNA) of various organisms is known to contain a small proportion of methylated nucleoside components,[1] in which methyl groups are located either on the base or on the sugar (at the $O^{2'}$ position) or on both (Attardi and Amaldi, 1970). Extensive analysis of the alkali-stable di- and trinucleotide sequences of rRNA from a variety of organisms (Singh and Lane, 1964; Lane, 1965; Nichols and Lane, 1966b; Gray and Lane, 1967; Wagner *et al.,* 1967; Tamaoki and Lane, 1968; Lane and Tamaoki, 1969; Choi and Busch, 1970; Lau and Lane, 1971; Maden *et al.,* 1972a; Klootwijk and Planta, 1973a) has shown that sugar methylation is markedly nonrandom and occurs at specific sites, with the proportion and distribution of $O^{2'}$-methyl groups in rRNA varying significantly among different organisms. The isolation of the dinucleotide m_2^6A-m_2^6Ap from alkaline hydrolysates of *Escherichia coli* rRNA (Nichols and Lane, 1966a) provided an early indication of specific and nonrandom base methylation in rRNA. Subsequent analyses of the methylated sequences in *E. coli* rRNA confirmed this clustering of N^6,N^6-dimethyladenosine residues and further showed that the 16S and 23S rRNA species are each homogeneous with respect to methylated sequences, with methyl-containing nucleosides being concentrated in a few loci (Fellner and Sanger, 1968; Fellner, 1969). Recent sequence studies of *E. coli* 16S rRNA have demonstrated that the majority of the methylated nucleoside components in this RNA species occur in the 3′-terminal quarter of the molecule, and that they seem to be localized in unpaired loop regions of the RNA (Fellner *et al.,* 1972; Ehresmann *et al.,* 1972).

Studies in eukaryotic systems have provided evidence

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[1] Abbreviations for nucleotide derivatives are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature ((1970) *Biochemistry 9,* 4022–4027). N, general abbreviation for ribonucleoside; A, C, G, U, ψ, adenosine, cytidine, guanosine, uridine, pseudouridine; Nm, $O^{2'}$-methylnucleoside; mN, base-methylated nucleoside; pN, nucleoside 5′-monophosphate; pNm, $O^{2'}$-methylnucleoside 5′-monophosphate; Nm-Np, alkali-stable dinucleotide; Nm-N, alkali-stable dinucleoside monophosphate; Nm-Nm-Np, alkali-stable trinucleotide. In defining sites of substitution, a superscript indicates the position on the heterocycle of the particular substitution in question, whereas a subscript indicates the level of substitution (*e.g.,* mono- or disubstitution). Thus, m_2^6A = N^6,N^6-dimethyladenosine.

that the methyl groups of rRNA arise by post-transcriptional modification of a macromolecular precursor which contains the nucleotide sequences of the mature rRNA species (Maden, 1971). Almost all of the methylation occurs at the level of the primary transcription product (45 S in mammals) (Greenberg and Penman, 1966; Zimmerman and Holler, 1967). In L cells and HeLa cells, the methylated sequences appear to be almost entirely conserved during the processing of the 45S precursor to the mature 28S and 18S RNA species (Wagner *et al.*, 1967; Tamaoki and Lane, 1968; Vaughan *et al.*, 1967; Maden *et al.*, 1972b). In contrast, Choi and Busch (1970) have concluded that some methylated sequences are lost during processing of nucleolar high molecular weight RNA (ribosomal precursor RNA) in Novikoff hepatoma cells. Although the biological function of modified nucleosides in rRNA is not yet understood, there is some indication that methylation of ribosomal precursor RNA may be a necessary condition for the formation of functional ribosomes (Vaughan *et al.*, 1967).

In addition to methylated nucleosides, the presence of pseudouridine in rRNA from various sources has been documented (Dunn, 1959; Lane and Allen, 1961a; Nichols and Lane, 1967; Dubin and Günlap, 1967; Amaldi and Attardi, 1968; Hadziyev *et al.*, 1968; Maden and Forbes, 1972). As in the case of methylation, the formation of ψ residues in rRNA has been shown to occur at the level of the primary transcription product (Jeanteur *et al.*, 1968; Maden and Forbes, 1972). A nonrandom distribution of ψ has been demonstrated by partial sequence analysis of rRNA from HeLa cells (Amaldi and Attardi, 1968; Maden and Forbes, 1972), yeast (Klootwijk and Planta, 1973b), and *E. coli* (Fellner and Sanger, 1968). In the latter organism, 6 out of the 10 pseudouridine residues of the 23S rRNA species are located in the methylated sequences released by digestion with T_1 RNase. A pseudouridine-containing alkali-stable trinucleotide sequence, Um-Gm-ψp, has been shown to be present in wheat embryo 18S + 26S rRNA in an amount 400 times greater than would be expected on a random distribution basis (Lane, 1965). These observations suggest a topographical correlation, and perhaps biosynthetic relationship (Lane, 1965), between methylated nucleosides and pseudouridine in rRNA. In addition, it is clear that formation of pseudouridine in rRNA, like methylation, is a highly specific process.

During recent analyses of the $O^{2'}$-methylnucleoside 5′-phosphates (pNm) present in snake venom hydrolysates of various types of RNA (manuscript in preparation), a compound having the properties of the 5′-monophosphate ester of $O^{2'}$-methylpseudouridine (*i.e.*, pψm) was isolated in trace amounts from the 18S + 26S ribosomal ribonucleates of wheat embryo. Although the $O^{2'}$-methyl derivative of pseudouridine has been identified as a component of the tRNA of a number of organisms, including wheat embryo (Hudson *et al.*, 1965), ψm had not previously been found in the rRNA of any organism. The study reported here was undertaken to eliminate the possibility that the pψm isolated from wheat embryo 18S + 26S rRNA could have originated from contaminating tRNA, and to examine the sequence distribution of ψm in wheat embryo rRNA.

Materials and Methods

Isolation and Purification of RNA. Total RNA was extracted from commercial wheat germ (milled from a mixture of hard Canadian spring wheat varieties) as previously described (Singh and Lane, 1964). The ribonucleates were fractionated according to differential solubility in 1 M NaCl at 0° into an *insoluble* portion, consisting primarily of the 26S and 18S RNA components of wheat embryo ribosomes; and a *soluble* portion, consisting of tRNA and 5S rRNA. These two fractions were further purified by published procedures (Singh and Lane, 1964; Cunningham and Gray, 1974). In order to ensure complete removal of any traces of low-molecular weight RNA species, the NaCl-insoluble RNA fraction was reprecipitated at least three times from 1 M NaCl.

Polyacrylamide Gel Electrophoresis. Electrophoresis of RNA samples in polyacrylamide gels was carried out by a modification of the method described by Loening (1967). The ratio of ammonium persulfate/acrylamide in these experiments was 0.06 g/g for 2.8% gels and 0.01 g/g for 5.0% gels, while the ratio of *N,N,N′,N′*-tetramethylethylenediamine/acrylamide was 0.08 ml/g for 2.8% gels and 0.045 ml/g for 5.0% gels. The buffer used for electrophoresis was "E" buffer (0.04 M Tris–0.02 M sodium acetate–0.001 M NaEDTA (pH 7.2)) of Bishop *et al.* (1967), containing 0.2% sodium lauryl sulfate.

To determine the proportions of the different size classes of RNA in freshly prepared wheat embryo NaCl-insoluble RNA, replicate samples of native or heat-denatured RNA were analyzed on 2.8% gels (see legend to Figure 4). Gels were scanned in a Joyce Loebl uv scanner attached to a Sargent Model SRLG recorder, set at high chart speed. The relative proportions of the different size classes of RNA were determined by weighing the portion of each tracing corresponding to the area occupied by each size class.

Enzymic Hydrolysis of RNA and Isolation of $O^{2'}$-Methylnucleoside 5′-Phosphates. The $O^{2'}$-methylnucleoside constituents of RNA were quantitatively assayed by a method to be described in detail elsewhere (manuscript in preparation). The method involves hydrolysis of RNA with a mixture of snake venom phosphodiesterase (*P*-diesterase) and 5′-nucleotidase to yield nucleosides (N) and $O^{2'}$-methylnucleoside 5′-phosphates (pNm). This hydrolytic procedure is based on the known resistance of pNm compounds to dephosphorylation by venom 5′-nucleotidase (Honjo *et al.*, 1964). After isolation of the pNm fraction by anion-exchange chromatography on DEAE-cellulose at pH 7.8, two-dimensional paper chromatography was used to resolve the individual components of this fraction.

For large-scale preparation of pψm, the pNm fraction isolated from 1 to 2 g of wheat embryo NaCl-soluble RNA or NaCl-insoluble RNA was chromatographed on DEAE-cellulose in the presence of 1 M formic acid (pH 1.8) (Lane and Allen, 1961b). This procedure resolved the pNm components into three subfractions, eluting in the order pCm + pAm (M-1), pGm (M-3), and pUm + pψm (M-4). (A fourth subfraction, M-2 (eluting between M-1 and M-3), is present in the elution profile of the pNm fraction from NaCl-soluble RNA. This subfraction contains the 5′-nucleotides of N^2,N^2-dimethylguanosine and a 3-substituted uridine derivative, tentatively identified as 3-(3-amino-3-carboxypropyl)uridine (manuscript in preparation)). To separate pψm from pUm, subfraction M-4 was submitted to paper chromatography in system A, in which nucleotide derivatives containing pseudouridine migrate more slowly than their uridine-containing analogs. The pψm isolated in this way was desalted by adsorption to and elution from charcoal (Gray and Lane, 1967).

Isolation and Analysis of Alkali-Stable Dinucleotides from Wheat Embryo NaCl-Insoluble RNA. Alkaline hy-

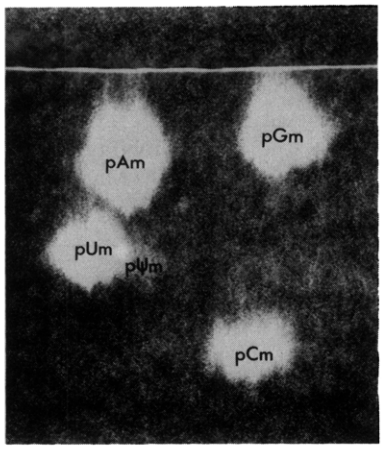

FIGURE 1: Ultraviolet contact photograph illustrating the resolution of the pNm fraction from wheat embryo NaCl-insoluble RNA. About 30 A_{260} units were spotted on ammonium sulfate-impregnated Whatman No. 1 paper for separation by two-dimensional chromatography (system A followed by system B; 24 hr in each solvent).

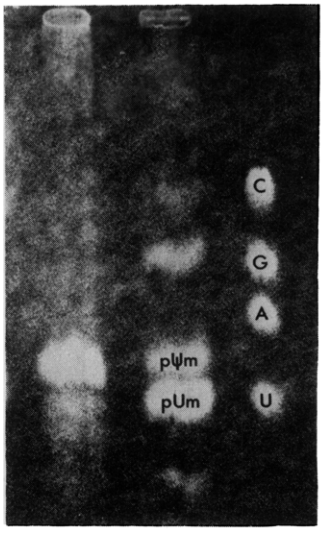

FIGURE 2: Ultraviolet contact photograph illustrating the chromatographic mobility (in system A) of the presumed pψm isolated from the pNm fraction of wheat embryo NaCl-insoluble RNA. This material (left track) corresponds to the trace of uv-absorbing material in pNm subfraction M-4 migrating more slowly than pUm in system A. The compound was concentrated by charcoal desalting, as described in the text. Middle track: pNm subfraction M-4 isolated from wheat embryo NaCl-soluble RNA. Right track: nucleoside marker.

drolysis of RNA and fractionation of the products were carried out as described by Lane (1965). The alkali-stable dinucleotide fraction (Nm-Np) was further resolved by chromatography on DEAE-cellulose at pH 1.8 (Gray and Lane, 1967). This procedure separates the alkali-stable dinucleotides into four subfractions (D-1 to -4), with Cm-Up, Um-Cp, Am-Up, and Um-Ap appearing in subfraction D-3 and Gm-Up, Um-Gp, and Um-Up present in subfraction D-4. Pseudouridine-containing dinucleotides appear in the same subfraction as their uridine-containing counterparts.

Each subfraction was treated with *E. coli* alkaline phosphatase (*P*-ase) and the resulting dinucleoside monophosphates (Nm-N) were desalted on charcoal (Gray and Lane, 1967) and quantitatively analyzed by two-dimensional chromatography, as described by Singh and Lane (1964). For sequence determination, individual Nm-N compounds were hydrolyzed with purified snake venom phosphodiesterase (Lane *et al.*, 1963). Hydrolysis mixtures contained 50 μl of Nm-N solution (2–5 A_{260} units), 50 μl of 0.5 M ammonium formate (pH 9.2), and 100 μl of purified *Vipera russelli* phosphodiesterase. The phosphodiesterase preparations used had activities in the range 0.2–1.2 units/ml (one unit of phosphodiesterase is that amount of enzyme which liberates 1 μmol of acid-soluble nucleotide/min from wheat embryo NaCl-insoluble RNA in the standard assay; for conditions, see Cunningham and Gray, 1974). In some cases, hydrolysis mixtures also contained 5 μl (1.7 units) of alkaline phosphatase (Worthington). After 24 hr at 37°, each hydrolysate was directly applied to untreated (system C) or ammonium sulfate-treated (system A) Whatman No. 1 chromatography paper, along with appropriate markers. The resolved products were characterized by R_F values and by uv absorption spectra and were quantitated spectrophotometrically.

Chromatographic and Electrophoretic Systems and Preparation of Markers. Systems used for descending paper chromatography were: (A) 95% ethanol–water (4:1, v/v), in conjunction with ammonium sulfate-impregnated Whatman No. 1 chromatography paper (Lane, 1963); (B) saturated ammonium sulfate–2-propanol (40:1, v/v), also used with amonium sulfate-treated paper (Singh and Lane, 1964); (C) 95% ethanol–1 M ammonium acetate (pH 9.0) (7:3, v/v), saturated with boric acid (Plesner, 1955).

Paper electrophoresis was carried out as described previously (Gray and Lane, 1967). The electrophoretic systems used were: (1) 1 M formic acid (pH 1.8); (2) 0.025 M ammonium formate (pH 9.0); (3) 0.025 M sodium tetraborate (pH 9.2).

Marker pψm and pUm were isolated from wheat embryo NaCl-soluble RNA by the procedure described above. Treatment of pUm with phosphatase was used to prepare a Um marker. Marker ψm was prepared by hydrolysis of the dinucleotide ψm-Up (isolated from an alkaline digest of wheat embryo NaCl-soluble RNA (Hudson *et al.*, 1965)) with a mixture of phosphodiesterase + phosphatase as above. The products (U + ψm) were separated by chromatography in system C, and ψm was recovered by charcoal desalting. Marker pψ and pU were isolated from a phosphodiesterase hydrolysate of wheat embryo NaCl-soluble RNA (Hudson *et al.*, 1965), while uridine and pseudouridine were commercial products.

Results

Identification of $O^{2'}$-Methylpseudouridine 5′-Phosphate in the pNm Fraction of Wheat Embryo NaCl-Insoluble RNA. The presence of $O^{2'}$-methylpseudouridine in wheat embryo rRNA was first suggested by the results of two-dimensional paper chromatography of the nucleoside monophosphate fraction isolated from snake venom hydrolysates of wheat embryo NaCl-insoluble RNA. This fraction contains $O^{2'}$-methylnucleoside 5′-phosphates, which are resistant to the 5′-nucleotidase present in venom (Honjo *et al.*, 1964). As shown in Figure 1, a faint uv-absorbing spot was consistently noted immediately to the right of pUm on two-dimensional chromatograms of the pNm fraction, in a position characteristic of pψm. The ultraviolet absorption spectrum of this minor component displayed an alkaline bathochromic shift, typical of pseudouridine and its derivatives.

When the pNm fraction from wheat embryo NaCl-insoluble RNA was further resolved by chromatography on DEAE-cellulose at pH 1.8, the nucleotide tentatively identi-

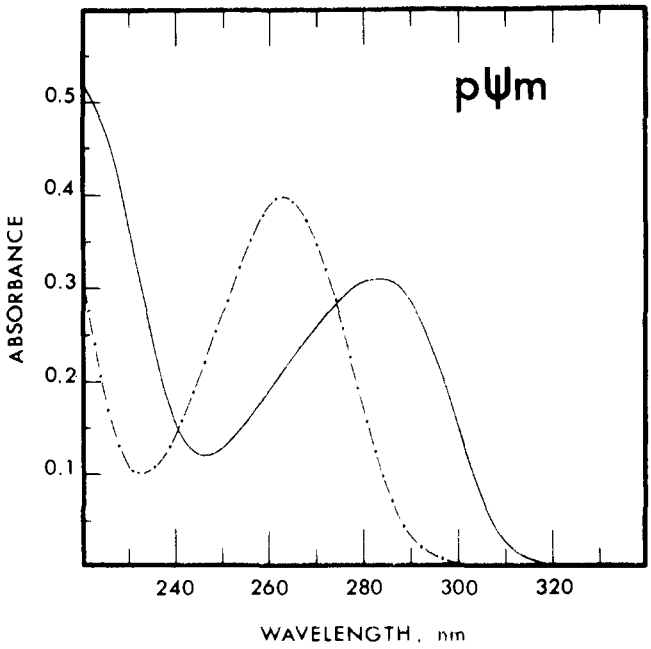

FIGURE 3: Ultraviolet absorption spectra of presumed pψm from wheat embryo NaCl-insoluble RNA. After isolation from pNm subfraction M-4 as described in the text, the compound was further purified by two-dimensional paper chromatography (system A followed by system B). The presumed pψm and an appropriate blank were eluted in 0.1 M HCl, and the acidic spectrum (— · · —) of the sample was recorded against the blank on a Bausch and Lomb Spectronic 505 spectrophotometer (λ_{max} 262 nm; λ_{min} 232 nm). The alkaline spectrum (——) was recorded after adding 10 M NaOH to the sample and blank until no further change in the spectrum occurred (λ_{max} 284 nm; λ_{min} 247 nm).

fied as pψm was located, as expected, in the subfraction containing pUm (*i.e.*, subfraction M-4). The presumed pψm could be separated from pUm by paper chromatography in system A. When the trace of uv-absorbing material migrating more slowly than pUm in this system was pooled from a number of chromatograms, concentrated by charcoal desalting, and rerun in system A, the bulk of the material had a mobility identical with that of a pψm marker (from wheat embryo NaCl-*soluble* RNA) and distinct from that of pUm (Figure 2). The ultraviolet absorption spectra of the compound at acidic and alkaline pH values (Figure 3) closely resembled those of pseudouridine 3′,5′-diphosphate (Tomasz and Chambers, 1965). The nucleotide migrated with marker pψm in a borate-containing solvent (system C; Table I), and could clearly be distinguished from pseudouridine 5′-phosphate (pψ) in this system. The electrophoretic properties of the compound were identical with those of a pUm marker (Table II), which can be distinguished from pU by its slower mobility (resulting from failure to complex with borate) in a borate-containing buffer (system 3). After treatment with alkaline phosphatase, the product had chromatographic and electrophoretic properties indistinguishable from those of a ψm marker (Tables I and II). Notably, the absence of a cis-diol grouping in the presumed pψm and its dephosphorylation product was indicated by their chromatographic and electrophoretic mobilities in borate-containing media.

TABLE I: Chromatographic Properties of $O^{2'}$-Methylpseudouridine 5′-Phosphate Isolated from Wheat Embryo NaCl-Insoluble RNA.

Compound	R_F in System A	R_F in System C
Presumed pψm	0.52	0.47
Markers		
pψm	0.54	0.48
pUm	0.60	0.52
pU	0.42	0.19
pψ	0.32	0.15
Presumed pψm + *P*-ase[a]	0.65	0.84
Markers		
ψm	0.64	0.84
Um	0.75	0.86
U	0.61	0.51
ψ	0.44	0.41

[a] Treatment of presumed pψm with alkaline phosphatase (*P*-ase) and recovery of the resulting nucleoside by charcoal desalting were carried out by published procedures (Gray and Lane, 1967).

TABLE II: Electrophoretic Properties of $O^{2'}$-Methylpseudouridine 5′-Phosphate Isolated from Wheat Embryo NaCl-Insoluble RNA.

Compound	Mobility Relative to Picrate Marker in System[a] 1	2	3
Presumed pψm	−0.63	−1.30	−1.15
Markers			
pUm	−0.63	−1.29	−1.15
pU	−0.60	−1.25	−1.30
Presumed pψm + *P*-ase	+0.07	−0.13	−0.32
Markers			
ψm	+0.01	−0.10	−0.29
Um	+0.05	−0.09	−0.29
U	+0.07	−0.09	−0.67
ψ	+0.03	−0.11	−0.77

[a] The picrate marker was assigned a mobility of −1.00 at all pH values, the minus sign indicating migration toward the anode.

The molar proportions of the $O^{2'}$-methylnucleoside constituents of wheat embryo NaCl-insoluble RNA, determined as their 5′-nucleotides, are listed in Table III. The proportions of pAm, pCm, pGm, and pUm were the same, within experimental error, for three different preparations of RNA. The values found in the present study agree closely with those previously determined by Lane (1965), using another method. All of the samples of wheat embryo NaCl-insoluble RNA analyzed contained ψm, at a level of about 0.015–0.020 mol %.

Search for Contaminating tRNA in the NaCl-Insoluble Fraction from Wheat Embryos. Because of the presence of $O^{2'}$-methylpseudouridine in wheat embryo tRNA (Hudson *et al.*, 1965), it was important to rigorously exclude the possibility that tRNA contamination could be the source of the pψm present in enzymic hydrolysates of wheat embryo NaCl-*insoluble* RNA. To account for the observed proportion of ψm in samples of 26S + 18S rRNA which had been multiply precipitated from 1 M NaCl, contaminating tRNA would have had to constitute 10–15% of the mass of these samples, a level of tRNA approximating that in total wheat

TABLE III: Molar Proportions of $O^{2'}$-Methylnucleoside 5'-Phosphates in Snake Venom Hydrolysates of Wheat Embryo NaCl-Insoluble RNA.[a]

pNm	RNA Preparation 1	2	3	Mean ± SD	Lane (1965)
pAm	0.49	0.48	0.50	0.49 ± 0.010	0.54
pCm	0.33	0.33	0.35	0.34 ± 0.010	0.35
pGm	0.44	0.41	0.42	0.42 ± 0.015	0.38
pUm	0.44	0.44	0.45	0.44 ± 0.005	0.47
pψm	0.016	0.017	0.019	0.017 ± 0.002	
Total				1.7	1.7

[a] Values are expressed as moles of pNm/100 mol of total nucleotides and are minimal values, being uncorrected for small losses which may have occurred during isolation of the pNm fraction and subsequent chromatographic analysis.

embryo RNA *before* salt fractionation. (This calculation is based on a ψm content of 0.12 mol % in wheat embryo NaCl-soluble RNA (Hudson *et al.*, 1965), of which tRNA constitutes about 85% (by mass), as determined by polyacrylamide gel electrophoresis.) When purified NaCl-insoluble RNA was resolved by electrophoresis in 2.8% polyacrylamide gels, there was no evidence of low-molecular-weight species migrating in the position of 5 S rRNA and tRNA (*cf.* Figure 4A and B). To examine the low-molecular-weight RNA region at higher resolution, wheat embryo NaCl-insoluble RNA was also electrophoresed in 5.0% gels. Even when a relatively large amount of NaCl-*insoluble* RNA was analyzed (Figure 5B), negligible uv-absorbing material was detected in the position occupied by tRNA in the gel containing NaCl-*soluble* RNA (Figure 5A). Denaturation of NaCl-insoluble RNA by heating and quick-cooling prior to electrophoresis did not lead to any significant increase in the amount of uv-absorbing material in the tRNA region (Figure 5C), even though this procedure did release the "satellite" RNA (located between 5 and 6 cm, Figure 5C) which has been shown to be noncovalently complexed to the 26S rRNA component of undenatured NaCl-insoluble RNA (Oakden *et al.*, 1972; Azad and Lane, 1973a,b). This result rules out the possibility that tRNA might have been complexed to ribonucleates of higher molecular weight. On the basis of these gel electrophoretic studies, it was concluded that tRNA could have constituted no more than 0.1% (by mass) of the NaCl-insoluble RNA from which pψm was isolated in this study. Thus, less than 1% of the pψm detected could have been derived from contaminating tRNA. Two tRNA-specific, modified nucleosides, 5-carboxymethyluridine and N^6-(*N*-threonylcarbonyl)adenosine, which are present in wheat embryo NaCl-soluble RNA at a level exceeding that of $O^{2'}$-methylpseudouridine (Gray and Lane, 1968; Gray, 1974), could not be detected in the NaCl-insoluble RNA fractions analyzed in this study, providing additional evidence against the presence of significant tRNA in these fractions.

Pseudouridine-Containing Dinucleotides in Alkaline Hydrolysates of Wheat Embryo NaCl-Insoluble RNA. The isolation of pψm from snake venom hydrolysates of wheat embryo NaCl-insoluble RNA implies that alkaline hydrolysis of this type of RNA should release ψm in the form of one or more alkali-stable dinucleotides (ψm-Np).

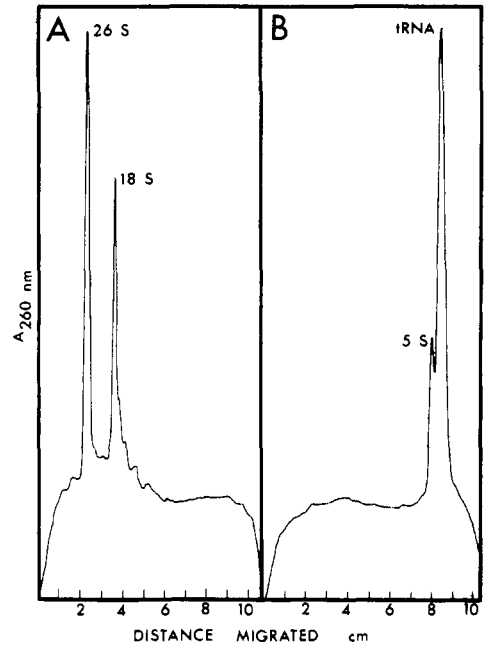

FIGURE 4: Polyacrylamide gel electrophoresis (2.8% gels) of purified wheat embryo NaCl-insoluble RNA (A) and NaCl-soluble RNA (B). Aliquots (20 μl) containing 0.4 A_{260} unit of insoluble RNA or 0.3 A_{260} unit of soluble RNA were applied to the gels, which were run at 5 mA/gel for 3 hr. Prior to electrophoresis, the NaCl-soluble RNA sample was heated for 5 min at 60° and quick-cooled, in order to eliminate small amounts of aggregates of 5S rRNA and/or tRNA which arise during purification of the soluble RNA fraction and which migrate more slowly than 5S rRNA and tRNA.

To detect and quantitate such dinucleotides, the Nm-Np fraction from wheat embryo NaCl-insoluble RNA was subfractionated by chromatography on DEAE-cellulose at pH 1.8 (Gray and Lane, 1967). After treatment with phosphatase, each of the resulting subfractions was analyzed by two-dimensional paper chromatography (Singh and Lane,

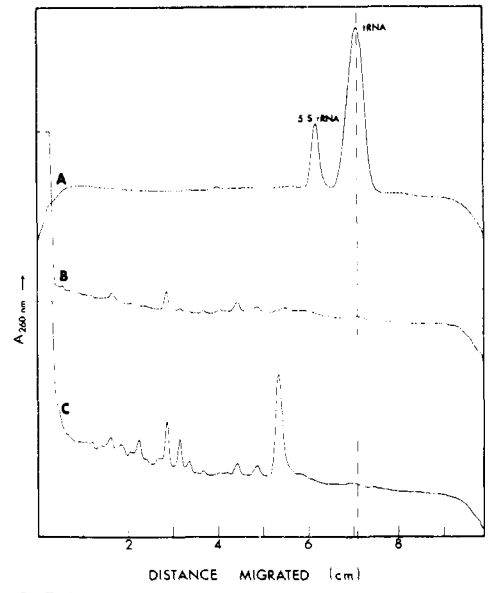

FIGURE 5: Polyacrylamide gel electrophoresis (5.0% gels) of purified wheat embryo NaCl-soluble RNA (heated) (A); NaCl-insoluble RNA (unheated) (B); and NaCl-insoluble RNA (heated) (C). Aliquots (20 μl) containing 0.3 A_{260} unit of soluble RNA or 3 A_{260} units of insoluble RNA were applied to the gels, which were run at 5 mA/gel for 2.5 hr. The peak at about 5.3 cm (C) represents the "satellite" rRNA which is specifically complexed to 26 S rRNA in the unheated sample (B) (Oakden *et al.* 1972; Azad and Lane, 1973a,b).

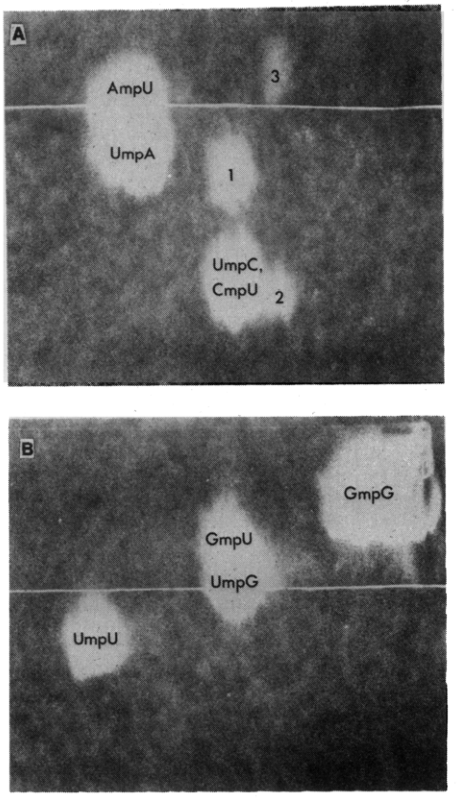

FIGURE 6: Ultraviolet contact photographs illustrating the resolution of phosphatase-treated, charcoal-desalted alkali-stable dinucleotide subfractions D-3 (A) and D-4 (B) from wheat embryo NaCl-insoluble RNA. Approximately 40–50 A_{260} units of each subfraction were separated by two-dimensional paper chromatography. The minor component **3** (a mixture of Gm-A and Am-G) is present in Nm-N subfraction D-3 due to a slight trailing of subfraction D-2 into D-3 during chromatography of the Nm-Np fraction at pH 1.8.

1964). Since pseudouridine-containing dinucleoside monophosphates migrate more slowly in the first-dimension solvent (system A) than their uridine-containing counterparts (Hudson *et al.*, 1965), the former are located to the right of the latter after chromatography in the second dimension. As shown in Figure 6A, minor uv-absorbing compounds having the chromatographic mobilities expected of ψm-A and/or Am-ψ (**1**) and ψm-C and/or Cm-ψ (**2**) were detected in subfraction D-3. In contrast, no material having the mobility of ψm-G and/or Gm-ψ or ψm-U and/or Um-ψ was found in subfraction D-4 after two-dimensional chromatography (Figure 6B).

The ultraviolet absorption spectra of **1** (Figure 7A) and **2** (Figure 7C), when compared with those of Am-U + Um-A (Figure 7B) and Cm-U + Um-C (Figure 7D), respectively, were consistent with the presence of pseudouridine rather than uridine in **1** and **2.** The acidic spectra of **1** and **2** were slightly shifted to longer wavelengths, relative to the acidic spectra of Am-U + Um-A and Cm-U + Um-C, respectively, while the alkaline spectra displayed distinct bathochromic shifts, diagnostic of pseudouridine.

To determine the sequences of **1** and **2,** these compounds were hydrolyzed with purified snake venom phosphodiesterase (in the presence and absence of phosphatase), and the products were identified and quantitated after resolution by paper chromatography. The results of these analyses, presented in Table IV, indicated that **1** had the sequence ψm-A, while **2** was Cm-ψ. There was no evidence from these experiments for the presence of the sequence isomers Am-ψ and ψm-C. Failure to detect either ψm-C or ψm-U, the two sequences contained in wheat embryo tRNA, conclusively eliminates tRNA as a possible source of the pψm detected in venom hydrolysates of NaCl-insoluble RNA.

The absolute molar proportions of the components of subfraction D-3 are listed in Table V. These values have been corrected for losses occurring at the charcoal desalting step, after alkaline phosphatase treatment, and so are directly comparable to values previously obtained by Singh and Lane (1964) for the molar proportions of Am-U + Um-A and Cm-U + Um-C in wheat embryo NaCl-insoluble RNA. Since the values obtained in the present study are identical with those previously reported, the molar proportions of ψm-A and Cm-ψ reported here are assumed to be a true reflection of the level at which these sequences occur in wheat embryo NaCl-insoluble RNA. Since the amount of ψm-Ap released by alkali hydrolysis (0.016 mol %) was equivalent to the amount of pψm released by venom hydrolysis (0.017 mol %), all of the $O^{2'}$-methylpseudouridine in wheat embryo NaCl-insoluble RNA appears to be confined to this single alkali-stable dinucleotide sequence.

The relative proportions of all of the alkali-stable dinucleotides from wheat embryo NaCl-insoluble RNA, determined in the present study, are listed in Table VI, along with the values previously determined by Singh and Lane (1964). It can be seen that there is good agreement between the two analyses. The two sequences identified in the present study, ψm-Ap and Cm-ψp, each constitute about 1 mol % of the total Nm-Np sequences of wheat embryo NaCl-insoluble RNA, as does Cm-Gp, the rarest sequence previously identified in this type of RNA.

$O^{2'}$-Methylpseudouridine as a Component of Wheat Embryo Cytosol rRNA. The isolation of ψm in the form of a 5′-nucleotide and also as part of an alkali-stable dinucleotide provides strong evidence that this modified nucleoside is a covalently linked constituent of a polyribonucleotide component of wheat embryo NaCl-insoluble RNA, the bulk

TABLE IV: Determination of Sequences of Alkali-Stable Dinucleoside Monophosphates **1** and **2.**

Expt	Nm-N	*P*-ase	Chromatog. in System	Products (nmol)[a]	Molar Ratio of Products
1	**1**	−	C	pA (191), ψm (208)	ψm: pA = 1.09
2	**1**	+	A	A (132), ψm (140)	ψm: A = 1.06
3	**2**	−	A	pψ (84.5), Cm (77.7)	Cm: pψ = 0.92
4	**2**	+	A	ψ (200), Cm (183)	Cm: ψ = 0.92

[a] In expt 1, the identity of the products was confirmed by rechromatography in system B. The products obtained in expt 2 and 4 were rechromatographed both in system B and in system C.

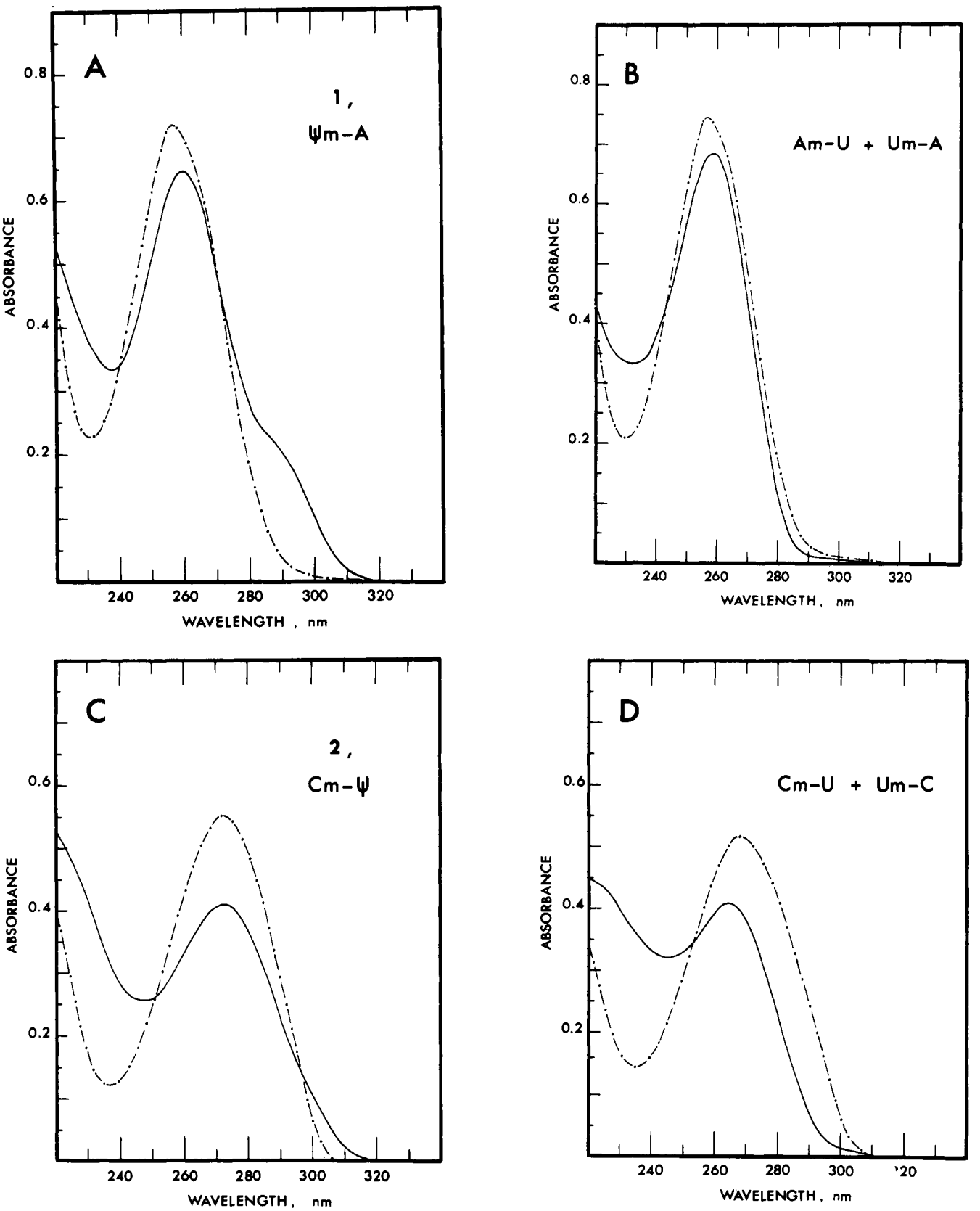

FIGURE 7: Ultraviolet absorption spectra of alkali-stable dinucleoside monophosphates from wheat embryo NaCl-insoluble RNA. The compounds in Nm-N subfraction D-3 (Figure 6A) were desalted on charcoal and their spectra recorded on a Bausch and Lomb Spectronic 505 spectrophotometer: (— · —) 0.1 N HCl; (—) 0.1 N NaOH.

Figure	Nm-N	Acidic		Alkaline	
		λ_{max}	λ_{min}	λ_{max}	λ_{min}
A	1 (Ψm-A)	257	230	260	238
B	Am-U + Um-A	257	229	259	233
C	2 (Cm-Ψ)	272	237	273	247
D	Cm-U + Um-C	268	236	264.5	246

TABLE V: Molar Proportions of Dinucleoside Monophosphates in Subfraction D-3 from Wheat Embryo NaCl-Insoluble RNA.[a]

Nm-N	Singh and Lane (1964)	This Study
Am-U	0.11	0.22
Um-A	0.11	
ψm-A	n.d.	0.016
Cm-U	0.094	0.17
Um-C	0.078	
Cm-ψ	n.d.	0.012

[a] Values are expressed as mol of Nm-N/100 mol of total nucleotides in the alkaline hydrolysate. The relative proportions of individual isomeric pairs was not determined in the present study. n.d. = not detected

of which is accounted for by the 18S and 26S RNA components of cytosol ribosomes. However, since NaCl-insoluble RNA is isolated by direct phenol extraction of whole embryos, it may contain RNA species which purify with 18S + 26S rRNA but which are not components of cytosol ribosomes (*e.g.*, mRNA, RNA from various subcellular organelles such as mitochondria and plastids). It is therefore pertinent to consider whether such additional RNA species could be the source of the ψm characterized in this study, or whether ψm is indeed a constituent of wheat embryo cytosol rRNA.

The 26S and 18S cytosol rRNA components of higher plants have apparent molecular weights of about 1.3×10^6 and 0.7×10^6, respectively, as determined by polyacrylamide gel electrophoresis (Loening, 1968). Assuming that an average nucleotide residue in rRNA has a molecular weight of 350, these values would correspond to chain lengths of about 3700 and 2000 nucleotides, respectively. End-group analysis of the satellite RNA from wheat embryo has indicated a chain length of about 200 nucleotides for this RNA species (Azad and Lane, 1973b), while the corresponding RNA from yeast is 158 nucleotides long (Rubin, 1973). Assuming that the chain lengths of 26 S, 18 S, and satellite RNA are 3700, 2000, and 150–200, respectively, a nucleotide residue occurring once per chain would constitute 0.027 (28 S), 0.050 (18 S), and 0.50–0.67 (satellite) mol % of the total nucleotide residues of each of these RNA species. From a knowledge of the proportion of the total NaCl-insoluble RNA which each of these species comprises, it is possible to estimate the level at which a particular nucleotide constituent should be present in the bulk NaCl-insoluble RNA, if such a constituent is present once per chain in one of the three RNA species. Measurement of the relative proportions of the various size classes of RNA in wheat embryo NaCl-insoluble RNA by polyacrylamide gel electrophoresis (Table VII) indicates that the 26 S, 18 S, and satellite RNA species account for about 42, 31, and 3%, respectively, of the mass of NaCl-insoluble RNA. Thus, if a particular nucleotide occurred once in 26S RNA but was not present in 18S or satellite RNA, such a constituent should account for 0.011 mol % (0.027 mol % × 0.42) of the total nucleotide constituents of bulk NaCl-insoluble RNA. The corresponding values would be 0.015 mol % and 0.015–0.020 mol % in the case of a nucleotide occurring once in either the 18S or satellite RNA species, respectively, but not in the remaining two species.

TABLE VI: Relative Proportions of the Alkali-Stable Dinucleotide Sequences of Wheat Embryo NaCl-Insoluble RNA.[a]

Subfraction	Sequence	Singh and Lane (1964)	This Study
D-1	Am-Ap	8.8	8.3
	Am-Cp	8.1	12.3
	Cm-Ap	4.8	
	Cm-Cp	5.4	5.8
D-2	Am-Gp	11.2	14.8
	Gm-Ap	3.1	
	Cm-Gp	0.9	6.9
	Gm-Cp	5.8	
D-3	Am-Up	7.8	15.3
	Um-Ap	8.5	
	ψm-Ap	n.d.	1.1
	Cm-Up	7.0	11.7
	Um-Cp	5.8	
	Cm-ψp	n.d.	0.9
D-4	Gm-Gp	7.8	9.5
	Gm-Up	3.0	8.1
	Um-Gp	6.1	
	Um-Up	5.9	5.3

[a] Values are expressed as mol of Nm-Np/100 mol of total alkali-stable dinucleotide sequences. The relative proportions of individual isomeric pairs were not determined in the present study. n.d. = not detectable.

It should be noted that the mass ratio of 26 S to 18 S which can be calculated from the data of Table VII (1.36) is considerably lower than the value of 1.85 expected if the 26S and 18S RNA species have chain lengths of 3700 and 2000, respectively. Since the true chain lengths of wheat embryo 26S and 18S RNA are not known with certainty at the present time, this assumed mass ratio may be high. On the other hand, it is known that under certain conditions wheat embryo 26S RNA can undergo a structural alteration with the formation of products the size of 18S RNA (McLennan and Lane, 1968). It has also been shown that wheat embryo NaCl-insoluble RNA contains a heterogeneous population of RNA species migrating on polyacrylamide gels between 26S RNA and 18S RNA and between 18S RNA and satellite RNA (Oakden *et al.*, 1972), and accounting for about 25% of the mass of NaCl-insoluble RNA (Table VII). (It is notable that the gel electrophoretic profiles of wheat embryo NaCl-insoluble RNA, obtained in this laboratory (Figure 4A), are essentially identical with those of Oakden *et al.* (1972) with respect to the mobilities and proportions of the minor RNA species comprising this "heterodisperse RNA fraction.") There is some evidence to suggest that the hetereodisperse RNA population (which appears to preexist in dormant embryos (Oakden *et al.*, 1972)) is comprised largely of discrete cleavage products of 26S and 18S rRNA. For example, species with apparently identical electrophoretic mobilities are present in RNA extracted from purified wheat embryo ribosomes, and increasing amounts of such discrete RNA species appear, at the expense of 26S and 18S rRNA, during storage of ribosomes at 4° (unpublished observations). In addition, it has been observed that the pattern of 3′-termini of the heterodisperse RNA fraction smaller than 18S RNA is similar to that of bulk NaCl-insoluble RNA (Azad and Lane, 1973b). For

TABLE VII: Proportions of Different Size Classes of RNA in Wheat Embryo NaCl-Insoluble RNA.

	Size Class (% of Total RNA in Gel; Mean ± SD, n = 3)						
	>26 S[a]	26 S (+satellite)	26 S (−satellite)	26 S − 18 S	18 S	<18 S	Satellite
Native RNA	3.0 ± 0.6	45.4 ± 1.7		6.5 ± 0.8	30.3 ± 1.1	14.7 ± 1.5	
Denatured RNA		(44.7)	42.0 ± 1.1	6.6 ± 0.7	30.9 ± 0.9	17.9 ± 1.5	2.7 ± 0.2

[a] The small proportion of material running more slowly than 26 S RNA in gels of native RNA represents rRNA aggregates (Oakden *et al.*, 1972).

the above reasons, the measured proportions of 18S RNA and particularly 26S RNA in wheat embryo NaCl-insoluble RNA may be low, and the actual mass ratio of intact + derived 26S RNA to intact + derived 18S RNA may be higher than that suggested by the data of Table VII. In any event, even in the extreme case in which one assumes that 26S + 18S rRNA together constitute 97% of the mass of NaCl-insoluble RNA, and are present in a 1.85:1 mass ratio, a nucleotide constituent occurring once in either species but not in the other (and not in satellite RNA) should be present at a level of 0.017 mol % in bulk NaCl-insoluble RNA.

Since the measured molar proportion of pψm (0.017 mol %) in NaCl-insoluble RNA is within the range of values calculated on the basis of the above assumptions (0.011–0.017 mol % in the case of 26S RNA, 0.015–0.017 mol % for 18S RNA, and 0.015–0.020 mol % for satellite RNA), we conclude that $O^{2'}$-methylpseudouridine, as part of the alkali-stable dinucleotide sequence ψm-Ap, occurs in only one of the three major species (26 S, 18 S, or satellite) of NaCl-insoluble RNA, at a frequency of not more than once per chain. The same conclusion is drawn in the case of Cm-ψp, which is present at a level of 0.012 mol % in alkaline hydrolysates of wheat embryo NaCl-insoluble RNA. These inferences are supported by the observation that ψm-Ap and Cm-ψp each constitute about 1% of the total alkali-stable dinucleotide sequences of wheat embryo NaCl-insoluble RNA (Table VI), the same proportion as Cm-Gp, which has been shown to occur once per chain in 18S RNA but to be absent from the 26S and satellite RNA species of wheat embryo (B. G. Lane, personal communication).

It is extremely unlikely that either ψm-Ap or Cm-ψp could be present in a component of wheat embryo NaCl-insoluble RNA other than 26S or 18S cytosol rRNA. For example, an RNA species having a chain length of 600 nucleotides would have to constitute about 10% of the mass of NaCl-insoluble RNA, in order to account for the observed level of these dinucleotides; or, if such an RNA component were present at a level of 1% in NaCl-insoluble RNA, ψm-Ap or Cm-ψp would have to occur about ten times per chain. The first possibility is readily eliminated by the gel electrophoretic studies of NaCl-insoluble RNA, and the second possibility is highly improbable. In support of this conclusion, we have found that ψm-Ap and Cm-ψp are present in the NaCl-insoluble portion of RNA extracted from the 18,000g supernatant of a wheat embryo homogenate, indicating that RNA species from subcellular organelles of wheat embryo are not the source of these sequences.

Discussion

Although analyses of both the alkali-stable dinucleotide sequences (Singh and Lane, 1964) and $O^{2'}$-methylnucleoside 5′-phosphates (Lane, 1965) from wheat embryo NaCl-insoluble RNA have been published, the presence of $O^{2'}$-methylpseudouridine in this type of RNA has not previously been reported. This is not surprising in view of the small amount of ψm in NaCl-insoluble RNA and the particular manner in which previous analyses were carried out. In the study of Singh and Lane (1964), the entire Nm-N fraction from wheat embryo NaCl-insoluble RNA was resolved by two-dimensional paper chromatography. Under these conditions, ψm-A probably migrated with Gm-U, while the small amount of Cm-ψ, which is barely separated from Cm-U + Um-C (Figure 6A), would likely not have been observed. In the present study, ψm-Ap and Cm-ψp were only detected after a preliminary anion-exchange separation of the Nm-Np fraction. The analysis of pNm derivatives by Lane (1965) utilized one-dimensional paper chromatography for resolution of these compounds, and, in the system used, the trace amount of pψm present would have migrated with and been obscured by the relatively large amount of pAm present. As reported here, pψm could only be detected in the pNm fraction from NaCl-insoluble RNA by two-dimensional paper chromatography of a relatively large amount of the pNm fraction, or by prior resolution of this fraction by anion-exchange chromatography, followed by one-dimensional paper chromatography.

$O^{2'}$-Methylpseudouridine represents the second example of an rRNA constituent containing a modification in both the base and sugar portions of the nucleoside. The first such example to be described, N^4,$O^{2'}$-dimethylcytidine, occurs once per 16S chain in *E. coli* rRNA (Nichols and Lane, 1966a,b; 1968). Another pseudouridine derivative, 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine, has recently been identified as a component of the 18S rRNA of Chinese hamster cells (Saponara and Enger, 1974). The biosynthesis of each of these modified components is of interest because multiple modifications in the same nucleoside residue suggest that separate enzymes may be involved in post-transcriptional modification at the same site during processing of ribosomal precursor RNA.

Because of the nature of the structural alterations involved, it would seem *a priori* that separate enzymes must catalyze pseudouridine formation and methylation during the biosynthesis of ψm. Depending upon the sequence of events, ψm biosynthesis in wheat embryo rRNA may be viewed as involving either the methylation of about 1% of the total ψ residues in this type of RNA or the conversion of about 3.5% of the total Um residues to ψm residues. Since a variety of methylases with differing specificities are known to carry out base and sugar methylation of transfer and ribosomal RNA, it seems more likely that a single enzyme

carries out pseudouridine formation in each type of RNA, and that a small proportion of the resulting ψ residues are then $O^{2'}$-methylated. Restriction of methylation to a limited number of the total pseudouridine residues in an RNA species could be determined by the primary sequence and/or tertiary structure in the vicinity of the susceptible ψ residue.

Although unmethylated ψ has been characterized as a component of an alkali-stable trinucleotide sequence (Um-Gm-ψp) in both wheat embryo (Lane, 1965) and yeast (Klootwijk and Planta, 1973a,b) rRNA, the isolation of Cm-ψp from wheat embryo NaCl-insoluble RNA represents the first reported occurrence of ψ in an alkali-stable dinucleotide. As discussed earlier, the quantitative data obtained in the present study suggest that Cm-ψp and ψm-Ap are localized in only one of the three major cytosol rRNA species (26 S, 18 S, and 26 S associated, or "satellite") of wheat embryo. Analysis of the methylated components of the wheat embryo satellite RNA species has indicated the absence of either Cm-ψp or ψm-Ap in this low-molecular-weight rRNA component (B. G. Lane, personal communication). Thus, each of these sequences must be present in one or the other of the high-molecular-weight, cytosol rRNA components. These sequences should therefore serve as distinctive markers in studies involving structural analysis of wheat embryo rRNA, and it is anticipated that their presence in cytosol rRNA will prove to be particularly valuable in a comparative study of $O^{2'}$-methylation in wheat embryo cytosol and mitochondrial NaCl-insoluble RNA, currently in progress in this laboratory.

The function of the sequences Cm-ψp and ψm-Ap in wheat embryo rRNA remains a matter for future investigation. It is tempting to speculate that sequences containing modified nucleotides that are unique to a particular rRNA species, and which occur only once per chain, may have a more specialized function than modified sequences which are not confined to a particular rRNA component, and which occur several times per chain. In this connection, it would be of interest to know whether the sequences described in this report occur in the rRNA of other organisms. Preliminary experiments in this laboratory indicate that $O^{2'}$-methylpseudouridine is present in the NaCl-insoluble RNA isolated from the hemoflagellate protozoan, *Crithidia fasciculata*, at about the same level as in wheat embryo NaCl-insoluble RNA (F. Bergez and M. W. Gray, unpublished observations). An investigation of the alkali-stable dinucleotide sequences of *Crithidia* NaCl-insoluble RNA is currently underway in order to establish whether ψm-Ap and Cm-ψp are present in the cytosol rRNA of this organism.

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Differential Effects of 3′-Deoxy Nucleosides on RNA Synthesis in Cotton Cotyledons†

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ABSTRACT: SDS and formamide polyacrylamide gel electrophoresis of RNA synthesized by germinating cotton cotyledons shows that the syntheses of the putative products of RNA polymerase I and III are inhibited by 3′dCyd and 3′dAdo *in vivo.* The synthesis of mRNA, considered the product of RNA polymerase II, is not inhibited by 3′dCyd. No mRNA-poly(A) is formed in the tissue treated with 3′dAdo, but its effect on mRNA synthesis distinct from its effect on polyadenylation of pre-mRNA is not measurable in these experiments.

In the course of investigating the processing of the putative stored mRNA in germinating cotton cotyledons (results to be published elsewhere), we have had occasion to test the effects of 3′dAdo[1] and 3′dCyd on the synthesis of different classes of RNA in this plant tissue. It has been reported previously (Siev *et al.,* 1969; Penman *et al.,* 1970; Abelson and Penman, 1972) that in HeLa cells both these inhibitors, probably by way of their triphosphorylated derivatives, truncate nucleolar rRNA synthesis. Yet neither affect the synthesis of heterogeneous nuclear RNA (HnRNA), although 3′dAdo prevents the appearance of mRNA in the cytoplasm, presumably through an inhibition of the poly(A) addition phase of mRNA processing. We report here the effects of these inhibitors on rRNA, 5S RNA, tRNA, and mRNA-poly(A) synthesis in a higher plant tissue, which, in view of the results obtained with HeLa cells, suggest that the difference in response of the cotton RNA polymerases to the 3′d nucleotide triphosphates *in vivo* may be a general characteristic of these enzymes.

The effect of the inhibitors on the synthesis of the several classes of RNA was determined by measuring the *in vivo* incorporation of radioactive precursors into RNA which had been fractionated by salt precipitation and chromatography on poly(U)-Sepharose and separated by gel electrophoresis.

Materials and Methods

Sepharose 4B was obtained from Pharmacia Fine Chemicals; poly(U), from Miles Laboratories; pancreatic and T_1 RNases were obtained from Worthington and Calbiochem, respectively. 3′dAdo was obtained from Sigma Chemical Co. and 3′dCyd was the kind gift of Dr. John C. Babcock of the Upjohn Company.

Germination and Radioactive Labeling of Cotton Cotyledons. Cotton embryos were removed from their seed coats, and placed in solutions of gramacidin D (0.5 mg/ml, used to prevent bacterial growth) with and without 3′dCyd or 3′dAdo (5 mM). These solutions were then shaken 30 min; the embryos were removed and placed in petri dishes between filter paper wetted with the gramicidin D solution with and without 3′dCyd or 3′dAdo. After 8 hr of germination at 25°, the embryos were transferred to identical milieu but containing $^{32}PO_4$ (100 μCi/ml) and [2-^{3}H]adenosine (100 μCi/ml) and incubated an additional 12 hr. At this point the embryos were removed and washed well with water, the axes were removed from cotyledons and discarded, and the cotyledons were homogenized.

Purification and Fractionation of RNA. Cotyledons were homogenized in an ice-cold solution of 0.1 M Tris-HCl (pH 7.8), 0.1 M NaCl, 0.001 M EDTA, and 0.5% SDS (2 ml/cotyledon pair) in a motor driven Duall homogenizer. The homogenate was diluted 1:1 with a 1:1 mixture of phenol and chloroform and shaken for 30 min at 4°. The phases were separated by centrifugation, and the phenol-chloroform layer along with the interface material was extracted with one-half volume of a solution containing 0.1 M Tris-HCl (pH 9), 0.001 M EDTA, and 0.5% SDS by shaking 30 min at room temperature. The phases were again separated,

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; 3′dAdo, 3′-deoxyadenosine; 3′dCyd, 3′-deoxycytidine; HnRNA, heterogeneous nuclear RNA.