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METHOD FOR ISOLATION OF 2′-O-METHYLRIBONUCLEOSIDES AND N^1-METHYLADENOSINE FROM RIBONUCLEIC ACID

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SUMMARY

N^1-methyladenosine and the 2′-*O*-methyl derivatives of adenosine, uridine, guanosine, and cytidine have been isolated from yeast soluble RNA. A nucleoside mixture obtained by enzymic hydrolysis of the RNA was resolved by means of chromatography on partition columns into major fractions. The minor components were then isolated by means of paper chromatography. This procedure permits detection of minute amounts of these nucleosides. 1 g of yeast soluble RNA yielded 1.2 mg of total 2′-*O*-methylated derivatives and 3.0 mg of N^1-methyladenosine. There is the suggestion that the ratio of 2′-*O*-methyladenosine to 2′-*O*-methyluridine and that of 2′-*O*-methylguanosine to 2′-*O*-methylcytidine may be close to unity.

INTRODUCTION

The presence of 2′(3′)-*O*-methylated ribonucleosides in RNA has been reported by SMITH AND DUNN[1]. BISWAS AND MYERS have characterized 2′-*O*-methylcytidine isolated from RNA of *Anacytstis nidulans*[2]. N^1-Methyladenine has also been found in RNA[3]. Detection of the small amounts of these methylated nucleosides in RNA solely by means of paper chromatography is difficult. This paper describes work which confirms the presence of the 2′-*O*-methylnucleosides in yeast soluble RNA and presents a method for their isolation. This procedure is based upon a preparative column procedure which first resolves an RNA digest into small fractions thereby facilitating final isolation of the minor constituents by means of paper chromatography.

EXPERIMENTAL

1 g of soluble yeast RNA[4] dissolved in 100 ml of 0.001 M $MgSO_4$ solution was hydrolyzed to its constituent nucleosides by treatment with 100 mg of whole snake venom (*Crotalus adamanteus*) and 25 mg of alkaline phosphatase (Worthington Corp., Freehold, N.J.). The pH was maintained at 9.2 by continuous addition of 1 N sodium hydroxide and after 7 h at 37° release of inorganic phosphate was complete. The solution was neutralized and lyophilized. This residue was suspended in 20 ml of water and stirred for 30 min while heating at 60° after which the solution was cooled to 4° and centrifuged at 30 000 × *g* for 30 min. This procedure eliminated much of the

protein. The clear supernatant was lyophilized to give a residue which was the starting material for the fractionation.

All the procedures on partition columns reported below were conducted according to the basic procedure of HALL[5]. The RNA hydrolysate was first resolved into major nucleoside fractions on a 150-g column of Celite-545–Microcel-E (9:1) (Johns-Manville Corp.) size 2.54 cm × 80 cm. The elution pattern is shown in Fig. 1. The

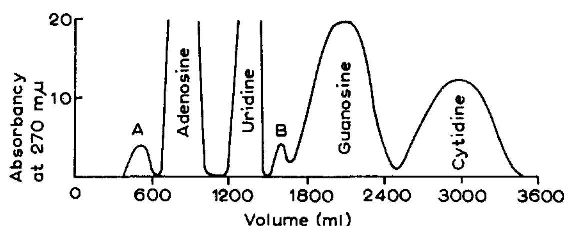

Fig. 1. Fractionation of a nucleoside mixture obtained by enzymic hydrolysis of yeast s-RNA. Column: 150 g of Celite-545–Microcel-E (9:1) (2.54 cm × 80 cm). Solvent 1, ethyl acetate–2-ethoxyethanol–2.0 % formic acid (4:1:2). When the uridine peak had been eluted, solvent changed to ethylacetate–*n*-butanol–H_2O (1:1:1).

first small peak (labelled A) contained N^1-methyladenosine, 2′-*O*-methyladenosine, and 2′-*O*-methyluridine as well as minute quantities of an unidentified nucleoside. The constituents of this peak were resolved on a second partition column (Fig. 2). The first peak of this column contained N^1-methyladenosine and 2′-*O*-methyladenosine and a trace amount of thymidine. These nucleosides were separated from each other by streaking on Whatman No. 3 MM paper and developing the paper in solvent system B. (Chromatography and subsequent elution of these nucleosides should be performed at 4° to prevent hydrolysis of the more acid-labile 2′-*O*-methyladenosine.) There is about 30 times as much N^1-methyladenosine as 2′-*O*-methyladenosine in this peak so it is necessary to streak heavily in order to see the latter component which travels just in front of the N^1-methyladenosine band. These two methylated adenosines have identical R_F values in several solvent systems and only in solvent system B is there sufficient difference in rate of migration for a separation. Another component of peak 1 (Fig. 2) was N^6-methyladenosine. It was obtained by paper chromatography of the nucleoside mixture from peak 1 in solvent system A. In this

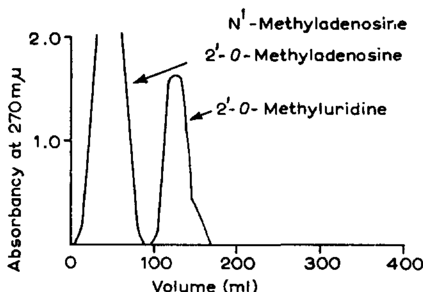

Fig. 2. Peak A of Fig. 1 fractionated on 50 g column of Celite-545 (1.9 cm × 45 cm). Solvent: *n*-butanol–H_2O–conc. NH_4OH (15:5:2).

system, this compound moves just behind the N^1-methyladenosine band. On the basis of spectrophotometric data the amount of this nucleoside was estimated to be 20 μg. Since N^1-methyladenosine is readily converted to N^6-methyladenosine[3] at alkaline pH's, it is likely that this rearrangement occurred to a limited extent during enzymic hydrolysis of the RNA or during paper chromatography in the alkaline solvent (A). Further studies are necessary to verify this possibility. The second peak from this column contained 2'-*O*-methyluridine and a small amount of an unidentified nucleoside. They were separated by paper chromatography in solvent system A. The unidentified nucleoside has the ultraviolet spectra shown in Fig. 3. The spectra

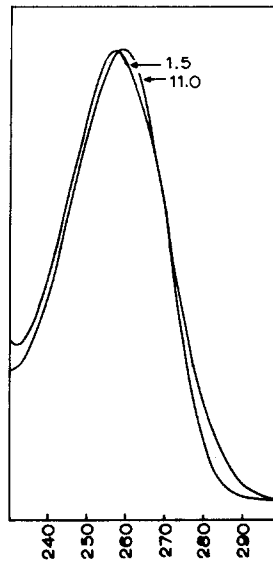

Fig. 3 Ultraviolet spectra at pH 2.5 and 11.0 of an unidentified nucleoside isolated from peak 2 of Fig. 2.

resembles that of adenosine but is shifted slightly to lower wavelengths. Mild acid hydrolysis of this nucleoside produces a base which resembles adenine and a sugar which by paper chromatography does not resemble ribose, deoxyribose or 2-*O*-methylribose. Since only 75 μg of this material was isolated, a more rigorous characterization was not possible. Whether this nucleoside is an artifact of the isolation procedure or a genuine constituent of RNA is a question presently under investigation.

The guanosine peak of Fig. 1 contained 2'-*O*-methylcytidine. Isolation of this nucleoside was facilitated by first fractionating the contents of the peak on a column as shown in Fig. 4. The peak labelled 2'-*O*-methylcytidine also contained N^1-methylguanosine. A pure sample of the 2'-*O*-methylcytidine was obtained by paper chromatography in the solvent system A. The uridine peak of Fig. 1 contained 2'-*O*-methylguanosine. Since a satisfactory solvent system for resolving this mixture on a partition column was not found, the contents of the uridine peak were chromatographed on sheets of Whatman No. 3 MM paper. After development for 48 h in solvent system A, the 2'-*O*-methylguanosine was found in a band moving just ahead of the uridine band. Sometimes separation was still incomplete, in which case the band

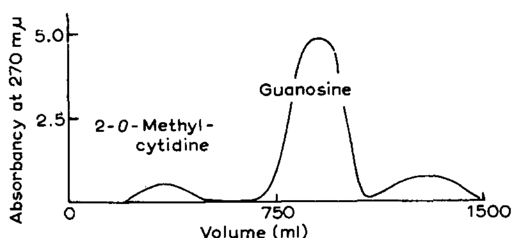

Fig. 4. Guanosine peak of Fig. 1 fractionated on 150 g column of Celite-545 (2.54 cm × 80 cm). Solvent: same as in Fig. 2.

containing 2′-*O*-methylguanosine was eluted, and the nucleosides rechromatographed in solvent system D. In this system 2′-*O*-methylguanosine runs just behind uridine.

Recovery of the methylated nucleosides from 1 g of yeast soluble RNA is shown in Table I. The values in this table, which represent minimal amounts of these

TABLE I

RECOVERY OF THE RIBONUCLEOSIDES FROM 1 g OF YEAST s-RNA

Weights calculated from spectrophotometric data.

2′-*O*-Methyladenosine	0.1 mg
2′-*O*-Methylcytidine	0.5 mg
2′-*O*-Methylguanosine	0.5 mg
2′-*O*-Methyluridine	0.1 mg
N^1-Methyladenosine	3.0 mg

nucleosides, permit two observations. Yeast soluble-RNA contains a 2′-*O*-methylated nucleoside for every 600 nucleosides, which means that only a few of the molecular species of soluble RNA contain these nucleosides. Further, these values, although not representing absolute accuracy, suggest that the molar ratio of 2′-*O*-methyladenosine to 2′-*O*-methyluridine and that of 2′-*O*-methylguanosine to 2′-*O*-methylcytidine is approximately one. If this result is borne out by more accurate analyses now in progress, there is the possibility that a species of nucleic acid exists which contains only 2′-*O*-methylated ribonucleosides. On the other hand the data of SMITH AND DUNN[1] indicate that the 2′-*O*-methyl derivatives can occur in combination with regular ribonucleosides.

The 2′-*O*-methylnucleosides were identified as follows: Electrophoretic mobility of these compounds at pH 9.2 is not changed by addition of borate to the buffer. The R_F values of these four derivatives are different from either the riboside or deoxyriboside as shown in Table II. The two purine nucleosides were then hydrolyzed with 0.1 N hydrochloric acid at 100° for 2 h. The two pyrimidine nucleosides were first hydrogenated over rhodium on alumina catalyst[6] to the dihydro compounds, then hydrolyzed with 0.1 N hydrochloric acid at 100° for 20 min. The sugars released by this treatment were compared with a synthetic sample of 2-*O*-methylribose[7] (we are indebted to Dr. G. R. BARKER for a gift of the precursor of this derivative) by paper chromatography and electrophoresis as shown in Table III. The ultraviolet spectra of each of the 2′-*O*-methylated nucleosides (read at pH values of 2, 7 and 11) were the same as those of the parent ribonucleosides. The N^1-methyladenosine was identified on the basis of its facile rearrangement to N^6-methyladenosine in dilute alkali[3].

TABLE II

PAPER CHROMATOGRAPHY OF NUCLEOSIDES

Solvents: A, *n*-Butanol–H_2O–conc. NH_4OH (86:14:5); B, isobutyric acid–H_2O–conc. NH_4OH (4:2:0.004); C, isopropanol–conc. HCl–H_2O (680:170:144); D, ETOH–*n*-propanol–H_2O(4:1:2) (upper phase).

Compound	$R_{guanosine}$ Solvent			
	A	*B*	*C*	*D*
Adenosine	5.16	1.56	1.33	3.00
2′-*O*-Methyladenosine	8.55	1.75	1.93	6.40
Deoxyadenosine	5.95	1.20	1.33	4.70
Cytidine	2.60	1.12	1.87	1.00
2′-*O*-Methylcytidine	5.16	1.40	2.55	2.38
Deoxycytidine	3.75	0.98	2.44	1.57
Guanosine	1.00	1.00	1.00	1.00
2′-*O*-Methylguanosine	2.60	1.36	1.95	2.66
Deoxyguanosine	1.65	0.85	1.00	1.57
Uridine	1.65	1.00	2.61	3.42
2′-*O*-Methyluridine	3.40	1.27	3.22	6.13
Deoxyuridine	4.16	1.21	2.80	5.15
N^1-Methyladenosine (isolated)	8.55	1.60	1.93	6.40
N^1-Methyladenosine (synthetic)	8.55	1.60	1.93	6.40

TABLE III

CHROMATOGRAPHY AND ELECTROPHORESIS OF THE SUGARS OF THE NUCLEOSIDES

Solvent E, *n*-Butanol–saturated boric acid solution (86:14). Electrophoresis 3 h at 22 V/cm; buffer (pH 9.2) containing 0.05 M borate.

Sugar from	R_{ribose} Solvent		Electrophoretic mobility ribose = 1.0
	D	*E*	
2′-*O*-Methyladenosine	2.18	2.16	0.33
2′-*O*-Methylcytidine	2.10	2.16	0.33
2′-*O*-Methylguanosine	2.20	2.16	0.33
2′-*O*-Methyluridine	2.20	2.16	0.33
Synthetic 2-*O*-Methylribose	2.10	2.16	0.33
Synthetic 3-*O*-Methylribose	2.45	3.77	—
N^1-Methyladenosine (isolated)	1.00	1.00	—

The rearranged product has an ultraviolet spectra identical with that of 6-methylaminopurine riboside. Hydrolysis of the nucleoside in 1 N hydrochloric acid for 15 min produced a sugar that has R_F values similar to D-ribose in the four solvent systems and a base that has ultraviolet spectra and R_F values on paper strips identical with those of 6-methylaminopurine.

DISCUSSION

Although the 2′-*O*-methyl derivatives could theoretically substitute for the deoxyribonucleosides, they have been found only in RNA. If they do occur in DNA, it is at very low levels, as demonstrated by an analysis of a 5-g sample of Ehrlich ascites DNA. This sample was hydrolyzed to its constituent nucleosides and fractionated as

previously described[5]. The eluate collected between the deoxyadenosine and deoxyguanosine peaks (see Fig. 1 of ref. 5) was evaporated to a volume of 0.2 ml. This was streaked in a 3-in wide band on Whatman No. 3 MM paper. After development in solvent system A, a band was obtained which contained 250 μg of a compound which corresponded to 2′-*O*-methylguanosine by means of paper chromatography, electrophoresis, and ultraviolet spectra. Since the starting DNA sample was contaminated with about 0.25 % of RNA, it is conceivable that this represented the source of 2′-*O*-methylguanosine. Further analytical work will be necessary to clarify this point.

The identity of all the nucleosides reported herein is based on spectral properties and a comparison on paper chromatography with authentic samples. Absolute assignment of structure will have to wait until sufficient amounts of material are obtained to permit more rigorous characterization.

NOTE ADDED IN PROOF

In succeeding experiments a variable ratio of N^1-methyl- to N^6-methyladenosine was obtained. This inconsistency may be traced to the labile rearrangement of N^1-methyl- to N^6-methyladenosine. The present method therefore is not satisfactory for precise determination of the ratio of these two compounds if both should be present in the same sample of nucleic acid.

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