The Alkali-stable Trinucleotide Sequences and the Chain Termini in 18S + 28S Ribonucleates from Wheat Germ*

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ABSTRACT: Alkali hydrolysates of the 18S + 28S ribonucleates from wheat germ contain seven characteristic alkali-stable trinucleotides, one of which contains 5-ribosyluracil as a constituent. The trinucleotides cumulatively account for 0.3 mole % of the total nucleotide residues in the 18S + 28S ribonucleates from wheat germ. The 2′-*O*-methyl ribonucleoside constituents which confer alkali stability on these trinucleotide sequences account for 10% of the total 2′-*O*-methyl ribonucleosides in the 18S + 28S ribonucleates from wheat germ while the remaining 90% of the 2′-*O*-methyl ribonucleosides participate in alkali-stable dinucleotide sequences (H. Singh and B. G. Lane, 1964, *Can. J. Biochem.* (formerly *Can. J. Biochem. Physiol.*) *42*, 1011). The 5′-phosphates of the 2′-*O*-methyl ribonucleosides have been recovered from venom phosphodiesterase hydrolysates in amounts equivalent to the quantities of 2′-*O*-methyl ribonucleosides which have been found as constituents of alkali-stable oligonucleotides in alkali hydrolysates of the ribonucleates. Studies of the end groups of 18S + 28S ribonucleates from wheat germ indicate an average chain length of 1200–1300 nucleotides based on the amounts of nucleosides and diphosphonucleosides recovered in equimolar quantities from alkali hydrolysates.

It has been shown that approximately 1.7% of the internucleoside phosphodiester linkages in the 18S + 28S ribonucleates from wheat germ are alkali-stable and remain intact as part of oligonucleotides after hydrolysis of ribonucleates in molar alkali for 90 hours at room temperature (Singh and Lane, 1964a; see also Glitz and Dekker, 1963; Lane and Allen, 1961). These oligonucleotides are chemically distinct from the slowly hydrolyzed normal oligonucleotides which are present in the hydrolysates at 24 hours but are absent after 90 hours (Singh and Lane, 1964a). The complete stability of a small fraction of internucleoside phosphodiester linkages was reported by Smith and Dunn (1959) to result from the 2′-*O*-methylation of a small proportion of the ribose residues in ribonucleate chains. Such methylation of ribose residues confers alkali stability on the internucleoside phosphodiester linkages to the adjacent 3′ position. The 2′-*O*-methyl nucleosides were shown by Smith and Dunn (1959) to be present in the ribonucleates from a variety of sources, including wheat germ. On the basis of studies relating to these observations and to the observations by Hall (1963, 1964), it was concluded that the alkali stability of 1.7% of the phosphodiester linkages in the 18S + 28S ribonucleates from wheat germ reflected the 2′-*O*-methylation of 1.7% of the constituent nucleoside residues.

Evidence was presented to show that the residues bearing a 2′-*O*-methyl substituent occur internally in the chains of the 18S + 28S ribonucleates from wheat germ, and this fact, in conjunction with the finding that more than 90% of the alkali-stable oligonucleotides are dinucleotides having the general structure NxpNp,[1] leads to the conclusion that about 90% of the 2′-*O*-methyl nucleosides are flanked by normal nucleosides in the ribonucleate chains. However, about 10% of the 2′-*O*-methyl nucleosides are part of trinucleotides having the general structure NxpNxpNp and thus occur in adjacent positions in ribonucleate chains.

The qualitative and quantitative examination of the alkali-stable dinucleotides has been published (Singh and Lane, 1964a) and it is the intention of this report to record the results of quantitative and qualitative analyses of the alkali-stable trinucleotides derived from the 18S + 28S ribonucleates from wheat germ, and to report the details of methodologically allied studies on the terminal residues of these ribonucleates as revealed by alkali hydrolysis.

Experimental

The techniques employed in this study were based on those described in an earlier report (Singh and Lane, 1964a) but owing to the minute quantity of trinucleotides in the hydrolysates it was necessary to conduct

* From the Department of Biochemistry, University of Alberta, Edmonton, Canada. *Received September 25, 1964.* The author wishes to acknowledge the participation of Dr. H. Singh in the exploratory work which led to the present investigation. The study was financially supported by a grant (MRC-MT-1226) of the Medical Research Council of Canada.

[1] The symbol N is used to designate a ribonucleoside residue and the symbol Nx is used to designate a 2′-*O*-methyl ribonucleoside residue. Oligonucleotides are abbreviated in accordance with the recommendations of the Journal of Biological Chemistry.

the isolation on a larger scale. The method of isolation, the sedimentation characteristics, and other properties of the 18S + 28S ribonucleates from wheat germ have been described (Singh and Lane, 1964a).

Alkali Hydrolysis of Ribonucleates. Ribonucleates (1 g) were dissolved in 25 ml of water and the resulting solution was mixed with 2.8 ml of 10 M sodium hydroxide solution. Hydrolysis was allowed to proceed for 90 hours at 22° before neutralization with concentrated formic acid.

Fractionation of Neutralized Alkali Hydrolysates. The neutralized hydrolysate was diluted to 1 liter with water and passed into a 12 × 4.5–cm column of DEAE-cellulose in the formate form. When the charging solution had passed into the column, elution was continued with 500 ml of 0.025 M Tris formate buffer, *p*H 7.8. The molarities of Tris formate buffers, *p*H 7.8, are expressed in terms of the total Tris concentration. The effluent from the charging and washing with 0.025 M Tris formate contained the nucleosides from chain ends of the ribonucleates. Elution of nucleoside monophosphates was effected with 1800 ml of a solution which was 0.085 M with respect to Tris formate buffer, *p*H 7.8, and 7 M with respect to urea. Elution of alkali-stable dinucleotides was accomplished with 1700 ml of an aqueous solution which was 0.17 M with respect to Tris formate, *p*H 7.8, and 7 M with respect to urea. Elution of alkali-stable trinucleotides together with nucleoside 2′,5′- and 3′,5′-diphosphates from chain ends of the ribonucleates was effected with 350 ml of 1 M pyridine formate, *p*H 4.5, which contained 79 ml of pyridine and 42 ml of concentrated formic acid per liter of solution. The elution with pyridine formate was begun after the column had been carefully washed with 1500 ml of water to remove nonvolatile Tris formate and urea from the interstices of the DEAE-cellulose. The pyridinium salts of the trinucleotides and diphosphonucleosides were obtained by direct evaporation of the column effluent in a "flash evaporator" at 37° and the salt-free residue was dissolved in 25 ml of 0.5 M ammonia and reevaporated to obtain ammonium salts of the trinucleotides and diphosphonucleosides.

TABLE I: Summary of the DEAE-Cellulose Column Fractionation of an Alkali Hydrolysate of the 18S + 28S Ribonucleates from Wheat Germ.[a]

Spectrophometric Units[b]	
Charge	= 27,600
N	= 36
Np	= 26,442
NxpNp	= 1,025
pNp + NxpNxpNp	= 136
Recovery	= 27,639

[a] Part of the ultraviolet absorption of the nucleoside fraction is contributed by nonnucleoside material. [b] (D_{260} × volume).

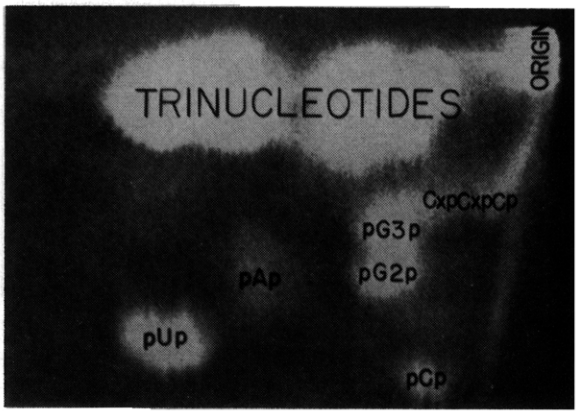

FIGURE 1: Ultraviolet photograph of a two-dimensional paper chromatogram depicting the separation of the nucleoside 2′,5′- and 3′,5′-diphosphates from alkali-stable trinucleotides.

A typical balance sheet of the column fractionation is shown in Table I. It is noteworthy that about 50% of the spectrophotometric units of the nucleoside fraction are contributed by buffer and column materials as well as by nonnucleoside materials from the ribonucleates themselves. The recovery of nucleosides was checked by the addition and recovery of ^{14}C-labeled adenosine to the neutralized hydrolysates and was found to be about 90% when desalting and concentrating was conducted by charcoal adsorption in the manner described previously (Lane and Allen, 1961).

Separation of Trinucleotides and Diphosphonucleosides. The nucleoside 2′,5′- and 3′,5′-diphosphates were separated from the trinucleotides by the two-dimensional paper chromatographic technique described previously (Singh and Lane, 1964a,b) except that separation in the second dimension was limited to a 5-hour run. The separation was performed with one-third of the total fraction eluted from DEAE-cellulose by 1 M pyridine formate, and an ultraviolet light photograph of such a chromatogram is shown in Figure 1. The remainder of the fraction eluted from DEAE-cellulose by 1 M pyridine formate was treated with column-purified *Escherichia coli* phosphomonoesterase to convert trinucleotides to trinucleoside diphosphates and to convert diphosphonucleosides to nucleosides. The digest with phosphomonoesterase was made 0.5 M with respect to sodium hydroxide to inactivate the phosphomonoesterase, and then neutralized and diluted before passage into a 5 × 2.5–cm DEAE-cellulose (formate) column. The nucleosides passed directly through the column and were recovered by charcoal adsorption while the trinucleoside diphosphates were recovered after washing the column with water, eluting with 1 M pyridine formate, *p*H 4.5, and converting to the ammonium salts as described earlier. The trinucleoside diphosphates were resolved by precisely the same two-dimensional paper chromatographic technique de-

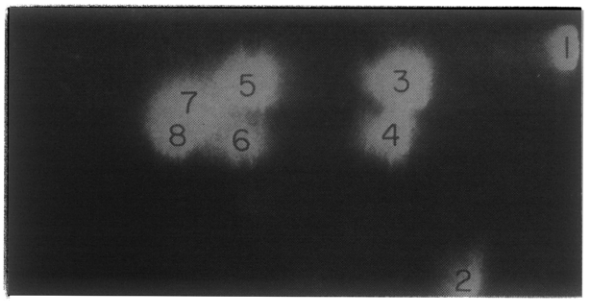

FIGURE 2: Ultraviolet photograph of a two-dimensional paper chromatogram showing the separation of the trinucleoside diphosphates. (1) Uncharacterized guanine-containing material, (2) CxpCxpC, (3) AxpGxpC, (4) CxpUxpG, (5) GxpAxpU, (6) UxpGxpψU, (7) GxpUxpU, (8) UxpGxpU. The chromatogram is overloaded too much for purposes of analysis but clearly shows CxpCxpC which is not easily seen in ultraviolet photographs when smaller quantities of material are spotted.

scribed earlier for dinucleoside phosphates (Singh and Lane, 1964a) and an ultraviolet photograph of such a chromatogram appears in Figure 2. The compounds which were eluted with 0.1 M hydrochloric acid accounted for 80–90% of the ultraviolet-absorbing material spotted on the chromatograms.

The qualitative identifications of the trinucleoside diphosphates were made by the methods described earlier (Singh and Lane, 1964b), which, in brief, consisted of treating the trinucleoside diphosphates with purified snake venom phosphodiesterase devoid of 5′-nucleotidase, and resolving the products with a borate-containing paper chromatographic solvent which cleanly separates the 2′-*O*-methyl nucleoside, the 2′-*O*-methyl nucleoside 5′-phosphate, and the normal 5′-nucleotide derived from each trinucleoside diphosphate. Schematically, the identification can be described as follows: N′xpN″xpN‴ → N′x + pN″x + pN‴. Where two trinucleoside diphosphates are unresolved during paper chromatography it is clear that the quantities of each can be obtained from the quantities of the degradation products produced by phosphodiesterase. The quantities of Nx, pNx, and pN from each trinucleotide were always within 10% of one another on a molar basis.

The treatment of alkali-stable dinucleoside phosphates with whole venom or with a combination of purified snake venom phosphodiesterase and purified *E. coli* phosphomonoesterase leads to the formation of the same end products: the normal ribonucleosides and the 2′-*O*-methyl ribonucleosides. As mentioned in an earlier report (Singh and Lane, 1964b), the deaminase activity of whole venom constituted a considerable drawback which could be avoided by using the combination of purified enzymes. The recent report by Honjo *et al.* (1964) has made it clear that, in the case of trinucleoside diphosphates, the treatments with whole venom and with a combination of purified enzymes are not equivalent even if the deaminase activity of whole venom is neglected, since the pNx compounds from the nonterminal positions of trinucleoside diphosphates are hydrolyzed to Nx compounds by *E. coli* phosphomonoesterase but not by whole venom. The characterization of trinucleoside diphosphates by conversion to the constituent nucleosides was incorrectly reported earlier (Singh and Lane, 1964b) since a combination of purified enzymes was employed in studies of trinucleoside diphosphates whereas the technique reported earlier, using whole venom, had been used only with dinucleoside phosphates and had been discarded in subsequent work with trinucleoside diphosphates in order to circumvent the difficulties encountered with whole-venom digestions.

Analysis of Phosphodiesterase Hydrolysates. Ribonucleates (50 mg) were dissolved in 5 ml of water and mixed with 2.5 ml of 1 M ammonium formate, *p*H 9.2, and 2.5 ml of purified snake venom phosphodiesterase devoid of contaminant 5′-nucleotidase and nuclease activities (Lane *et al.*, 1963). After incubation at 37° for 24 hours, the digest was adjusted to *p*H 7 with formic acid, diluted to 100 ml, and passed into a 12 × 2.5-cm DEAE-cellulose (formate) column. The column was washed with 0.025 M Tris formate buffer, *p*H 7.8, and the nucleosides present in the effluent from charging and washing were recovered by charcoal adsorption. The nucleoside 5′-phosphates comprising more than 99.5% of the hydrolysis products were eluted with 500 ml of a solution which was 0.085 M with respect to Tris formate, *p*H 7.8, and 7 M with respect to urea. The column was washed with water to remove nonvolatile Tris formate and urea and then nucleoside diphosphates (pN>p)[2] were eluted with 1 M pyridine formate, *p*H 4.5, and converted to the ammonium salt forms by reevaporation from 0.5 M aqueous ammonia. The 5′-nucleotides were desalted by adsorption on DEAE-formate, washing with water, and elution with 1 M pyridine formate, *p*H 4.5. The pyridine formate was evaporated and the 5′-nucleotides were recovered as the ammonium salts by evaporation from 1 M ammonia.

The nucleosides, 5′-nucleotides (including pseudouridine-5′-phosphate), and the diphosphonucleosides were resolved by the two-dimensional paper chromatographic technique mentioned earlier.

In order to separate the 2′-*O*-methyl nucleoside 5′-phosphates (pNx) from the normal 5′-nucleotides (pN), the hydrolysates were fractionated as before except that the elution of mononucleotides was begun with a Tris formate–Tris borate buffer in 8 M urea, which caused the pNx compounds to be eluted as a small peak preceding the pN compounds. The eluent was made by dissolving 12.1 g of Tris and 6.1 g of boric acid in 1 liter of a solution which was

[2] The formula pN>p is an abbreviation for nucleoside diphosphates which have a phosphomonoester substituent at the 5′ position of ribose and an internal cyclic phosphodiester substituent between the 2′ and 3′ positions of ribose (Fraenkel-Conrat and Singer, 1962).

0.06 M with respect to Tris formate and 8 M with respect to urea. The final *p*H value of the solution was 8.3. The pNx compounds were desalted in the manner described for pN compounds. The pNx compounds were readily resolved by one-dimensional paper chromatography (Lane, 1963) as shown in Figure 3. A small amount of pC from the pN fraction was included with pNx in order to ensure complete recovery of pGx, the last of the pNx compounds to be eluted.

Results

Analysis of Snake Venom Phosphodiesterase Hydrolysates of the 18S + 28S Ribonucleates from Wheat Germ. The acquired susceptibility of alkali-stable dinucleotides to periodate oxidation when treated with phosphomonoesterase and the evidence that *O*-methylation occurs at the 2′ rather than the 3′ position of ribose (Smith and Dunn, 1959; Hall, 1963; Honjo *et al.*, 1964; Singh and Lane, 1964b) support the structural assignments made for the dinucleotides and are consistent with the view that there is a 3′–5′-phosphodiester linkage between the 2′-*O*-methyl ribonucleoside and normal ribonucleoside constituents of the alkali-stable dinucleotides. Similar studies have shown that the stable trinucleotides also contain 3′–5′-phosphodiester linkages between the 2′-*O*-methyl and normal ribonucleoside constituents. The release of the normal nucleoside constituent as a 5′-nucleotide by treatment of dinucleoside phosphates and trinucleoside diphosphates with venom phosphodiesterase supports these findings in the case of the dinucleotides and trinucleotides, respectively. It has been shown that the non-terminal 2′-*O*-methyl ribonucleoside constituent of the trinucleotides is released as a 2′-*O*-methyl nucleoside 5′-phosphate after treatment of trinucleoside diphosphates with phosphodiesterase, and thus a 3′–5′-phosphodiester linkage between the constituent 2′-*O*-methyl ribonucleosides of trinucleotides is also indicated.

The bulk of the 2′-*O*-methyl ribonucleosides appears as terminal residues of stable dinucleotides and trinucleotides after alkali hydrolysis of ribonucleates, and do not bear 5′-phosphoryl substitutions. Earlier evidence showed that the 2′-*O*-methyl ribonucleosides were not terminal groups of ribonucleate chains and it was concluded that they occur internally and bear 5′-phosphoryl substituents in the intact ribonucleate polymers as indicated by Smith and Dunn (1959). Further work has verified this assumption, since complete hydrolysis with purified venom phosphodiesterase releases 2′-*O*-methyl nucleoside 5′-phosphates from the 18S + 28S wheat germ ribonucleates in quantities equivalent to the amounts of 2′-*O*-methyl ribonucleosides found as part of alkali-stable oligonucleotides after alkali hydrolysis. The compounds in phosphodiesterase digests which are thought to be 2′-*O*-methyl nucleoside 5′-phosphates are not susceptible to periodate oxidation and are completely dephosphorylated to compounds identical with the 2′-*O*-methyl nucleosides by treatment with *E. coli* alkaline phosphomonoesterase. The 5′-nucleotidase of snake venom does not hydrolyze the presumed 2′-*O*-methyl nucleoside 5′-phosphates in accordance with the discovery of Honjo *et al.* (1964).

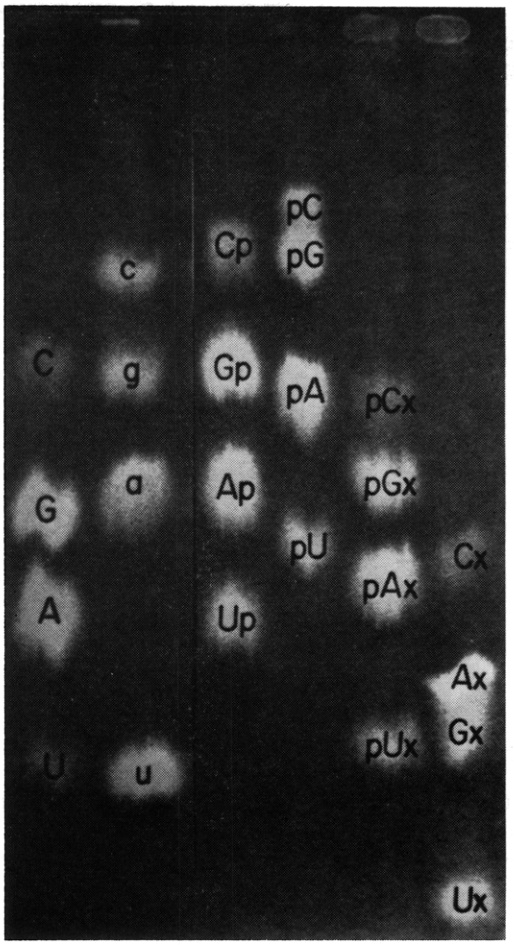

FIGURE 3: Ultraviolet photograph of a paper chromatogram showing the movement of pNx and Nx compounds in relation to the movement of other nucleate derivatives using a paper chromatographic system which has been described in detail elsewhere (Lane, 1963). The bases are indicated by lower-case lettering a, g, c, u and the ribonucleosides are indicated by upper-case lettering A, G, C, U while all other symbols conform to standard usage.

An analysis for the 5′-nucleotides and minor components of venom phosphodiesterase hydrolysates is shown in Table II. The small amounts of nucleosides and diphosphonucleosides result from trace hydroxide ion–catalyzed cleavages occurring during the hydrolysis with venom phosphodiesterase at *p*H 9.2 (Lane *et al.*, 1963). Evidence to be presented in a separate report shows that such end groups are artifactual in the sense that they are absent from the 18S + 28S ribonucleate preparations prior to incubation at the slightly alkaline *p*H conditions needed for venom phosphodiesterase

TABLE II: Analysis of a Phosphodiesterase Hydrolysate of the 18S + 28S Ribonucleates from Wheat Germ.[a]

Component	Amount (moles/100 moles nucleotides)
pA	23.2
pG	30.1
pC	24.8
pU	18.1
pψU	1.8
pAx	0.54
pGx	0.38
pCx	0.35
pUx	0.47
A	0.054
G	0.047
C	0.028
U	0.031
pA>p	0.028
pG>p	0.025
pC>p	0.049
pU>p	0.053

[a] There was no oligonucleotide residue in the hydrolysate and no evidence for the presence of any components other than those tabulated.

action (unpublished observations, J. Diemer, B. McLennan, and B. G. Lane). It is noteworthy that improvements in technical aspects of end-group measurements have resulted in recoveries of diphosphonucleosides in quantities roughly equal to the amounts of nucleosides in phosphodiesterase hydrolysates, in contrast with earlier studies (Singh and Lane, 1964a) where poor recovery was experienced because pN>p compounds adsorbed very poorly to charcoal during desalting of column effluents.

Analysis of the Native End Groups of 18S + 28S Ribonucleates from Wheat Germ by Hydrolysis with Alkali. The end groups of low molecular weight s-RNA account for more than 2% of the constituent nucleoside residues and can be obtained in nearly quantitative recovery as nucleosides and nucleoside diphosphates after alkali hydrolysis of s-RNA (Bell *et al.*, 1963). The end groups of high molecular weight ribosomal ribonucleates account for less than 0.2% of the constituent nucleoside residues. A preliminary examination showed that, although the end groups which appear as nucleosides in alkali hydrolysates could be obtained in nearly quantitative recovery, the quantity of nucleoside diphosphates did not exceed 30% of the quantity of nucleosides owing to difficulties encountered in separating nucleoside diphosphates from the much larger quantity of oligonucleotides present in the alkali hydrolysates of high molecular weight ribonucleates from wheat germ (Lane and Allen, 1961). Tomlinson and Tener (1963) showed that the inclusion of urea in eluents used for anion-exchange fractionation on DEAE-cellulose permitted separation on the basis of net charge, and this technique proved most valuable in the separation of trace quantities of nucleoside diphosphates from the bulk of the alkali-stable oligonucleotides. The nucleoside diphosphates were recovered in amounts equivalent to about 60% of the quantity of nucleosides in the initial experiments with urea-containing eluents (Singh and Lane, 1964a), but further work has shown that the employment of larger DEAE-cellulose columns, as in the present study, permits the recovery of nucleoside diphosphates in amounts equivalent to the quantity of nucleosides in the alkali hydrolysates of 18S + 28S wheat germ ribonucleates.

The results of end-group analyses appear in Table III and indicate a mean chain length of 1200–1300

TABLE III: End-Group Analysis of the 18S + 28S Ribonucleates from Wheat Germ.[a]

Component	Preparation 1 (mole/100 moles nucleotides)	Preparation 2 (mole/100 moles nucleotides)
A	0.012	0.016
G	0.024	0.024
C	0.021	0.019
U	0.021	0.019
Total	0.078	0.078
pAp	0.008	0.007
pGp	0.031	0.032
pCp	0.020	0.018
pUp	0.023	0.020
Total	0.082	0.077

[a] The values have been corrected to quantitative recovery, the actual recoveries being 85–90% of the values quoted.

nucleotide residues. It should be commented that the analysis for nucleosides is most easily performed on 24-hour hydrolysates. A small amount of glycosyl-N bond splitting occurs in molar alkali at room temperature and care must be exercised to separate the resulting bases from the nucleosides derived from end groups. The most prominent of the bases is adenine, which can reach as much as 0.014 mole/100 moles of nucleotides in 90-hour hydrolysates. The small amount of bases present makes it preferable to use two-dimensional chromatography in the separation of the nucleosides of 90-hour hydrolysates to avoid introducing small errors into the values for nucleosides, whereas the quantity of bases in 24-hour hydrolysates can be ignored and one-

dimensional chromatography is sufficient. On the other hand, the analysis for nucleoside diphosphates is facilitated with a 90-hour rather than a 24-hour hydrolysis time, since the presence of slowly hydrolyzed normal oligonucleotide material at 24 hours interferes with the paper chromatographic resolution of the nucleoside diphosphates. The quantities of nucleosides obtained by treatment of the mixture of trinucleotides and diphosphonucleosides with phosphomonoesterase (Bell *et al.*, 1963) agreed precisely with the results of direct analysis for diphosphonucleosides and could be used to circumvent the type of problem encountered with 24-hour hydrolysates.

Alkali-Stable Trinucleotides of Alkali Hydrolysates of 18S + 28S Ribonucleates from Wheat Germ. The qualitative composition of the trinucleotide fraction was invariant for the six preparations of wheat germ ribonucleates which were examined. The same seven trinucleotides were always found, together with some uncharacterized material remaining at the origin of two-dimensional chromatograms. It is apparent, however, from the quantitative distributions of the trinucleotides which were obtained for three preparations (Table IV) that there was significant scatter in the relative amounts of the trinucleotides found in the ribonucleate preparations from different batches of wheat germ. The most prominent variation was observed with the trinucleotide, CxpCxpCp, which showed nearly a 3-fold variation among the different preparations. The possibility was considered that this trinucleotide might have been lost in some instances as part of the dinucleotide fraction during separations on DEAE-cellulose, since it would be the most easily eluted trinucleotide and might be highly sensitive to small changes of conditions as a consequence of possible shifting of the amino pK_a values to higher values in the presence of 8 M urea (Stockx and Vandendriessche, 1963). Some support for this view was obtained when it was found that there was some CxpCxpCp in the latter one-third of the dinucleotide fraction in the case of preparation 2; however, this amounted to only one-seventh of the quantity which was recovered in the trinucleotide fraction and could not explain the observed discrepancy between preparations 2 and 3. It should be stated, however, that none of the other trinucleotides could be detected in the latter one-third of the dinucleotide fraction, and for this reason it may be significant that there was even a small amount of CxpCxpCp found since it exhibits by far the greatest quantitative variation. Further examination of this point would seem warranted but the minute quantities available for study make systematic experimentation difficult.

The spectral ratios for the different trinucleotides at acidic, neutral, and alkaline *p*H values corresponded very closely to predicted values based on the spectral ratios (250/260 mμ, 280/260 mμ, and 290/260 mμ) which can be calculated from the molar extinction values of the normal ribonucleosides which comprise the corresponding normal trinucleotides. It is noteworthy that the quantities of six trinucleotides are 20–100 times greater than would be expected for a random occurrence of the constituent nucleosides in the ribonucleates as a whole. In the case of the trinucleotide, UxpGxpΨUp, the quantity recovered is more than 300 times greater than statistical randomness would predict.

TABLE IV: Trinucleotides Recovered from Alkali Hydrolysates of the 18S + 28S Ribonucleates from Wheat Germ.[a]

Trinucleotide	Preparation 1	2	3 (mole/100 moles nucleotides)	Calculated for Random Distribution
AxpGxpCp	0.014	0.016	0.013	0.0005
GxpAxpUp	0.013	0.014	0.012	0.0004
GxpUxpUp	0.023	0.023	0.014	0.0003
CxpCxpCp	0.009	0.011	0.022	0.0002
CxpUxpGp	0.013	0.012	0.009	0.0005
UxpGxpUp	0.011	0.011	0.014	0.0003
UxpGxpψUp	0.013	0.009	0.012	0.00003
Total	0.096	0.091	0.096	0.002

[a] The calculated quantities were estimated on the basis of a random occurrence of the constituent nucleosides in the particular sequences. For example, the quantity of AxpGxpCp, based on a random distribution of the constituents, was 100 × 0.0054 × 0,0038 × 0.248 = 0.0005. The molar extinction coefficients for the trinucleotides were obtained by adding the molar extinction coefficients (260 mμ) of the constituent nucleosides of the corresponding normal trinucleotides with no correction for hypochromicity. The values have been corrected for an estimated 85% recovery during the isolation procedures.

The uncharacterized material remaining at the origin of two-dimensional chromatograms has spectral properties similar to guanosine, does not contain deoxyribose, and yields material with properties similar to 2′-*O*-methyl guanosine when degraded enzymically. The uncharacterized material accounted for about 10% of the ultraviolet-absorbing material (260 mμ) spotted for two-dimensional separation.

The variability in the quantitative proportions of the different trinucleotides is greater than was encountered in earlier work with alkali-stable dinucleotide sequences and could be related to technical factors as discussed earlier. However, since there is, on the average, only one trinucleotide sequence per ribonucleate chain, it would be anticipated that fractionation of the 18S + 28S ribonucleates which might occur during the isolation procedures would have a more pronounced effect on the proportions found for trinucleotide sequences than it would have on the proportions found for dinucleotide sequences which occur with an average frequency of about fifteen sequences per ribonucleate chain.

Discussion

There are sixty-four possible alkali-stable trinucleotide sequences having the general structure NxpNxpNp, in which N is any of the four major ribonucleosides and Nx is the 2′-*O*-methyl derivative of any of the four major ribonucleosides. The present study has shown that only six of these trinucleotide sequences are present in measureable amounts in the 18S + 28S ribonucleates from wheat germ. A trinucleotide sequence containing the minor ribonucleoside 5-ribosyluracil was also detected. It is noteworthy that the amounts of the seven trinucleotides which have been identified are 20–300 times greater than would be expected for a random distribution of N and Nx residues in ribonucleate chains, and thus the presence of very much smaller quantities of other trinucleotides might be conjectured but would not have been detected by the techniques used in the present study. The study of trinucleotide sequences has amplified the earlier conclusions arising from an examination of dinucleotide sequences (Singh and Lane, 1964a) by showing that there is not only a nonrandomness with respect to the amounts of different sequences but also that there is a nonrandomness with respect to the spatial distribution of 2′-*O*-methyl ribonucleosides in the 18S + 28S ribonucleates from wheat germ. A nonrandomness of spatial distribution is evident from the observation that there is about one trinucleotide sequence per thousand nucleotide residues, whereas one would predict that there would only be $0.017 \times 0.017 \times 1 = 0.0003$ trinucleotide sequence per nucleotide, or one trinucleotide sequence for about three thousand nucleotide residues. The actual quantity of trinucleotide sequences corresponds to about one such sequence per ribonucleate chain based on the mean chain length of 1200–1300 nucleotides from end-group measurements.

The isolation of the 2′-*O*-methyl nucleoside 5′-phosphates in an amount very similar to the quantity of 2′-*O*-methyl ribonucleosides found in the alkali-stable oligonucleotides of alkali hydrolysates is consistent with the view that all internucleoside phosphodiester linkages involving the 2′-*O*-methyl nucleoside constituents are 3′–5′-phosphodiester bonds as in the case of the normal nucleosides of ribonucleate chains. It is noteworthy that the 5′-phosphates of the constituent nucleosides and *O*-methyl nucleosides account for about 99.7% of the total constituent nucleoside residues of the 18S + 28S ribonucleates after phosphodiesterase hydrolysis, and this supports the widely held view that there are only 3′–5′-phosphodiester linkages in these ribonucleates.

There can be little valid discussion of the significance of the analytical data on the alkali-stable oligonucleotides at this time except insofar as there appears to be an interesting correlation between the occurrence of 2′-*O*-methyl ribonucleosides and the 5-ribosyluracil content of some ribonucleate preparations. The wheat germ 18S + 28S ribonucleates are relatively rich in 5-ribosyluracil (Glitz and Dekker, 1963) and contain an abundance of 2′-*O*-methyl ribonucleosides, whereas the ribosomal ribonucleates of *E. coli* have indiscernible amounts of 5-ribosyluracil and 2′-*O*-methyl ribonucleosides (Dunn *et al.*, 1963). This correlation suggests the interesting possibility that the free N-1 of the 5-ribosyluracil might serve as a potential transfer agent for activated methyl groups to the 2′ position of ribose residues which are suitably disposed spatially.

Finally, it might be commented that the end-group data may have significance for current studies of the biosynthesis of 18S + 28S ribonucleates. The end groups detected by alkali hydrolysis could result from the pyrophosphorolytic condensation of ribonucleoside triphosphates (Kornberg, 1957) during the terminal addition to, or primed biosynthesis of, the ribonucleate chains,[3] and/or from the endonucleolytic cleavage of a polynucleotide precursor in such a way as to produce a new 5′-phosphomonoester group on one of the resulting chains and no phosphomonoester group on the other chain end produced by the scission. The latter possibility would be pertinent to recent suggestions that ribosomal ribonucleates may arise from fragmentation of a macromolecular precursor (Rake and Graham, 1964). It may be significant that the preparations examined in the current work had roughly equal weights of the 18S and 28S components when observed in the analytical ultracentrifuge but had mean chain lengths by end-group analysis of 1200–1300 nucleotides, a figure which is more in agreement with what would be expected for the 18S component itself, on the basis of physicochemical estimates of weight-average molecular weight. This suggests the possibility that the 28S component might be composed of chains having the same length and end-group characteristics as the 18S component, otherwise the number-average molecular weight of the 18S + 28S ribonucleates is substantially lower than the weight-average molecular weight and indicates a polydispersity not generally attributed to these ribonucleates.

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[3] If it is assumed that ribonucleoside triphosphates are the immediate precursors of all nucleotide residues, both terminal and nonterminal, in ribonucleate chains, then the 5′-phosphomonoester terminus of s-RNA and the 18S + 28S ribonucleates must result from an as yet undefined pyrophosphate release from a triphosphate group.

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The Chemistry of Pseudouridine. IV. Cyanoethylation*

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ABSTRACT: Acrylonitrile reacts with adenosine, guanosine, uridine, cytidine, and pseudouridine at *p*H 11.5. However, at *p*H 8.8 selective cyanoethylation of pseudouridine is possible. The product is 1-cyanoethylpseudouridine. The mono- and dicyanoethyl derivatives of pseudouridine, pseudouridine-2′(3′)-phosphate, and pseudouridine-2′,3′-cyclicphosphate have been prepared. RNAase is active on 1-cyanoethylpseudouridinecyclicphosphate, but not on the 1,3-dicyanoethyl derivative. Selective cyanoethylation of uracil to give 1-cyanoethyluracil is described. Benzoylation of uracil to give a mixture of 1- and 3-benzoyluracil is also reported.

During the course of our studies on the chemistry of pseudouridine we observed that acrylonitrile reacted with the pyrimidine ring to give a monocyanoethyl derivative (Chambers *et al.*, 1963). The most remarkable feature of this reaction was that it appeared to be specific for the 1 position of pseudouridine. If cyanoethylation were really specific for pseudouridine and if this specificity could be maintained under mild conditions, then this reaction offered a potential route for selective modification of the pseudouridine residues in s-RNA. Therefore, we studied the cyanoethylation reaction in more detail.

In aqueous ethanol at *p*H 7 there was no detectable reaction between acrylonitrile and pseudouridine after 24 hours at room temperature. In dilute ammonium hydroxide (*p*H 8) a single new product was formed, but the reaction was slow and even after 64 hours 28% pseudouridine remained (Table I). Addition of NH_4OH and a further 24-hour incubation gave only a slight increase in yield. Additions of both NH_4OH and acrylonitrile finally brought the reaction near completion (Table I).

These results demonstrated that the reaction could be run under mild conditions. They also indicated that it was *p*H dependent and that ammonia was reacting with acrylonitrile. In order to inhibit this side reaction, the ammonium hydroxide was replaced by triethylammonium acetate buffer and the *p*H was raised to 8.8. Under these conditions the formation of monocyanoethylpseudouridine was rapid and after 3 hours the reaction was essentially complete (Figure 1). During this time no dicyanoethylpseudouridine could be detected. However, after 30 hours some of the disubstituted product had formed. Adenosine, guano-

TABLE I: Cyanoethylation of Pseudouridine.

Time (hr)	Additions[a]	Ψ[b]
0–64	None	28
64–88	NH_4OH	23
88–96	NH_4OH + acrylonitrile	15
96–120	NH_4OH + acrylonitrile	5

[a] The reaction was carried out in dilute NH_4OH at *p*H 8 with a 10-fold excess of acrylonitrile. Further additions were made as indicated (see Experimental for details). [b] Ψ = pseudouridine. Values are per cent of starting material remaining.

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† Health Research Council Career Scientist of the City of New York (Investigatorship I 200).