Moffatt, J. G., and Khorana, H. G. (1961), *J. Am. Chem. Soc. 83*, 649.
Nirenberg, M. W. (1963), *Methods Enzymol 6*.
Nirenberg, M. W., Jones, O. W., Leder, P., Clark, B. F. C., Sly, W. S., and Pestka, S. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 549.
Nirenberg, M. W., and Leder, P. (1964), *Science 145*, 1399.
Rammler, D. H., and Khorana, H. G. (1962), *J. Am. Chem. Soc. 84*, 3112.
Richards, E. G., Flessel, C. P., and Fresco, J. R. (1963), *Biopolymers 1*, 431.
Sevag, M. G., Lackman, D. B., and Smolens, J. (1938), *J. Biol. Chem. 124*, 425.
Simha, R. (1945), *J. Chem. Phys. 13*, 188.
Singer, M. F., and Guss, J. K. (1962), *J. Biol. Chem. 237*, 182.
Speyer, J. F., Lengyel, P., Basilio, C., Wahba, A. J., Gardner, R. S., and Ochoa, S. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 559.
Szer, W., and Ochoa, S. (1964), *J. Mol. Biol. 8*, 823.
Tomlinson, R. V., and Tener, G. M. (1962), *J. Am. Chem. Soc. 84*, 2644.

The Alkali-Stable Dinucleotide Sequences and the Chain Termini in Soluble Ribonucleates from Wheat Germ*

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ABSTRACT: There are sixteen possible alkali-stable dinucleotides having the general structure NxpNp 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. Quantitative and qualitative data show that at least thirteen of these dinucleotides are detectable in alkali hydrolysates of wheat germ soluble ribonucleates (s-RNA). Two additional dinucleotides which contain the 2′-*O*-methyl derivative of the minor ribonucleoside, pseudouridine, have been identified. The proportions of the different alkali-stable dinucleotide sequences in wheat germ s-RNA contrast sharply with the corresponding proportions found for the 18S + 28S RNA from the same source. The alkali-stable dinucleotide sequences cumulatively account for about 2.6 mole % of the constituent nucleotides of wheat germ s-RNA, and, since the chain termini of s-RNA which appear as nucleosides and nucleoside diphosphates in alkali hydrolysates also cumulatively account for 2.6 mole % of the constituent nucleotides, there is an average of one alkali-stable dinucleotide sequence per s-RNA chain.

Smith and Dunn (1959a) isolated alkali-stable dinucleotides from the alkali hydrolysates of several ribonucleate specimens of animal, plant, and microbial origin. It was shown that there was 1 mole of *O*-methylribose per mole of dinucleotide and all available evidence supports their initial proposition that the sugar is 2′-*O*-methylribose (Smith and Dunn, 1959a; Hall, 1964; Honjo *et al.*, 1964; Singh and Lane, 1964a; Broom and Robins, 1965). The 2′-*O*-methylation confers alkali stability on the internucleoside phosphodiester bond which is linked to the adjacent 3′ position of the *O*-methyl nucleoside constituents of ribonucleate chains.

An examination of 18S + 28S RNA from wheat germ has shown that the *O*-methyl nucleoside constituents occur internally in ribonucleate chains (Singh and Lane, 1964b; Lane, 1965). About 90% of the *O*-methyl nucleosides in wheat germ 18S + 28S RNA are flanked by normal ribonucleosides in the ribonucleate chains and can be recovered as part of alkali-stable dinucleotides, NxpNp,[1] while the remaining 10% occur in clusters of two and can be recovered as part of alkali-stable trinucleotides, NxpNxpNp, after hydrolysis of the ribonucleates by alkali. The *O*-methyl nucleosides participate in all of the sixteen possible alkali-stable dinucleotide sequences and in at least six of the sixty-four possible alkali-stable trinucleotide sequences which can result from permuting the four principal ribonucleosides and their *O*-methyl derivatives (Lane, 1965).

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[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 abbreviations 18S + 28S RNA and s-RNA refer to the 18S + 28S ribonucleates and amino acid acceptor ribonucleates, respectively.

It is the intention of this report to record the results of an examination of the alkali-stable dinucleotide sequences of wheat germ s-RNA and to compare these findings with the results of the corresponding analyses of the 18S + 28S RNA from the same source. The results of methodologically allied studies of the terminal residues of wheat germ s-RNA will also be considered in this report.

Experimental Section

Preparation of Wheat Germ s-RNA. Freshly milled wheat germ of the Thatcher variety was generously provided by Ogilvie Flour Mills Co. of Edmonton. The ribonucleates were extracted from 125 g of wheat germ in an aqueous emulsion composed of 500 ml of water-saturated phenol and 500 ml of 0.05 M potassium phosphate buffer, pH 7. The emulsion was shaken for 15 minutes at room temperature and then centrifuged at 2000 rpm in an International centrifuge for 10 minutes at 4°. The aqueous layers from seventeen extractions (2125 g wheat germ) were combined and made 3 M with respect to sodium chloride. After a period of 18 hours at 0–4°, the suspension was centrifuged to collect the precipitate of 18S + 28S RNA. Two volumes of 95% ethanol were added to the supernatant solution in order to precipitate the s-RNA. Further purification of the s-RNA was achieved by the method of Glitz and Dekker (1963). The yield of s-RNA was about 3 g from 2125 g of wheat germ and the product had an extinction coefficient of 160–190 at 260 mμ when dissolved to give a 1% aqueous solution.

Fractionation of Neutralized Alkali Hydrolysates of Wheat Germ s-RNA. A 5% aqueous solution of the s-RNA (1.7 g dissolved in 35 ml of water) was made 1 M with respect to alkali by the addition of 10 M sodium hydroxide. The solution was allowed to stand at 22° for 90 hours before neutralization with concentrated formic acid.

The neutralized alkali-hydrolysate of the s-RNA was diluted with water to reduce the salt concentration to 0.025 M and the diluted solution was poured into a 26- × 4.5-cm DEAE-cellulose column (formate form). The nucleoside, nucleoside monophosphate, and dinucleotide fractions were separated following the method of Singh and Lane (1964b). After the elution of the dinucleotide fraction, the column was washed thoroughly and the nucleoside diphosphates were eluted with 1 M pyridinium formate, pH 4.5, and converted to ammonium salts as described earlier (Lane, 1965). The nucleosides were desalted by charcoal adsorption and the alkali-stable dinucleotides were desalted by readsorption to DEAE-cellulose (formate) and elution with volatile 1 M pyridinium formate, pH 4.5. The salt-free residue recovered after evaporating the pyridinium formate was reevaporated from 0.5 M ammonia to convert the pyridinium to ammonium salts. The results of a typical fractionation are summarized in Table I.

TABLE I: Summary of the DEAE-Cellulose Column Fractionation of an Alkali Hydrolysate of Wheat Germ s-RNA.

Spectrophotometric Units[a]	
Charge =	36,400
N =	617
Np =	34,300
NxpNp =	900
pNp =	600
Recovery =	36,417

[a] (D_{260} × volume).

Separation of Nucleosides and Diphosphonucleosides. An aliquot of the nucleoside fraction (about 1.5 μmoles) was separated into the component nucleosides by one-dimensional paper chromatography (Lane, 1963). The adenosine, cytidine, and uridine bands were spectrophotometrically pure, but the "guanosine" band had an unusual spectrum. The material in the "guanosine" bands of several chromatograms was pooled and cleanly separated into three bands by paper chromatography using the borate solvent of Plesner (1955). The three bands had R_F values of 0.21, 0.52, and 0.65, and were found to contain guanosine, 2-amino-6-hydroxy-5-methylformamido-4-phosphoribosylaminopyrimidine (Lawley and Brooks, 1963), and adenine, respectively. The adenine is a breakdown product of adenylate and appears in measurable amounts only after extended periods of hydrolysis in 1 M alkali. The pyrimidine, a breakdown product of 7-methylguanylate, does not adsorb to DEAE-cellulose at pH 6–7 and thus appears in the nucleoside fraction of anion-exchange separations. In order to obtain a reliable value for cytidine diphosphate, a relatively large amount (about 3 μmoles) of the nucleoside diphosphate fraction was separated by the two-dimensional paper chromatographic system described in earlier reports (Singh and Lane, 1964a,b).

Separation of Alkali-Stable Dinucleotides. An aliquot of the alkali-stable dinucleotide fraction was passed into a 25- × 1-cm DEAE-cellulose column (formate form). The dinucleotides were separated into three fractions at pH 2 (Lane and Allen, 1961a). The first two fractions were eluted with 1 M formic acid, pH 2, and the last fraction was removed by 1 M pyridine formate, pH 4.5. The pH value of the first two fractions was adjusted to 4.5 by the addition of pyridine, and the fractions were concentrated into small volumes by evaporation in a flash evaporator at 37°. Separation of the dinucleotides into three fractions prior to paper chromatography facilitated the quantitative recovery of those minor dinucleotides which comprised less than 2% of the alkali-stable dinucleotide fraction. The first fraction contained AxpAp, CxpCp, and CxpAp; the second fraction contained AxpGp, GxpAp, GxpCp, CxpUp, UxpAp, UxpCp, and ψUxpCp; and the third fraction contained GxpGp, GxpUp, UxpGp, UxpUp, and ψUxpUp.

Aliquots of each dinucleotide fraction containing about 3 μmoles in 200 μl were treated with 50 μl of *Escherichia coli* phosphomonoesterase at pH 9.2 before separation by the same two-dimensional chromatographic technique described earlier (Singh and Lane, 1964a,b). The photographs of two-dimensional paper chromatographic separations of the alkali-stable dinucleoside phosphates from 18S + 28S RNA have been published but ψUx-containing dinucleoside phosphates were not present. It should be noted, therefore, that the ψUx-containing dinucleoside phosphates from s-RNA moved slightly behind the corresponding Ux-containing compounds in the first dimension and slightly ahead of them in the second dimension. This chromatographic mobility is not unexpected because of the retarding effect of ψU in the first-dimensional solvent system. For instance ψU and pψU have the same mobilities as A and pA, respectively, and move well behind U and pU, respectively, in the first-dimensional solvent system (see Lane, 1965).

TABLE II: End-Group Analysis of s-RNA from Wheat Germ.[a]

Component	Quantity (mole/100 moles nucleotides)
Adenosine	1.00
Guanosine	0.024
Cytidine	0.22
Uridine	0.050
Total	1.3
pAp	0.049
pGp	1.16
pCp	0.013
pUp	0.074
Total	1.3

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

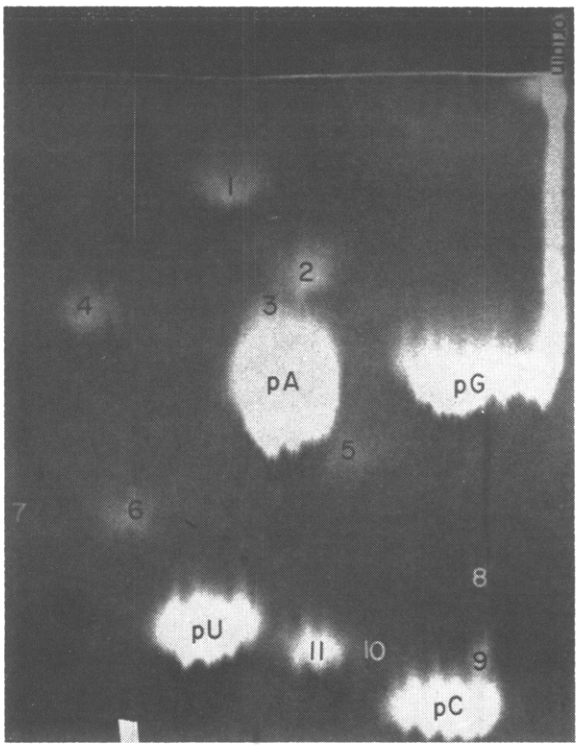

FIGURE 1: Ultraviolet photograph of a two-dimensional chromatogram showing the separation of 5′-mononucleotides produced by snake venom phosphodiesterase hydrolysis of wheat germ s-RNA. The numbered areas contain the following minor components: (1) pN^2,N^{2}diMepG, (2) p1MeG, (3) pN^{2}MeG, (4) pN^{6}MeA, (5) pI, (6) p5MeU, (7) pUx, (8) p7MeG, (9) p1MeA, (10) p5MeC, (11) pψU. Spots 7, 8, and 10 did not photograph well but were easily seen by direct inspection of the paper chromatogram with an ultraviolet lamp. The chromatogram is overloaded with respect to pG in order to obtain measurable amounts of the minor components. Spectral analysis of small sections of the ultraviolet absorbing band between pG and the origin indicated that it contained only pG. The chromatogram was developed in the first dimension (horizontal) and the second dimension (vertical) according to the methods described in an earlier report (Singh and Lane, 1964a).

The individual dinucleoside phosphates were characterized spectrophotometrically and by degradation with purified snake venom phosphodiesterase to their component 2′-*O*-methyl nucleosides and normal 5′-nucleotides. The mixed dinucleoside phosphates were also characterized by periodate degradation to bases and 2′-*O*-methyl nucleoside 3′-monophosphates (Neu and Heppel, 1964).

Analysis of Snake Venom Phosphodiesterase Hydrolysates of Wheat Germ s-RNA. The 2′-*O*-methyl nucleoside 5′-phosphates were measured by complete digestion of the s-RNA with whole venom. The s-RNA (50 mg), dissolved in 5 ml of water, was mixed with 2.5 ml of 1 M ammonium formate buffer, pH 9.2, and 2.5 ml of a 2.5% aqueous solution of Russell's viper venom, and then incubated at 37° for 24 hours. The normal 5′-nucleotides produced by the phosphodiesterase were dephosphorylated by the 5′-nucleotidase present in the snake venom, but the 2′-*O*-methyl nucleoside 5′-phosphates were unaffected (Honjo *et al.*, 1964). The nucleosides were separated from the 2′-*O*-methyl nucleoside 5′-phosphates by passage through a 3- × 2.5-cm DEAE-cellulose column (formate form). The 2′-*O*-methyl nucleoside 5′-phosphates were eluted, desalted, and separated by one-dimensional paper chromatography (Lane, 1963). The

TABLE III: Proportions of Normal and Alkali-Stable Dinucleotide Sequences in Wheat Germ s-RNA.

Alkali-Stable Dinucleotides	Observed[a] (moles/100 moles of total alkali-stable dinucleotide sequences)	Calculated[b]	Normal Dinucleotides	Calculated[c] (moles/100 moles of total dinucleotide sequences)
AxpAp	0.3	0.08	ApAp	3.9
AxpGp	0.1	0.1	ApGp	5.8
AxpCp	0.0	0.1	ApCp	5.2
AxpUp	0.0	0.07	ApUp	3.1
GxpAp	3.1	7.9	GpAp	5.8
GxpGp	18.3	11.7	GpGp	8.6
GxpCp	7.8	10.5	GpCp	7.7
GxpUp	4.9	6.3	GpUp	4.6
CxpAp	4.5	6.7	CpAp	5.2
CxpGp	0.0	9.9	CpGp	7.7
CxpCp	6.3	8.9	CpCp	6.9
CxpUp	20.0	5.3	CpUp	4.1
UxpAp	0.6	5.0	UpAp	3.1
UxpGp	7.6	7.5	UpGp	4.6
UxpCp	9.5	6.7	UpCp	4.1
UxpUp	8.6	4.0	UpUp	2.5
ψUxpAp	0.0	2.0	ψUpAp	0.54
ψUxpGp	0.0	3.0	ψUpGp	0.79
ψUxpCp	2.1	2.7	ψUpCp	0.71
ψUxpUp	6.3	1.6	ψUpUp	0.42
Totals	100.0	100.0		85.4

[a] The molar extinction coefficients for the dinucleotides were obtained by adding the molar extinction coefficients (260 mμ) of the constituent nucleosides of the corresponding normal dinucleotides without correction for hypochromicity. The alkali-stable dinucleotides account for about 2.6 mole % of the constituent nucleotides in wheat germ s-RNA, and consequently the *O*-methyl nucleotide constituents of the dinucleotides comprise about 1.3 mole % of the total nucleotide constituents of the s-RNA. [b] The calculated quantities were estimated on the basis of a random occurrence of the constituent nucleosides in the sequences listed, using the data of Table IV to obtain nucleoside frequencies (*f*). For example, the proportion of GxpCp was $100 \times [f(\text{GxpCp})/f(\text{NxpNp})]$ or, $100 \times [f(\text{Gx}) \times f(\text{C})/f(\text{Nx}) \times f(\text{N})] = 100 \times (0.0047 \times 0.263/0.0130 \times 0.911) = 10.5\%$. [c] The calculated quantities were estimated as described. Since each nonterminal nucleotide constituent of a ribonucleate chain participates in two dinucleotide sequences, the moles of a given dinucleotide sequence per 100 moles of nucleotides are nearly equivalent to the moles of dinucleotide sequences per 100 moles of total dinucleotide sequences in the chains. The chain length and heterogeneity of s-RNA are sufficiently great to allow the possibility of randomness in those dinucleotide sequences containing only the four major ribonucleosides, although this possibility is perhaps questionable in the case of dinucleotide sequences containing trace components.

2′-*O*-methyl nucleoside 5′-phosphates were also analyzed by the method described in an earlier report, in which case hydrolysis was achieved with purified venom phosphodiesterase rather than with whole venom (Lane, 1965). The results with whole venom were similar to results obtained with purified phosphodiesterase although the paper chromatograms were cleaner when the purified enzyme was employed.

The amounts of other ribonucleate constituents were determined by hydrolysis with purified snake venom phosphodiesterase as described in an earlier report of work with 18S + 28S wheat germ ribonucleates. An aliquot containing 5–6 μmoles of nucleotide material was separated by two-dimensional paper chromatography (Singh and Lane, 1964a,b) and an ultraviolet photograph of such a chromatogram is shown in Figure 1. The major nucleoside 5′-phosphates, pseudouridine 5′-phosphate, and the nucleoside 5′-phosphates with methyl substituents on the heterocycle were spectrophotometrically characterized at pH values 1, 7, and 13. The amounts of the methylguanine nucleotides were determined by rechromatography of the

material eluted from the first two-dimensional chromatographic separation.

Results

The analysis for terminal groups of wheat germ s-RNA is presented in Table II and the data can be used to calculate a mean chain length of about eighty nucleotide residues for the preparation.

The amounts of the alkali-stable dinucleotide sequences in wheat germ s-RNA are shown in Table III, and are based on the large-scale isolation described earlier which was specifically designed to quantitatively analyze for those dinucleotides present in trace quantity. Other analyses performed on a smaller scale in the course of refining the experimental techniques showed no important variations from the values in Table III. The values for those components present in minute amounts were not considered reliable in small-scale experiments and any quantitative variations in the proportions of the major dinucleotides were ascribable to the partial fractionation which can occur during the isolation of s-RNA on a large scale.

The amounts of the different alkali-stable dinucleotide sequences which could be expected for a statistically random distribution of their component nucleosides in ribonucleate chains are also presented in Table III. The calculations are based on the proportions of the major and minor components obtained in an analysis of snake venom phosphodiesterase hydrolysates of wheat germ s-RNA shown in Table IV where the figures have been normalized to 100%. Available evidence suggests that there is an essentially random distribution of the four principal ribonucleosides in those ribonucleate preparations which have been examined without extensive fractionation (Bautz and Heding, 1964; Miura, 1964), and the calculated values for normal dinucleotide sequences involving only the four principal nucleosides are included in Table III for comparative purposes. For purposes of comparison with the data on s-RNA in Table III, the corresponding data for wheat germ 18S + 28S RNA are shown in Table V.

The phosphodiesterase degradation of the individual dinucleoside phosphates from both 18S + 28S RNA and s-RNA gave unambiguous results. The periodate degradation of the bulk dinucleoside phosphates from 18S + 28S RNA gave satisfactory results in the sense that the proportions found for the four bases and four 2′-*O*-methyl nucleoside 3′-phosphates were in substantial agreement with expectations based on dinucleotide proportions, although recoveries were only about 70%. The periodate degradation of the bulk dinucleoside phosphates from s-RNA gave little or no uracil, and, although the reasons for this are not yet clear, a side reaction favored by a large proportion of NxpU compounds (40% of the total in the case of s-RNA) may be implicated.

TABLE IV: Analysis of a Phosphodiesterase Hydrolysate of s-RNA from Wheat Germ.[a]

Component	Quantity (moles/100 moles of total nucleotides)
pA	19.8
pAx	0.005[b]
p1MeA	0.18
pN^{6}MeA	0.73
A	0.039
pG	29.3
pGx	0.47
p1MeG	0.73
p7MeG	0.18
pN^{2}MeG	0.29
pN^2,N^{2}diMeG	0.55
G	0.058
pI	0.090
pC	26.3
pCx	0.40
p5MeC	1.37
C	0.039
pU	15.7
pUx	0.30
p5MeU	0.64
U	0.029
pψU	2.7
pψUx	0.12

[a] There was no oligonucleotide residue in the hydrolysate. The nucleosides do not arise by dephosphorylation and their origin has been explained elsewhere (Lane, 1965). The nucleosides and p1MeA were recovered for chromatographic separation by recovering the material of the hydrolysate which did not adsorb to DEAE-cellulose during routine column fractionations (Lane, 1965). The rate of conversion of p1MeA to pN^{6}MeA at pH 9.2 in phosphodiesterase digestions can fully account for all of the pN^{6}MeA found in the analysis. The recovery of p7MeG is certain to be low since it is also partially destroyed at pH 9.2 (Lawley and Brooks, 1963). Spectral identification of minor components was based on the data of Dunn and co-workers (Littlefield and Dunn, 1958; Smith and Dunn, 1959b). [b] This figure is based on the dinucleotide analysis of alkali hydrolysates because the scale of the phosphodiesterase analysis (50 mg) was too small to provide a reliable estimate for pAx.

Discussion

The first indication that pGp was the principal nucleoside diphosphate in alkali hydrolysates of ribonucleates emerged from studies by Lane and Butler (1959) who isolated and characterized pGp from alkali hydrolysates of yeast ribonucleates but did

TABLE V: Proportions of Normal and Alkali-Stable Dinucleotide Sequences in Wheat Germ 18S + 28S RNA.

Alkali-Stable Dinucleotides	Observed[a] (moles/100 moles of total alkali-stable dinucleotide sequences)	Calculated[b]	Normal Dinucleotides	Calculated (moles/100 moles of total dinucleotide sequences)
AxpAp	8.8	7.5	ApAp	5.4
AxpGp	11.2	9.7	ApGp	7.0
AxpCp	8.1	8.0	ApCp	5.8
AxpUp	7.8	5.9	ApUp	4.2
GxpAp	3.1	5.3	GpAp	7.0
GxpGp	7.8	6.8	GpGp	9.1
GxpCp	5.8	5.6	GpCp	7.5
GxpUp	3.0	4.1	GpUp	5.4
CxpAp	4.8	4.8	CpAp	5.8
CxpGp	0.9	6.3	GpGp	7.5
CxpCp	5.4	5.2	CpCp	6.2
CxpUp	7.0	3.8	CpUp	4.5
UxpAp	8.5	6.5	UpAp	4.2
UxpGp	6.1	8.4	UpGp	5.4
UxpCp	5.8	6.9	UpCp	4.5
UxpUp	5.9	5.1	UpUp	3.3
Totals	100.0	100.0		92.8

[a] The molar amounts of the dinucleotides were estimated as described in the legend of Table III. The alkali-stable dinucleotides account for approximately 3 mole % of the total nucleotide residues in 18S + 28S RNA, and consequently the *O*-methyl nucleotide constituents of these dinucleotides comprise about 1.5 mole % of the total nucleotide residues. The precise absolute quantities of these components in alkali hydrolysates are not quoted in the absence of the requisite information on the hypochromicities of the individual dinucleotides. [b] The calculated quantities were estimated as described in the legend of Table III using the data from phosphodiesterase hydrolysis of 18S + 28S RNA which were reported in an earlier publication (Lane, 1965). The calculated values presume that a statistically random proportion of the pNx compounds recovered by phosphodiesterase hydrolysis participates in alkali-stable dinucleotide sequences, although this is not strictly true on the basis of studies of the alkali-stable trinucleotide sequences which are found in amounts exceeding random proportions (Lane, 1965).

not detect measurable amounts of pAp, pCp, or pUp. This observation was quickly followed by reports that pGp was the only nucleoside diphosphate in alkali hydrolysates of the s-RNA from several sources (Singer and Cantoni, 1960; Herbert and Canellakis, 1960; Zillig *et al.*, 1960), although it was noted by Lane and Allen (1961b) that small amounts of pAp, pCp, and pUp were present in alkali hydrolysates of wheat germ s-RNA. The quantitative data of this report show that pAp, pCp, and pUp constitute about 10% of the nucleoside diphosphates in alkali hydrolysates of wheat germ s-RNA, and this result is consonant with the discovery of a similar quantity of adenine, cytosine, and uracil bases at the 5′-phosphomonoester termini of yeast s-RNA (Bell *et al.*, 1963; Ralph *et al.*, 1963).

The presence of small amounts of pAp, pCp, and pUp in addition to the much larger quantity of pGp in the alkali hydrolysates of s-RNA is not perhaps surprising since there is no known requirement for a guanine base at the 5′-phosphomonoester terminus of s-RNA. On the other hand, the presence of small amounts of guanosine and uridine, in addition to the principal nucleosides cytidine and adenosine, in alkali hydrolysates of s-RNA might seem more conceptually tenuous because of the specific requirement of a -Cp-CpA terminal sequence for the amino acid acceptor function of s-RNA. The appearance of uridine and guanosine after alkali hydrolysis could simply reflect a loss of the -CpCpA terminal sequence of some s-RNA chains; nevertheless, it seems pertinent to recall the observation by Harbers and Heidelberger (1959) that there are enzymes capable of effecting a terminal addition of a -UpUpG sequence to ribonucleate chains. In any event, it is true, in practice, that both 18S + 28S RNA (Lane, 1965) and s-RNA yield four nucleosides and four nucleoside diphosphates after alkali hydrolysis.

Hydrolysis of 18S + 28S RNA from wheat germ by venom phosphodiesterase or by alkali indicates that

about 1.7% of the constituent nucleosides have a 2′-*O*-methyl substituent (Singh and Lane, 1964b; Lane, 1965). Hydrolysis of wheat germ s-RNA by venom phosphodiesterase or by alkali reveals that about 1.3% of the constituent nucleosides bear a 2′-*O*-methyl substituent. The analyses for 2′-*O*-methyl nucleoside 5′-phosphates have shown that the proportions of the *O*-methyl nucleoside constituents bear no obvious relation to the proportions of the normal ribonucleoside constituents in the case of either the 18S + 28S RNA or the s-RNA of wheat germ, and the *O*-methyl nucleosides do not therefore arise by random *O*-methylation of the constituent normal ribonucleosides. The proportions of the *O*-methyl nucleosides in 18S + 28S RNA are distinctly different from those found for the s-RNA of the same source, and this is particularly apparent in the case of Ax, which is the most abundant *O*-methyl nucleoside in 18S + 28S RNA but is barely detectable in s-RNA. Similar conclusions can be drawn from the observations by Hall on the *O*-methyl nucleoside composition of several ribonucleate preparations from different sources (Hall, 1963).

The analyses of the alkali-stable dinucleotide sequences have shown not only that *O*-methylation is nonrandom but that the *O*-methyl nucleoside constituents are nonrandomly distributed with respect to the normal ribonucleoside constituents in both 18S + 28S RNA and s-RNA from wheat germ. All of the sixteen possible alkali-stable dinucleotide sequences involving the four principal ribonucleosides and their *O*-methyl derivatives have been found in 18S + 28S RNA, but three of these sequences (AxpC, AxpU, CxpG) have not been found, and two (AxpA, AxpG) have been found in only trace quantity, in wheat germ s-RNA. No clear pattern has emerged which is common to both 18S + 28S RNA and s-RNA from wheat germ, but it is perhaps noteworthy that the sequences GxpGp and CxpUp contain the largest proportions of Gx and Cx, respectively, in both types of ribonucleate.

Earlier studies have shown that there is an average of about fifteen alkali-stable dinucleotide sequences and one alkali-stable trinucleotide sequence per chain in the 18S + 28S RNA from wheat germ. On the other hand, there is an average of about one alkali-stable dinucleotide sequence per chain and no detectable alkali-stable trinucleotide sequences in wheat germ s-RNA.

The isolation of a compound with the properties expected for 2′-*O*-methylpseudouridine was first reported by Hall (1964) who discovered it in a number of ribonucleate specimens and found that it accounted for approximately 1% of the pseudouridine in s-RNA. The compound identified as *O*-methylpseudouridine in the present study was isolated by phosphodiesterase digestion of ψUxpU and ψUxpC. It exhibited the expected enhancement of mobility with respect to pseudouridine when chromatographed on paper in organic solvents, it had the same ultraviolet spectral properties as pseudouridine at acid, neutral, and alkaline pH values, and it failed to complex with borate in the paper chromatographic system described by Plesner (1955).

Whereas Ax, Gx, Cx, and Ux account for only 0.03, 1.2, 1.3, and 1.7%, respectively, of the parent ribonucleosides, it is notable that ψUx accounts for about 4% of the pseudouridine in wheat germ s-RNA. It seems significant that the quantity of ψUxpUp in wheat germ s-RNA greatly exceeds random expectations (Table III) and this points, once again, to a possible relation between pseudouridine and *O*-methylation as mentioned in an earlier report (Lane, 1965).

An unidentified component which accounts for 0.15 mole % of the constituent nucleotides of wheat germ s-RNA has been found in the alkali-stable dinucleotide fraction of all preparations examined. It has spectral properties similar to thymidine and appears to contain 2 moles of monoester phosphate. The possibility that it might be related to 1,5-diribosyluracil diphosphate is now under investigation.

The studies of alkali-stable dinucleotide sequences in heterogeneous ribonucleate preparations have pointed to a considerable degree of nonrandomness in the degree of *O*-methylation of ribonucleosides and in the distribution of *O*-methyl nucleosides in ribonucleate chains. Further studies will be directed toward the biogenesis of *O*-methyl groups and the examination of alkali-stable dinucleotide sequences in homogeneous preparations of s-RNA.

References

Bautz, E. K. F., and Heding, L. (1964), *Biochemistry 3*, 1010.

Bell, D., Tomlinson, R. V., and Tener, G. M. (1963), *Biochem. Biophys. Res. Commun. 10*, 304.

Broom, A. D., and Robins, R. K. (1965), *J. Am. Chem. Soc. 87*, 1145.

Glitz, D. G., and Dekker, C. A. (1963), *Biochemistry 2*, 1185.

Hall, R. H. (1963), *Biochim. Biophys. Acta 68*, 278.

Hall, R. H. (1964), *Biochemistry 3*, 876.

Harbers, E., and Heidelberger, C. (1959), *Biochim. Biophys. Acta 35*, 381.

Herbert, E., and Canellakis, E. S. (1960), *Biochim. Biophys. Acta 42*, 363.

Honjo, M., Kanai, Y., Furukawa, Y., Mizuno, Y., and Sanno, Y. (1964), *Biochim. Biophys. Acta 87*, 698.

Lane, B. G. (1963), *Biochim. Biophys. Acta 72*, 110.

Lane, B. G. (1965), *Biochemistry 4*, *212*.

Lane, B. G., and Allen, F. W. (1961a), *Can. J. Biochem. Physiol. 39*, 721.

Lane, B. G., and Allen, F. W. (1961b), *Biochim. Biophys. Acta 47*, 36.

Lane, B. G., and Butler, G. C. (1959), *Can. J. Biochem. Physiol. 37*, 1329.

Lawley, P. D., and Brooks, P. (1963), *Biochem. J. 89*, 127.

Littlefield, J. W., and Dunn, D. B. (1958), *Biochem. J. 70*, 642.

Miura, K. (1964), *J. Biochem. (Tokyo) 55*, 452.
Neu, H. C., and Heppel, L. A. (1964), *J. Biol. Chem. 239*, 2927.
Plesner, P. (1955), *Acta Chem. Scand. 9*, 197.
Ralph, R. K., Young, R. J., and Khorana, H. G. (1963), *J. Am. Chem. Soc. 84*, 1490.
Singer, M. F., and Cantoni, G. L. (1960), *Biochim. Biophys. Acta 39*, 182.
Singh, H., and Lane, B. G. (1964a), *Can. J. Biochem. 42*, 87.
Singh, H., and Lane, B. G. (1964b), *Can. J. Biochem. 42*, 1011.
Smith, J. D., and Dunn, D. B. (1959a), *Biochim. Biophys. Acta 31*, 573.
Smith, J. D., and Dunn, D. B. (1959b), *Biochem. J. 72*, 294.
Zillig, W., Schachtschabel, D., and Krone, W. (1960), *Z. Physiol. Chem. 318*, 100.

Biochemical Correlates of Respiratory Deficiency. IV. Composition and Properties of Respiratory Particles from Mutant Yeasts*

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ABSTRACT: Respiratory deficient mutant yeasts (strains p_8, p_{13}, p_{12}, and a cytoplasmic mutant) have been studied in comparison with wild-type yeast. The mutant yeasts differ from the wild type in that they contain at most only traces of cytochromes *a* and a_3 and relatively low amounts of cytochromes *b*, *c*, and c_1 (with the exception of mutant p_{12} which contains normal amounts of cytochromes *c*, c_1, and *b*). Respiratory particles prepared from the mutant yeasts contain no cytochrome *a* or a_3 and very low amounts of cytochromes *b*, *c*, and c_1 (again with the exception of the electron-transport particles from mutant p_{12} which contain appreciable amounts of cytochromes *c* and c_1 but no cytochrome *b*). In addition, the respiratory particles from the mutant yeasts contain highly elevated levels of non-heme iron normal concentrations of copper, and low values of flavin and coenzyme Q as compared to the electron-transport particles prepared from wild-type yeast. Respiratory particles prepared from wild-type yeast harvested in the logarithmic phase of growth resemble the mutant respiratory particles in that the content of non-heme iron is high and levels of flavin and coenzyme Q are low compared to levels found in ETP from wild-type yeast in the stationary phase. The ETP's from the mutant yeasts show only traces of activity as DPNH and succinic oxidases and cytochrome *c* reductases, but are able to function as indophenol reductases with DPNH and succinate as substrates.

Previously, we have reported the isolation of electron-transport particles[1] (ETP) from wild-type *Saccharomyces cerevisiae* and from a cytoplasmic mutant strain (Mahler *et al.*, 1964a) and established that, although the latter is respiratory deficient (cannot grow aerobically on nonfermentable substrates), the primary dehydrogenases for D-lactate, L-lactate, succinate, and diphosphopyridine nucleotide (DPNH) are all present (Mahler *et al.*, 1964a). Furthermore, the ETP prepared from the cytoplasmic mutant, although lacking oxidase activity for these substrates, binds or reacts with an antiserum prepared against ETP from wild-type or respiratory-competent cells (Mahler *et al.*, 1964b), suggesting that at least some of the protein components of the two ETP's are similar. In the present communication, the composition and enzymatic properties of ETP prepared from several respiratory-deficient mutant strains and from wild-type *S. cerevisiae* will be reported. The mutants examined included both cytoplasmic (Ps) and segregational (or genic) ($P \times s^+/s^-$) types (Sherman, 1963; Sherman and Slonimski, 1964). The mutant strains were grown in media containing several different carbohydrates, *i,e.*, sucrose and galactose, to rule out possible differences due to glucose repression (Ephrussi *et al.*, 1956; Slonimski, 1956; Strittmatter, 1957; Polakis *et al.*, 1964; Tustanoff and Bartley, 1964a,b; Schatz, 1963).

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[1] Abbreviations used in this work: ETP, electron-transport particle; DPNH, diphosphopyridine nucleotide, reduced form.