Preparation, Purification and Analyses of Thirteen Alkali-Stable Dinucleotides from Yeast Ribonucleic Acid

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Of the 16 alkali-stable dinucleotides known to be obtained by hydrolysis of commercial yeast RNA with alkali, 13 were prepared in quantities of the order of 10 mg or more. The samples, with only one exception, contain at least 90% of dinucleotide, and spectroscopic constants and nucleotide-sequence determinations, although not conclusive, indicate a high degree of purity of these products. The small dinucleotide fraction in 150 g of RNA hydrolysed with alkali (1-2% of the total nucleotides) was separated from the mononucleotides by stepwise ion-exchange chromatography on DEAE-cellulose columns and resolved into seven fractions containing from one to four different dinucleotides by electrophoresis on paper at pH 3.0. These fractions were resolved into their constituent dinucleotides by chromatography in ammonium sulphate. Contamination of the products by impurities from the paper was minimized by washing it before using it for chromatography or electrophoresis and, by using a thick grade of paper (Whatman no. 17), it was possible to handle and purify relatively large quantities of nucleotides.

Smith & Dunn (1959) discovered a number of alkali-stable dinucleotides in alkali-hydrolysed RNA obtained from higher plants and animals and described them tentatively as dinucleotides containing a 5'-linked ribonucleotide and a 3'(or 2')-linked 2'(or 3')-*O*-methylribonucleotide. This chemical constitution has not been finally established. However, it is reasonable to assume that the compounds have the general structure P1xP2p, where P1 and P2 are purine or pyrimidine nucleosides and x indicates that P1 is a β -D-ribofuranoside modified by 2'(or 3')-*O*-methylation. All relevant information given in the present paper and elsewhere supports this view. For known compounds the symbols A, G, C or U replace P1 and P2 as appropriate.

Subsequent work has confirmed the existence of such stable dinucleotides and their degradation products in both alkali and enzyme hydrolysates of tRNA and rRNA from a number of sources. Chemical analyses support the suggested structure of these compounds and evidence, which will be discussed later, has accumulated to show that they occur in definite positions in the polynucleotide chain. The present work was initiated on the assumption that it is unlikely that any component of RNA is fortuitous and that consequently the components in question have a definite and necessary role in the structure and function of the RNA from which they come. It then followed that these

compounds should be prepared pure in the classical chemical sense so that their physical, chemical and enzymological properties could be studied rigorously. In the present paper the preparation and preliminary analysis of 13 such compounds from commercial yeast RNA is described. They were obtained in quantities varying from about 10 mg to about 50 mg.

METHODS

Hydrolysis. Commercial yeast RNA was hydrolysed in 50 g batches by heating at 55°C for 48 h in 2 l of 1.1M-diethylamine. The hydrolysate was centrifuged to remove solid matter and evaporated *in vacuo* at 50°C to about 100 ml, then made to 500 ml and ethanol (3 vol.) was added. Flocculation of the gelatinous precipitate, consisting mainly of guanylic acid, was accelerated by adding a little saturated NaCl solution. After standing overnight at 4°C the precipitate was removed by centrifuging and washed twice with a little cold ethanol-water (3:1, v/v). The chief purpose of the ethanol precipitation was to remove DNA and certain guanine derivatives that were found to interfere in the separation of individual dinucleotides at a later stage. However, with the RNA samples used, precipitation of the guanylic acid component of the hydrolysate occurred too and was nearly quantitative. This conferred an added advantage by rendering the hydrolysate easier to handle in ion-exchange chromatography. Ethanol was removed from the supernatant by distillation *in vacuo* at 40°C.

Ion-exchange chromatography. The mononucleotides

remaining in the mixture were separated from the dinucleotides by stepwise elution from DEAE as described by Singh & Lane (1964a). DEAE-cellulose washed with 0.5M-KOH followed by water and equilibrated with 0.02M-ammonium formate, pH 5, was packed in a column (3 cm × 20 cm) by applying five stepwise increases of pressure up to 0.34 atm. A 5–10 g sample of the nucleotide mixture was passed through the column in 1.5–2.5 l of water at pH 5 at a flow rate of about 1.5 ml/min. Adsorbed mononucleotides were eluted by washing with 5–6 l of 0.02M-ammonium formate, pH 5, at about the same flow rate. Formate was removed from the column by washing with 2–3 l of 0.01M-NH₄HCO₃, pH 8.6, at a flow rate of 5 ml/min and the dinucleotide fraction was then eluted by washing with M-NH₄HCO₃, pH 8.6, at a flow rate of 2–3 ml/min. NH₄HCO₃ was removed by repeated evaporation *in vacuo* at 45°C.

To ensure complete breakdown of alkali-labile oligonucleotide material, the dinucleotide fraction obtained from a 50 g batch of RNA was hydrolysed again in 50 ml of 1M-diethylamine for 48 h at 55°C and, after removal of diethylamine by evaporation, the ion-exchange separation was repeated.

Electrophoresis on paper. (a) The apparatus of Markham & Smith (1952a) was used for small-scale electrophoresis; (b) larger-scale electrophoretic separations were done on paper laid on a thin glass sheet cooled on the underside by a precision-ground plate of dural through which tap water circulated. The glass sheet was stuck to the plate with silicone fluid [MS 200/350 cs. (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) has suitable properties]. The electrophoresis paper was covered above by a sheet of thin glass lifted clear of it by paper wicks, which connected it to the electrode vessels. This allowed for some top cooling of the paper by evaporation into the small space thus provided. The plate measured 30 cm × 63 cm and permitted a run of about 50 cm on strips of paper up to about 25 cm in width. Whatman papers of grades nos. 1, 2, 3MM or 3MC and 17 were used, according to the quantities of material to be handled. The thinner papers were wetted by dipping in the buffer and the surplus liquid was removed by blotting with filter paper when they were in position on the plate. Thick papers, usually Whatman no. 17, were positioned dry on the plate and sprayed evenly with buffer from an atomizer. Excess of buffer was removed by blotting with filter paper, using a rubber roller to obtain even pressure. Wicks connecting the electrode vessels to the flat paper were usually made of strips of filter paper. Some control of water flow into the paper was obtained by varying the thickness of the wicks, by covering them with strips of dialysis tubing (Visking Viscose), or both. By using appropriate buffers voltage gradients up to about 80 V/cm could be applied. From 1 µg to 25 mg of a single nucleotide component in a mixture could be separated and detected in a single separation with this equipment. For example, in a mixture of 1 µg of each Gp, Ap, Cp and Up (all samples of mononucleotides used were mixtures of the 2'- and 3'-esters) were completely separated in 30 min on Whatman no. 3MM paper in 0.2M-ammonium formate buffer, pH 3, in a voltage gradient of 70 V/cm. On Whatman no. 17 paper the same nucleotides were completely separated from a mixture containing about 6 mg of each in 1–1½ h at 80 V/cm on a strip 25 cm wide.

Chromatography on paper. The following solvents were

used for chromatography on paper: A1, propan-2-ol-water (6:3, v/v) and A2, propan-2-ol-water (7:3, v/v) both with NH₃ in the vapour phase (Markham & Smith, 1952b); B, saturated (NH₄)₂SO₄-0.5M-sodium acetate-propan-2-ol (75:19:2, by vol.) (Markham & Smith, 1951) and C, propan-1-ol-aq.NH₃ (sp.gr. 0.88)-water (50:10:35, by vol.) (Neu & Heppel, 1964). Large quantities of nucleotides could be handled on Whatman no. 17 paper. Thus in solvent A2 single components containing 10 mg of nucleotide material could be separated from much larger quantities of other nucleotides in a single overnight run on a sheet of paper 27 cm wide. In solvent B and on the same width of paper, 15 mg of the very slow-moving adenylic acid was completely resolved into its 2'- and 3'-isomers in a 40 h run in ascending chromatography.

Paper washing. Compounds separated by repeated runs on paper were intended to be isolated and purified. Consequently it was desirable to remove as much soluble matter as possible from the paper before it was used. This was achieved by washing with 0.2M-formic acid or 0.5M-NH₃ for 24 h by descending chromatography followed by washing with water until the water running from the paper was neutral. This took 1–2 days. Under laboratory conditions chromatography paper continuously breaks down to yield water-soluble products. Strips (2 cm × 27 cm) of Whatman no. 17 paper were eluted 9 months after washing by irrigation with 5 ml of water. The eluates contained 5–10 µg of water-soluble solid/cm² of paper. The elution was by no means exhaustive. Paper eluted immediately after washing and drying yielded about one-tenth the quantity of solid that could be removed from paper that had been kept for some time after washing.

Once dried, freshly washed chromatography paper releases water-soluble material that gives a positive reaction with Bial's orcinol reagent for pentose. Unwashed Whatman no. 3MM paper gave the equivalent of 1.2–1.6 µg of pentose/cm² when eluted with water (0.2–0.5 ml/cm² of paper), one-quarter to one-third of this being present in the first 0.01 ml of the eluate. Papers washed with water, dil. formic acid followed by water or dil. NH₃ followed by water and chromatographic solvents, so as to yield no pentose-reacting material on elution with water before drying, again yielded quantities of pentose-like material of the same order as did unwashed paper immediately after drying. This tended to increase slowly if the paper was stored at room temperature. When treated with the pentose reagent (Militzer, 1946) the material gave a solution with the same absorption spectrum as that obtained from ribose. However, unlike ribose and other sugars, it was adsorbed by charcoal under the same conditions as nucleotides and, also like nucleotides, could be recovered from the charcoal by elution with ammoniacal ethanol. With the anion-exchange gel Biogel TE-2, which contains no cellulose or cellulose derivatives, designed specifically for the separation of nucleotides, the ion-exchange behaviour of the pentose-like material was compared with that of nucleotides. The material was adsorbed on an equilibrated column of Biogel TE-2 from 0.01M-NH₄HCO₃, pH 8.6, and eluted by higher concentrations of the same buffer, as in the method of Rushizky & Sober (1962), in which DEAE-cellulose is used. Some of the material was eluted by the range of buffer concentrations required to elute adenylic acid (about 0.1M) but more than

half of it required 1M buffer or even higher concentrations. It is clear that when, as is often the case, nucleotides are isolated by a number of steps involving the use of paper chromatography and other commonly used techniques mentioned here, the unknown material, which is generated by chromatography paper and has some of the properties of uronic acids but is not glucuronic acid, may be accumulated in the preparation.

In view of the properties described above the removal of this material presents some difficulty. However, it was found that it could be separated from mono- and dinucleotides by electrophoresis on glass paper (Whatman GF81) at pH 8.6 in NH_4HCO_3 buffer. It moved nearly twice as fast and in the same direction as mono- and dinucleotides under the combined but opposed effects of electrophoresis and endosmosis.

Electrophoresis on glass-fibre paper. Glass-fibre paper has been used as a supporting medium for the electrophoresis of borate complexes of mono- and oligosaccharides (Bourne, Foster & Grant, 1956; Fuller & Northcote, 1956). This supporting medium is also suitable for the separation of mononucleotides by electrophoresis at pH 3.5. Thus, under these conditions, with 0.2M-ammonium formate buffer, Ap, Gp, Cp and Up were separated from a mixture in 15 min with a voltage gradient of 60 V/cm. Electroendosmosis towards the cathode was so great that the nucleotides moved in the reverse direction to that of their normal electrophoretic migration. The current was high so that runs had to be of short duration and the rate of flow of cooling water in the plate had to be higher than is usually needed for electrophoresis on paper.

Washing of glass paper. Glass paper was washed by irrigation in a chromatography tank for 24 h with water and then overnight with aq. 90% ethanol. Eluates from the washed paper contained a small amount of solid, some of which could be removed by filtration through a MF-type Millipore filter, pore size 0.22 μm . The filtrate contained no material with specific absorption in the u.v. region and no material containing phosphorus or pentose.

Desalting. Large amounts of $(\text{NH}_4)_2\text{SO}_4$ in eluates from chromatograms obtained with solvent B were precipitated by the addition of 2 vol. of ethanol, standing for 6 h at -20°C , filtration on a sintered-glass filter and washing the crystals with aq. 66% ethanol at 0°C until free from nucleotide. The filtrate was evaporated *in vacuo* to a small volume and the ethanol precipitation repeated. The remaining small quantity of $(\text{NH}_4)_2\text{SO}_4$ was removed by the method of Rushizky & Sober (1962). Small amounts of salt could often be removed by electrophoresis in 0.1M- NH_4HCO_3 buffer, pH 8.6.

Total phosphorus. Samples were incinerated by the method of Bartlett (1959), i.e. samples (10–20 μl) were heated in a small tube at 150 – 160°C with 0.25 ml of 5M- H_2SO_4 for 3 h, then 0.025 ml of aq. 30% (v/v) H_2O_2 was added and the heating continued for a further 1½ h. The reagent of Chen, Torribara & Warner (1956) (2.5 ml) was added and the blue colour read at 820 nm after 1½ h at 37°C .

Pentose. This was determined by the method of Militzer (1946). For pyrimidine nucleotides the method of Trim, Baker & Leah (1964) was used.

Drying. Samples of ammonium salts of dinucleotides were freeze-dried and then dried to constant weight at room temperature in high vacuum (<0.0005 mmHg).

Ultraviolet-absorption spectra. These were measured on the Cary model 14 recording spectrophotometer.

Nucleotide sequences. These were determined by the method of Neu & Heppel (1964) by using cyclohexylamine to cleave the periodate-oxidized dinucleoside monophosphate, which was obtained by dephosphorylation of the dinucleotide with *Escherichia coli* phosphomonoesterase at pH 8.6. The procedure of Neu & Heppel (1964) was not followed beyond this stage but was replaced by the following steps. The mixture was submitted to electrophoresis by method (b) at pH 3.0 in 0.1M-ammonium formate buffer on Whatman no. 3MM paper at 60 V/cm for 45 min. This removed some u.v.-absorbing material not derived from nucleotides and left the base and nucleotide near the starting line. These compounds were eluted with water from the dried paper and run again in the same electrophoresis apparatus at pH 8.6 in 0.05M- NH_4HCO_3 buffer at about 60 V/cm for 30 min. The compounds were eluted with water and determined spectrophotometrically. In some cases the separation of base and nucleotide was improved by repeating the electrophoresis. The two compounds were then run with appropriate base and nucleotide markers in electrophoresis at pH 3 in 0.01M-ammonium formate buffer for 45 min at 60 V/cm. Bases and nucleotides were further identified by chromatography, electrophoresis and measurement of absorption spectra at pH 7. The nucleotides were then prepared for analysis by electrophoresis at pH 8.6 in 0.05M- NH_4HCO_3 buffer on Whatman 3MM paper at 60 V/cm for 30 min, eluted with water, concentrated to a small volume and submitted to electrophoresis at pH 8.6 in 0.05M- NH_4HCO_3 buffer at 60 V/cm on Whatman GF81 glass-fibre paper for 15 min. The bands were eluted with water and filtered through a Millipore filter (type MF, pore size 0.22 μm). U.v.-absorption spectra of the solutions were measured at pH 2, 7 and 12, and samples taken for the determination of total phosphorus and pentose.

RESULTS AND DISCUSSION

The dinucleotide fraction from 150 g of commercial yeast RNA was obtained by column chromatography. About 20% of this fraction migrated faster than UpUp on electrophoresis at pH 8.6. Samples (20–25 mg) of the dinucleotide fraction were run for 3 h with a voltage gradient of 20–25 V/cm on a sheet (22 cm $\times$ 50 cm) of Whatman no. 17 paper. The dinucleotides travelled close together in a broad slow band preceded by three bands containing most of the contaminating material. More contaminants were removed by chromatography of the eluate in solvent A2 on sheets of washed Whatman no. 17 paper. The nucleotides were eluted with water and evaporated to a small volume containing about 50 mg of nucleotide/ml.

On electrophoresis at pH 3, the 16 possible dinucleotides, each containing pairs of the four major ribonucleotides (Ap, Gp, Cp and Up) should separate into ten bands as shown in Table 1. The corresponding alkali-stable dinucleotide would behave similarly. In practice this resolution is not

Table 1. *Electrophoretic mobilities of mono- and di-nucleotides at pH 3.0 and mobility in chromatography solvent B*

Electrophoretic mobilities of mono- and di-nucleotides were calculated from the dissociation constants of monoribonucleotides (Smith, 1955) and 2'-*O*-methylation was assumed to have a negligible effect. Mobilities were measured by running in 0.1M-ammonium formate buffer, pH 3, for 70 min with a voltage gradient of about 80 V/cm (electrophoresis method *b*) with authentic samples of the mononucleotides and samples of the alkali-stable dinucleotides purified during the present work. Each dinucleotide was applied as a small spot, containing about 3 μ g of material and flanked on each side by a marker containing about 3 μ g of each mononucleotide at a distance of less than 1cm, to Whatman 3MM paper. Mobilities are expressed relative to that of Up, taken as 1.0. Chromatography in solvent B was ascending and R_F values are relative to that of Up.

Compound	Band		Relative electrophoretic mobility at pH 3.0		Relative R_F in solvent B
	From calculations	Found by experiment	Calculated	Measured	
Cp			0.04	0.17	
Ap			0.17	0.26	
Gp			0.83	0.72	
Up			1.00	1.00	
CxpCp	1	1'	0.06	0.23	0.79
AxpCp	2		0.14	0.30	0.27
CxpAp			0.14	0.29	0.39
AxpAp	3	2'	0.23	0.37	0.071
					0.081
CxpGp	4		0.61	0.65	0.64
GxpCp		3'	0.61	0.62	0.50
AxpGp	5		0.70	0.64	0.20
GxpAp			0.70	0.64	0.25
CxpUp	6	4'	0.73	0.80	0.75
UxpCp			0.73	0.80	0.75
AxpUp	7		0.82	0.74	0.24
UxpAp		5'	0.82	0.77	0.38
GxpGp	8		1.17	—	—
GxpUp	9		1.28	1.08	0.50
UxpGp		6'	1.28	1.13	0.68
UxpUp	10		1.40	1.30	0.75

achieved and, although the order of movement of nucleotides is not changed, the relative mobilities differ more or less from the calculated values. About 2.5mg of dinucleotide mixture was loaded on to a strip of Whatman 3MM paper (10cm $\times$ 58cm) about 12cm from one end and run by electrophoresis method (*a*) for 3h with a voltage gradient of about 25V/cm. This separated the dinucleotides into seven bands (Table 1; 1'–7') and a battery of ten electrophoresis units permitted the processing of 75mg of dinucleotide mixture per day. This rate of operation could be easily doubled by using Whatman no. 17 paper and one electrophoresis plate (method *b*), for the capacity per paper would be increased more than tenfold and the running time would be decreased to 1h.

Bands 1'–7' were eluted separately with water, pooled, concentrated and desalted by the method of Rushizky & Sober (1962). The values in Table 1 show that once resolved into groups according to their electrophoretic mobilities at pH 3, the individual dinucleotides may be resolved by chroma-

tography in ammonium sulphate (solvent B). Accordingly the desalted dinucleotide bands 1'–7' were run on freshly washed Whatman no. 17 paper in solvent B by descending chromatography. Runs were usually of short duration in this comparatively fast-moving solvent (8–18h). However, AxpAp, which would be expected to move very slowly, was run for 72h during which time it was resolved into two components identical in other respects. The two bands were close together and were presumably the 2'- and 3'-ribose phosphate isomers of AxpAp, which would be expected to result from the alkali hydrolysis of rRNA. Each band was desalted and rerun twice in solvent B to resolve the two components completely.

Electrophoresis at pH 3 in 0.5M-acetic acid separated small amounts of nucleotide contaminants from the samples. This was followed by electrophoresis at pH 8.6 in 0.05M-ammonium hydrogen carbonate, which separated further small amounts of nucleotide contaminants.

Desalted dinucleotide preparations were dried

at 50°C and dissolved in a small volume of water to give a concentration of 30–120mg/ml. Ethanol (2vol.) was added and the solution cooled to –10°C for 1h. Any precipitate remaining when the solution was allowed to warm up to room temperature was removed by centrifuging and washed three times at –10°C with a small volume of ethanol–water (2:1, v/v). This was repeated until there was no further precipitate on addition of ethanol. With some of the dinucleotides the ethanol was evaporated at 50°C and the dinucleotide was dissolved in the original volume of water. Acetone (2–4vol.) was added and the solution brought to –10°C. Any precipitate remaining when the solution was brought back to room temperature was removed by centrifuging. To solutions in either ethanol–water or acetone–water, 90% formic acid was added in the proportion of 0.1ml of acid/ml of the initial aqueous solution at –10°C. This amount of acid brought the pH of the water to 1.8. After standing at –10°C for 1h the precipitated dinucleotide was removed by centrifuging at –20°C and washed three times with the appropriate organic solvent–water mixture without added formic acid. Ammonia was added in small excess to dissolve the precipitate and any insoluble material was removed by centrifuging.

Both ethanol and acetone removed some non-

nucleotide material from the preparation of UxpUp dissolved in water but the dinucleotide remained soluble in the acidified mixture and this method was not used for its preparation. The purity of this sample was increased by electrophoresis on washed Whatman GF81 glass-fibre paper in 0.05M-ammonium hydrogen carbonate buffer, pH 8.6. Strips (20cm wide) were loaded with about 2mg of dinucleotide and a potential of 70V/cm was applied for 15min. The dinucleotide was readily eluted quantitatively from the paper with water. Colloidal material from the paper, probably silica, coagulated on concentration at 45°C and was removed by filtration through the Millipore filter (type MF, pore size 0.22μm).

The solutions of ammonium salts of nucleotides were usually filtered through a sintered-glass filter and, after concentration to a small volume at 45°C, freeze-dried and finally dried to constant weight at room temperature *in vacuo* at a pressure of less than 0.0005mmHg. The solids were dissolved to known concentrations between 2.5 and 3.0mg/ml in water and stored at –20°C. The alkali-stability of all samples was confirmed by treating samples (100μg) with 20μl of m-potassium hydroxide for 96h at room temperature. The potassium hydroxide was removed by electrophoresis in 0.05M-ammonium hydrogen carbonate buffer, pH 8.6, for

Table 2. *Precipitation and analysis of dinucleotides*

All methods are described in the text.

Compound	Precipitation			Dry weight (mg)	Phosphorus		Pentose (mol/2mol of phosphate)
	Concn. in aqueous soln. (mg/ml)	Precipitant	Precipitant/water ratio (v/v)		%	as % of calculated value	
CxpCp	5	Ethanol	2:1	16.9	8.5	96	0.0
AxpCp	7	Ethanol	2:1	35.0	8.3	96	0.37
CxpAp	9	Ethanol	2:1	10.2	8.4	97	1.20
AxpAp1	3	Ethanol	2:1	7.8	7.9	94	1.32
AxpAp2	4	Ethanol	2:1	9.0	7.8	93	1.40
GxpGp							0.61
CxpGp	5	Ethanol	2:1	7.1	7.8	92	1.30
GxpAp	10	Acetone	2:1	18.4	7.9	97	1.26
AxpGp	12	Acetone	2:1	35.7	8.1	99	1.20
CxpUp) UxpCp)	10	Acetone	4:1	20.2	8.6	96	0.09
AxpUp	15	Acetone	4:1	44.4	8.6	99	0.42
UxpAp	9	Ethanol	2:1	10.2	8.0	93	1.22
GxpUp	10	Acetone	3:1	20.7	8.0	95	0.43
UxpGp	8	Acetone	3:1	24.0	8.1	96	1.20
UxpUp				9.6	7.6	85	1.36
A	5	Acetone	4:1	9.4	8.1		0.48
B	12	Acetone	4:1	7.2	8.1		0.50
C	12	Acetone	4:1	9.0	7.6		1.08
D	5	Acetone	2:1	7.6	8.2		0.96

Table 3. *Ultraviolet absorption of alkali-stable dinucleotides*

About 20 μ l of suitably diluted dinucleotide solution was added (1) to about 2 ml of 0.01M-HCl, (2) to about 2 ml of Sørensen's phosphate buffer, pH 7.0, or (3) to about 2 ml of 0.01M-NaOH. The absolute dilutions and quantities of dinucleotide solution and reagents were arranged to give 37.5 μ M solutions of dinucleotide at (1) pH 2.0, (2) pH 7.0 and (3) pH 12.0. Equivalent solutions containing equimolar quantities of the two corresponding mononucleotides were prepared in the same way and used for comparison. U.v.-absorption spectra were measured between 220 and 310 nm on all solutions. ϵ is extinction per mole of nucleotide residue, calculated per 31g of P/l.

Com- pound	pH	$\lambda_{\min}$ (nm)	$\lambda_{\max}$ (nm)	$10^{-3} \epsilon$			Extinction ratios							
				at 220 nm	at $\lambda_{\min}$	at $\lambda_{\max}$	E_{230}/E_{260}	E_{240}/E_{260}	E_{250}/E_{260}	E_{270}/E_{260}	E_{280}/E_{260}	E_{290}/E_{260}	E_{300}/E_{260}	
Cp	2	239	278	8.10	1.80	12.7	0.50	0.26	0.48	1.62	1.83	1.25	0.29	
	7	251	270	8.60	6.67	8.90	1.04	0.97	0.88	1.17	0.89	0.26	0.01	
CxpCp	12	251	270	9.10	6.67	8.85	1.06	0.97	0.87	1.16	0.87	0.25	0.01	
	2	239	278	8.50	1.90	13.2	0.56	0.28	0.47	1.67	1.96	1.39	0.35	
Cp + Ap	7	249	268	8.47	6.25	8.47	1.05	0.95	0.85	1.17	0.87	0.32	0.04	
	12	249	268	8.47	6.28	8.44	1.04	0.94	0.86	1.15	0.88	0.34	0.04	
CxpAp	2	233	266	6.60	2.87	9.70	0.32	0.38	0.73	0.99	0.76	0.45	0.11	
	7	226	261	6.35	4.92	11.1	0.46	0.59	0.81	0.83	0.39	0.09	0.01	
AxpCp	12	228	261	6.95	6.10	10.1	0.51	0.61	0.83	0.83	0.40	0.10	0.02	
	2	234	264	7.86	3.32	10.6	0.33	0.39	0.73	0.98	0.76	0.48	0.13	
Ap	7	227	261	7.13	5.09	10.5	0.50	0.62	0.83	0.81	0.42	0.13	0.03	
	12	228	262	7.13	5.09	10.5	0.49	0.60	0.81	0.83	0.43	0.13	0.02	
AxpAp	2	234	264	8.15	3.34	10.8	0.34	0.40	0.74	0.97	0.75	0.45	0.13	
	7	227	261	6.66	4.99	10.9	0.47	0.59	0.81	0.83	0.42	0.12	0.02	
Cp + Gp	12	226	261	6.70	5.16	11.0	0.48	0.59	0.83	0.81	0.41	0.12	0.02	
	2	228	257	7.10	3.20	14.5	0.24	0.48	0.89	0.68	0.20	0.03	0.00	
AxpGp	7	226	258	5.10	2.45	15.0	0.19	0.43	0.81	0.62	0.13	0.00	0.00	
	12	227	260	5.70	2.65	15.0	0.19	0.39	0.77	0.66	0.14	0.00	0.00	
CxpGp	2	229	257	7.75	3.50	14.8	0.24	0.44	0.84	0.70	0.23	0.05	0.01	
	7	225	256	5.30	2.60	13.8	0.22	0.46	0.88	0.63	0.17	0.02	0.01	
AxpAp	12	227	257	5.40	2.70	14.0	0.21	0.42	0.84	0.68	0.19	0.03	0.02	
	2	228	257	7.80	3.50	14.9	0.25	0.47	0.87	0.68	0.23	0.05	0.014	
Cp + Gp	7	227	257	5.80	2.68	14.0	0.21	0.43	0.83	0.66	0.20	0.03	0.015	
	12	227	257	5.80	2.86	14.0	0.22	0.44	0.83	0.68	0.21	0.03	0.018	
CxpGp	2	231	276	5.47	3.06	9.6	0.37	0.50	0.80	1.10	1.13	0.77	0.22	
	7	224	256	5.90	5.42	10.0	0.61	0.83	1.02	0.95	0.77	0.27	0.02	
GxpCp	12	231	268	9.77	5.90	9.1	0.68	0.74	0.89	1.05	0.73	0.17	0.02	
	2	232	276	6.38	3.57	10.5	0.38	0.48	0.79	1.06	1.08	0.73	0.23	
Ap + Gp	7	224	254	6.11	5.55	10.1	0.65	0.87	1.04	0.92	0.74	0.33	0.05	
	12	230	268	10.71	6.19	10.0	0.65	0.71	0.87	1.03	0.73	0.22	0.04	
AxpGp	2	230	274	6.35	3.58	10.0	0.39	0.53	0.83	1.07	1.04	0.67	0.21	
	7	225	255	5.84	5.30	9.7	0.60	0.82	1.00	0.93	0.69	0.28	0.05	
GxpAp	12	230	268	10.56	6.18	9.9	0.65	0.74	0.89	1.02	0.71	0.20	0.03	
	2	228	256	5.10	2.85	12.3	0.25	0.55	0.94	0.72	0.42	0.22	0.06	
Ap + Up	7	224	255	4.10	2.83	13.9	0.28	0.62	0.96	0.71	0.38	0.13	0.02	
	12	229	259	8.20	3.62	12.1	0.30	0.49	0.84	0.78	0.35	0.06	0.02	
AxpUp	2	227	256	6.20	3.24	13.1	0.27	0.54	0.93	0.71	0.42	0.23	0.07	
	7	225	256	4.40	2.87	13.2	0.26	0.54	0.93	0.70	0.38	0.14	0.03	
GxpUp	12	228	258	8.50	3.57	12.9	0.28	0.50	0.86	0.76	0.36	0.06	0.01	
	2	227	256	6.16	3.24	13.1	0.27	0.54	0.92	0.70	0.42	0.22	0.07	
Ap + Up	7	225	256	4.71	2.97	13.1	0.27	0.57	0.93	0.72	0.40	0.16	0.03	
	12	228	259	8.76	3.73	12.9	0.29	0.50	0.85	0.80	0.38	0.06	0.01	
AxpUp	2	229	258	5.70	2.97	11.3	0.27	0.49	0.84	0.74	0.27	0.09	0.02	
	7	228	260	4.63	2.60	12.6	0.21	0.44	0.77	0.72	0.22	0.02	0.01	
UxpAp	12	232	260	7.80	5.33	10.3	0.52	0.54	0.81	0.71	0.18	0.03	0.02	
	2	229	258	6.17	2.81	11.4	0.25	0.44	0.82	0.72	0.27	0.05	0.01	
UxpAp	7	228	260	4.94	2.41	11.6	0.21	0.41	0.79	0.72	0.26	0.03	0.01	
	12	230	260	6.87	4.88	10.9	0.42	0.55	0.83	0.69	0.18	0.03	0.01	
UxpAp	2	229	257	6.12	2.82	11.7	0.26	0.48	0.86	0.72	0.27	0.05	0.01	
	7	228	258	5.23	2.52	11.7	0.22	0.46	0.83	0.71	0.25	0.03	0.01	
UxpAp	12	230	258	7.48	5.08	10.8	0.47	0.66	0.84	0.69	0.21	0.04	0.02	

Table 3 (continued).

Com- pound	pH	$\lambda_{\min.}$ (nm)	$\lambda_{\max.}$ (nm)	$10^{-3} \epsilon$	$10^{-3} \epsilon$	$10^{-3} \epsilon$	Extinction ratios							
				at 220nm	at $\lambda_{\min.}$	at $\lambda_{\max.}$	E_{230}/E_{260}	E_{240}/E_{260}	E_{250}/E_{260}	E_{270}/E_{260}	E_{280}/E_{260}	E_{290}/E_{260}	E_{300}/E_{260}	
Gp + Up	2	228	258	3.80	2.40	9.6	0.27	0.58	0.89	0.78	0.52	0.25	0.07	
	7	225	255	3.90	3.10	11.1	0.34	0.69	1.00	0.82	0.52	0.16	0.02	
	12	237	260	10.10	5.55	8.50	0.70	0.67	0.88	0.89	0.47	0.08	0.02	
GxpUp	2	228	257	4.54	2.54	10.4	0.26	0.53	0.89	0.77	0.50	0.26	0.09	
	7	226	255	4.12	3.04	11.0	0.31	0.63	0.97	0.81	0.52	0.19	0.03	
	12	232	260	10.80	5.84	9.5	0.62	0.67	0.89	0.89	0.48	0.08	0.02	
UxpGp	2	228	258	4.53	2.59	10.9	0.25	0.49	0.86	0.78	0.52	0.28	0.10	
	7	226	255	3.89	2.87	11.2	0.29	0.62	0.98	0.79	0.51	0.18	0.03	
	12	233	260	10.81	5.74	9.7	0.61	0.65	0.87	0.87	0.48	0.09	0.02	
Up	2	229	261	4.50	2.40	10.0	0.24	0.47	0.78	0.80	0.32	0.03	0.00	
	7	230	260	5.10	2.65	10.1	0.26	0.42	0.77	0.82	0.32	0.03	0.00	
	12	241	260	10.10	5.77	7.5	1.03	0.77	0.85	0.77	0.26	0.03	0.01	
UxpUp	2	230	260	4.92	2.28	9.12	0.25	0.44	0.80	0.76	0.31	0.03	0.00	
	7	229	260	4.92	2.28	9.12	0.25	0.43	0.78	0.79	0.35	0.04	0.00	
	12	241	260	9.20	5.52	7.00	1.00	0.79	0.86	0.79	0.29	0.04	0.02	

30min at 100V/cm on Whatman 3MM paper. The eluted dinucleotide was run in 0.2M-ammonium formate buffer, pH3, for 2-4h at 20V/cm. No hydrolysis of internucleotide linkages was detected.

The methods of precipitation, yields and phosphorus and pentose contents of the purified compounds are listed in Table 2. Relative R_F values in ammonium sulphate (solvent B) and relative electrophoretic mobilities at pH3 are given in Table 1. In solvent A1 all the alkali-stable dinucleotides containing guanine (CxpGp, GxpCp, AxpGp, CxpAp, UxpGp and GxpUp) move at or near to the rate for guanylic acid, which is the slowest-moving mononucleotide in that solvent. Cp, Ap and Up move together as a faster band and the remaining alkali-stable dinucleotides (CxpCp, CxpAp, AxpCp, AxpAp, CxpUp, UxpCp, AxpUp, UxpAp and UxpUp) move in the same region. This is not in agreement with the results of Smith & Dunn (1959) who found 11 of the 16 alkali-stable dinucleotides in the guanylic acid band obtained by chromatography of alkali-hydrolysed RNA in solvent A1.

The components A, B, C and D will not be described in detail. They were not dinucleotides although they had many properties in common with dinucleotides. They are most likely to be trinucleotides such as have been shown to occur in yeast nucleic acid by Singh & Lane (1964a) and Gray & Lane (1967).

Constants calculated from u.v.-absorption spectra measured at pH2, 7 and 12 are recorded in Table 3 together with data on corresponding mixtures of the appropriate pair of corresponding mononucleotides for comparison. Values of $\epsilon_{\max.}$, $\epsilon_{\min.}$ and ratios of extinction at 240, 250, 270, 280 and

290nm relative to that at 260nm are in agreement with values given by Toal, Rushizky, Pratt & Sober (1968) for the corresponding diribonucleotides.

Results of sequence determinations made by a method based on the procedure of Neu & Heppel (1964) are summarized in Table 4. In many cases a high recovery of terminal base, residual nucleotide or both was obtained. However, the quality of some of the absorption spectra of degradation products was not good, and chromatography and electrophoresis showed the presence of small amounts of compounds related to the main products. Treatments that improved the quality of the spectra led to the loss of a part of the material so that a strictly quantitative interpretation of the results cannot be attempted. However, it appeared that, with the exception of AxpGp, which was shown by this procedure to be considerably contaminated with GxpAp, the dinucleotides were either homogeneous or only contaminated to a small extent with other nucleotides.

AxpGp contained up to 30% of GxpAp. Chromatography in solvent B (either ascending or descending) failed to separate the two compounds. Following Lane & Butler's (1959) separation of ApGp and GpAp on a DEAE-cellulose column by elution with sodium acetate buffer, pH4.6, the cationic buffer piperazine dihydrochloride (approx. 0.01M) adjusted to pH4.6 with sodium hydroxide (Smith & Smith, 1949) and a linear gradient of 0.02-0.04M-potassium chloride, total volume 21, were used to effect a separation. About 10mg of dinucleotide mixture was loaded on to a column (10cm x 1.5cm) of DEAE-cellulose equilibrated with 0.01M-piperazine dihydrochloride buffer, pH4.6. The nucleotides were resolved into two

Table 4. *Sequence determination of alkali-stable dinucleotides by a method based on that of Neu & Heppel (1964)*

Dinucleotide	Percentage of total released by alkaline phosphomonoesterase	Terminal base	Percentage of terminal base recovered	Residual nucleotide	Percentage of residual nucleotide recovered
CxpCp	50	Cytosine	84	Cxp	83
CxpAp	50	Adenine	42	Cxp	77
AxpCp	48	Cytosine	77	Axp	77
AxpAp	50	Adenine	44	Axp	71
AxpAp	51	Adenine	49	Axp	77
CxpGp	49	Guanine	32	Cxp	71
GxpAp	48	Adenine	56	Gxp	56
CxpUp					
UxpCp	50	—	—	—	—
AxpUp	46	Uracil	77	Axp	75
UxpAp	49	Adenine	35	Uxp	64
GxpUp	48	Uracil	70	Gxp	50
UxpGp	50	Guanine	30	Uxp	66
UxpUp	48	Uracil	60	Uxp	64

Table 5. *Ultraviolet extinction and pentose content of presumed 2'-O-methyl ribonucleotides related to their phosphate content*

Extinctions were measured at $\lambda_{\max}$. of the corresponding ribonucleotide and pentose and phosphate were determined by the methods described in the text. $\epsilon:P$ is effectively the ratio of the observed molecular extinction coefficient and that of the corresponding ribonucleotide.

Mononucleotide	Dinucleotide of origin	$\epsilon:P$	Pentose/phosphate molar ratio	
			(a) before bromination	(b) after bromination
Cxp	CxpCp	1.06	0.03	0.30
	CxpAp	1.09	0.08	0.33
	CxpGp	1.09	0.05	0.30
	CxpUp	1.10	0.05	0.35
Axp	AxpCp	1.03	0.33	—
	AxpAp	1.11	0.35	—
	AxpAp	1.04	0.36	—
	AxpUp	1.09	0.35	—
Gxp	GxpAp	1.10	0.33	—
	GxpUp	1.10	0.37	—
Uxp	UxpAp	1.11	0.04	0.41
	UxpGp	1.03	0.03	0.37
	UxpUp	1.08	0.04	0.39

overlapping peaks, recovered by the method of Rushizky & Sober (1962) and purified for analysis as described above.

The mononucleotide obtained by the periodate degradation of each dinucleotide was purified and analysed. Most of the spectra (at pH 2, 7 and 12) were distorted by the continued presence of material carried over from the degradation. However, $\lambda_{\max}$, $\lambda_{\min}$, and the E_{250}/E_{260} and E_{280}/E_{260} ratios were in good agreement with the values for the

corresponding pure ribonucleotides. Table 5 shows that molecular extinction relative to phosphorus content is high, as would be expected with nucleotide material contaminated with u.v.-absorbing impurities. The pentose content was the same for each mononucleotide irrespective of the dinucleotide from which it was obtained and, although appreciable, was low compared with those to be expected from the corresponding ribonucleotide.

After the discovery of this class of dinucleotide

by Smith & Dunn (1959), Singh & Lane (1964a) established the existence of the 16 possible alkali-stable dinucleotides derived from Ap, Gp, Cp and Up and their tentatively designated 2'-*O*-methyl derivatives in whole yeast RNA, and Hall (1964) obtained pseudouridine modified by probable 2'(or 3')-*O*-methylation from tRNA, thus showing the possible existence of alkali-stable dinucleotide sequences involving 2'(3')-*O*-methylpseudouridylic acid in RNA. Nichols & Lane (1966) showed that *E. coli* rRNA yields an alkali-stable dinucleotide in which P1 is 6-*N*-methyl-2'-*O*-methylcytidine and P2 is cytidine.

Thus the existence of at least 18 different alkali-stable dinucleotide sequences in RNA is almost certain and it seems highly probable that others will be found. Analysis of the nucleotide composition of separate and mixed tRNA species shows that a particular 2'-*O*-methyl nucleotide may only be present in one or a few specific tRNA species and only at the rate of one nucleotide/RNA molecule composed of 70–80 nucleotides; thus certain alkali-stable dinucleotide sequences may be present in a very small proportion of the whole cell RNA and would consequently be difficult to find by existing methods of analysis.

Although the functional effect and consequent biological significance of the structural modification conferring alkali-stability is not yet known there is already evidence that it probably occurs at definite sequential positions in the polynucleotide chain. The existing direct evidence is summarized by Sober (1968); and all analyses to date of total tRNA (Hall, 1964, 1965; Hudson, Gray & Lane, 1965; Nichols & Lane, 1966; Gray & Lane, 1967; Morisawa & Chargaff, 1968), of rRNA (Singh & Lane, 1964a; Nichols & Lane, 1966), and total RNA (Singh & Lane, 1964b), support the more definite evidence obtained with single molecular species of tRNA.

Borek & Srinivasan (1966) have reviewed the now clear evidence that the methylation of bases in tRNA and rRNA (Svensson, Boman, Erikson & Kjellin, 1963; Starr, 1963) occurs by methylation of bases at definite sites in preformed polynucleotides by groups of highly specific enzymes. It seems likely that an entirely analogous process must occur in the methylation of ribose to produce the alkali-stable dinucleotide sequences under discussion. Since the pattern of *O*-methylation is different in tRNA and the two larger components of rRNA (5S RNA does not appear to be methylated at all) a new and subtle complication of RNA function is added to that created by the modification of bases by both methylation and other enzyme-mediated changes of chemical structure. This adds urgency to the need to understand the structural and functional changes produced by these modifications.

The main purpose of the present work was to obtain comparatively large quantities of pure alkali-stable dinucleotides, to the possible detriment of obtaining quantitative recoveries. Nevertheless it was surprising to find no GxpGp, since Singh & Lane (1964a) found it as one of the more abundant alkali-stable dinucleotides in commercial yeast RNA, and Morisawa & Chargaff (1968) showed that it was by far the most abundant of the 12 alkali-stable dinucleotides found in whole yeast tRNA.

There are a number of possible explanations of this apparent loss. The most likely is that the dinucleotide was adsorbed on the large quantity of guanylic acid gel that was precipitated with ethanol at the beginning of the preparation. For it is known (Shapiro, 1968) that guanine and its derivatives bind strongly to themselves and to other substances, largely by virtue of the large number of hydrogen-bonding sites present in the guanine molecule. This base, Gp, pG and guanosine 5'-phosphoramidate tend to precipitate as gels (Chambers, Shapiro & Kurkov, 1960; Michelson, Massoulié & Gushlbauer, 1967) and deoxyguanosine and guanosine oligonucleotides aggregate to form ordered secondary structures (Ralph, Connors & Khorana, 1962). This binding phenomenon may also explain the discrepancy between our observations on the rate of movement of pure alkali-stable dinucleotides in chromatography on paper in solvent A1 and those of Smith & Dunn (1959), who found that most of the dinucleotides travelled at the same rate as guanylic acid in that solvent. For in this case the dinucleotides may have become associated with the large amount of Gp with which they were mixed. An association of this kind could equally explain the failure of repeated efforts in the present work to separate GxpGp from the ethanol-precipitated guanylic acid.

An alternative but less likely explanation is that the component of the alkali-stable dinucleotide fraction described as GxpGp by other workers may be some other derivative of guanine having some of the properties of GxpGp. A number of such components were encountered in the material under investigation.

The analyses show that the compounds isolated in the course of the present work were of a high degree of purity. However, the determinations of sequence were not sufficiently quantitative for it to be certain that the main component was not, in some cases, contaminated with its sequence isomer and further work will be necessary to ensure that this is not so. The procedure of Neu & Heppel (1964), on which the determination of sequence was based, is a considerable improvement on the previously described methods of periodate degradation of nucleosides. These authors published results on the degradation of a number of oligonucleotides,

three dinucleoside monophosphates (equivalent to dinucleotides) and one dinucleotide. They claimed a recovery of 80–100% of the terminal base and about 80–90% of the residual nucleotide, measured as the nucleoside after dephosphorylation with phosphomonoesterase. Both products were measured by determination of extinctions at absorption maxima. When the method was extended to a wider range of dinucleotides in the present work quantitative recoveries of base and nucleotide were not achieved. In the present work some of the interfering substances were removed by electrophoresis, which was necessary for the separation of the base and nucleotide product of each degradation. This entailed considerable losses, particularly of the purine bases, which are poorly soluble in water. However, the results show that recoveries from some of the dinucleotides were high enough to suggest that they were pure.

In some of the less successful analyses difficulties were probably introduced by the large relative concentrations of reagents employed in the method. These may have been responsible for the trailing bands and apparent displacement of part of the products into separate minor bands. The minor bands could be identified as related to major bands and would, in most cases, have accounted for the lower yield of the expected products. In a few cases, as was confirmed by other methods, the minor bands contained products of degradation of contaminating nucleotide material.

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