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A General Procedure for the Isolation of "Minor" Nucleosides from Ribonucleic Acid Hydrolysates*

Ross H. Hall

ABSTRACT: A general procedure for the isolation of minor components of ribonucleic acid (RNA) is described. Samples of RNA are hydrolyzed enzymatically to a mixture of nucleosides and the hydrolysate is resolved into major fractions corresponding to the major ribonucleosides by means of column partition chromatography, neutral solvents being used throughout. These major fractions are resolved into a number of subfractions by further column partition chromatography using different solvent systems. The minor nucleosides contained in each of these subfractions can be readily isolated and purified by means of paper chromatography. Eighteen minor nucleosides were obtained from a sample of yeast s-RNA. Seventeen of these have been detected previously in this and in other laboratories. The eighteenth nucleoside, which had not been previously detected, appears to consist of adenine and two molecules of ribose. It has been tentatively assigned the structure 9[2′(3′)-*O*-ribosyl-β-D-ribofuranosyl]adenine.

More than twenty minor nucleosides have been detected in the hydrolysates of samples of RNA isolated from a variety of sources. The question as to whether all these compounds are genuine constituents of RNA must be assessed on the basis of the evidence available for each compound. Many of the compounds, especially the methylated base constituents, have been isolated from several sources under different conditions. Possibly the most convincing evidence is that some of these compounds have been isolated as components of oligonucleotides obtained from partial enzymatic digests of RNA (see, for example, Staehelin, 1964). Some of the more recently described constituents have not yet been isolated as nucleotides but as will be described in this paper they and the previously known minor constituents, eighteen in all, have been isolated under mild conditions from an enzymatic digest of yeast s-RNA.

There are several classes of minor components, of which the one comprising the methylated bases contains the largest number of known members, and in fact it appears that the majority of the theoretically possible methylated derivatives of the common bases have already been found. The biological significance of the methylated bases has not been established but results of research in several laboratories[1] have shed considerable light on their origin and possible function. These minor constituents arise by direct methylation of the intact RNA molecule and, what is most significant, the methylation appears to be highly specific. Thus, the methylated bases are not distributed randomly in an individual RNA molecule, but appear to be specifically located. These findings have led to speculation that the methylated bases may provide for a recognition mechanism and this possibility has been investigated in the case of a bacteriophage-host system (Gold *et al.*, 1964).

Essentially nothing is known about either the origin or the biological function of the other types of minor components, but subtle modifications of the physical and chemical properties conferred on individual species of RNA molecules by these compounds can be visualized. For example, Honjo *et al.* (1964) have found that 2′-*O*-methylribonucleoside 5′-phosphates are resistant to the action of bull semen and snake venom 5′-nucleotidases. This suggests that analogous modifications in substrate specificity toward RNA hydrolytic enzymes could be expected at points where such compounds are located.

It is doubtful that the number of minor constituents of RNA known at present even closely approaches the

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[1] Much of this work is summarized in a recent review by Srinivasan and Borek (1964).

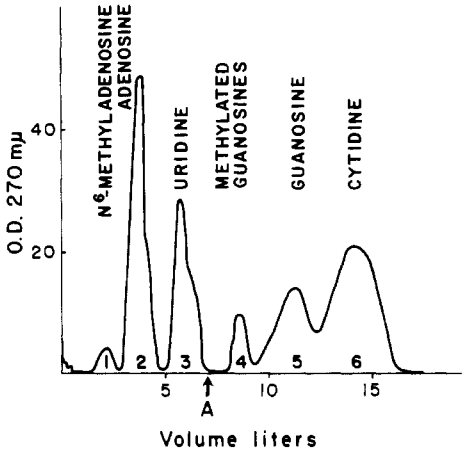

FIGURE 1: Major fractionation of a nucleoside mixture obtained by enzymic hydrolysis of yeast s-RNA. Column, 770 g of Celite 545–Microcel E (9:1), 5.08 × 80 cm. Sample weight 5.375 g, of which 2.875 g was mixed nucleosides. Solvent F, ethyl acetate–2-ethoxyethanol–water (4:1:2); solvent G, ethyl acetate–1-butanol–ligroine–water (1:2:1:1). Solvent F was passed through the column until the uridine fraction had been eluted; then solvent G was started and continued to end of run.

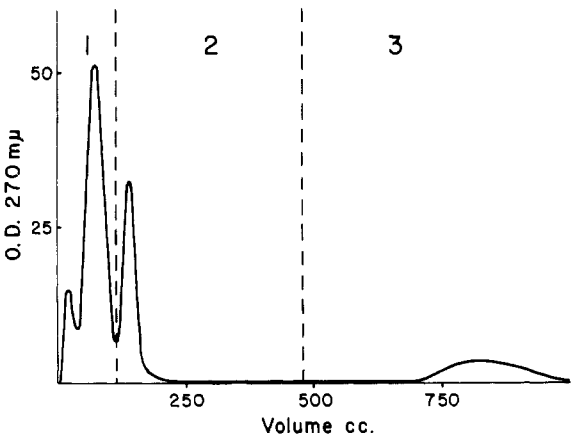

FIGURE 2: Subfractionation of material corresponding to major fraction 1. Column, 150 g of Celite 545 mixed with 72 cc of lower phase. Sample dissolved in 9 cc of lower phase and mixed with 18 g of Celite 545. Solvent system H.

total number that actually exist. As part of a broad study of the minor constituents of nucleic acids in this laboratory, a continued search for minor constituents of nucleic acids is being conducted. In order that "labile" constituents of RNA may be detected it is essential that such a search be carried out using the mildest possible techniques. Harsh hydrolytic procedures involving acids or alkali should be avoided. In our procedure, enzymes are used to hydrolyze the RNA to the nucleosides which are more amenable to mild separation techniques, such as partition chromatography, than either the nucleotides or free bases. Another compelling reason for resolving RNA constituents at the nucleoside level is that differences in the sugar portion may be revealed. The isolation of nucleosides containing sugars other than ribose has demonstrated the usefulness of this approach. Earlier, reports from this laboratory have described the isolation of a number of previously unknown minor nucleosides from RNA hydrolysates (Hall, 1963a–d; 1964a,b). This paper describes a basic procedure for isolating minor nucleosides and also reports the presence in a digest of yeast s-RNA of a previously undetected nucleoside which appears to consist of adenine and two residues of ribose. It has been tentatively assigned the structure 9[2′(3′)-*O*-ribosyl-β-D-ribofuranosyl]adenine (See Figure 7).

Experimental

General. All solvents used were reagent grade and were redistilled through a 50-cm column containing a Goodloe Packing.[2] The OD at 270 mμ of the redistilled 2-ethoxyethanol in a 1-cm cell was 0.18 and that of the other solvents was less than 0.10.

The Celite 545[3] was washed successively with 6 N hydrochloric acid, water, and ethanol, and dried at 100° for 16 hours (Hall, 1962a). The Microcel E[3] was used as received from the manufacturer.

Paper Chromatography. Whatman No. 1 paper was used for purposes of comparative identification and Whatman No. 3MM paper was used for the isolation of the minor components.

Solvent Systems. (A) 1-butanol–water–concd ammonium hydroxide (86:14:5); (B) 2-propanol–1% aqueous ammonium sulfate (2:1); (C) 2-propanol–concd hydrochloric acid–water (680:170:144); (D) 2-propanol–water–concd ammonium hydroxide (7:2:1); (E) ethyl acetate–1-propanol–water (4:1:2); (F) ethyl acetate–2-ethoxyethanol–water (4:1:2); (G) ethyl acetate–1-butanol–ligroin (bp 66–75°)–water (1:2:1:1); (H) 1-butanol–water–concd ammonium hydroxide (3:1:0.05); (I) ethyl acetate–glacial acetic acid–water (5:1:2); (J) ethyl acetate–1-butanol–water (1:1:1). *Note:* Solvents A through E were used for paper chromatography. Solvents E through J are all two-phase systems and were used for partition chromatography on columns.

Isolation Procedure. The original enzymatic digest was broken down first by column chromatography into six major fractions, four of which correspond to the four major nucleosides (Figure 1). Each of the major fractions can be separated by means of further column chromatography into subfractions. The minor nucleosides were isolated from these subfractions by means of

[2] Scientific Glass Apparatus Co., Bloomfield, N.J.
[3] Trade names of the Johns-Manville Co.

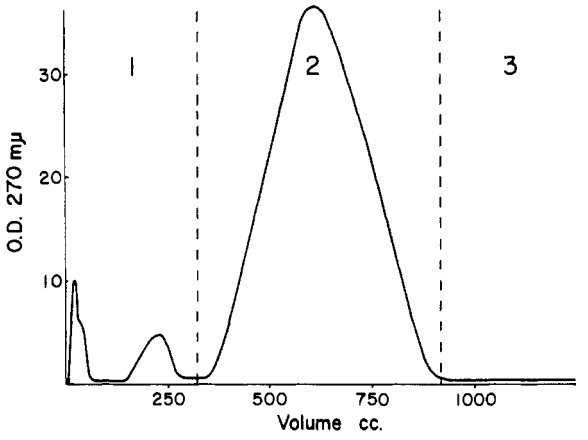

FIGURE 3: Subfractionation of material corresponding to major fraction 3. Column, 150 g of Celite 545 mixed with 60 cc of lower phase. One-fourth of this material was dissolved in 10 cc of lower phase and mixed with 25 g of Celite 545. Solvent system I.

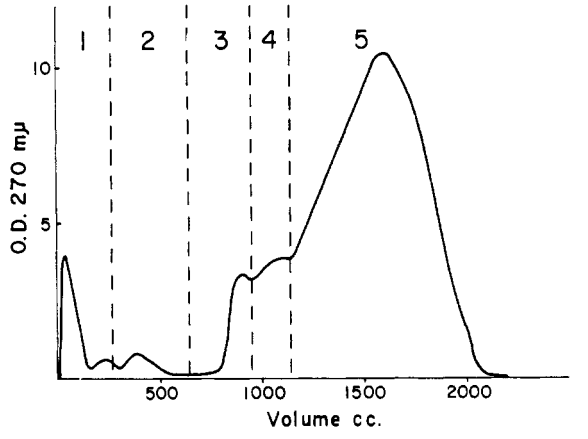

FIGURE 4: Subfractionation of material corresponding to major fraction 4. Column, 150 g of Celite 545 mixed with 66 cc of lower phase. Sample dissolved in 11 cc of lower phase and after filtering the solution was mixed with 22.5 g of Celite 545. Solvent system E.

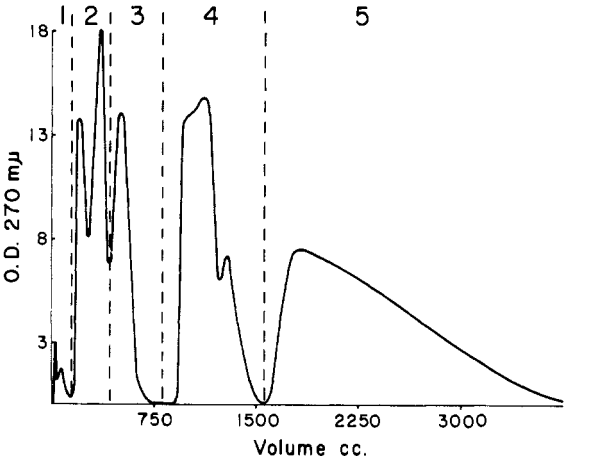

FIGURE 5: Subfractionation of material corresponding to major fraction 5. Column, 150 g of Celite 545 mixed with 72 cc of lower phase. The sample was dissolved in 30 cc of hot water which was filtered and kept at 4° for several days. The precipitate, consisting mostly of guanosine, was filtered off and the filtrate was lyophilized. The residue was dissolved in 10 cc of lower phase and mixed with 22 g of Celite 545. Solvent system H.

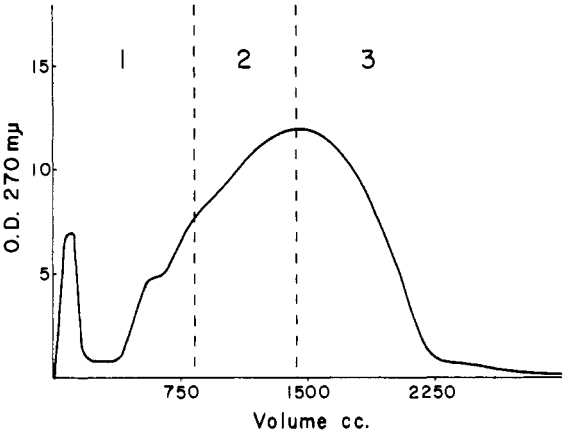

FIGURE 6: Subfractionation of material corresponding to major fraction 6. Column, 150 g of Celite 545 mixed with 65 cc of lower phase. One-fourth of the material was dissolved in 14 cc of lower phase and mixed with 32 g of Celite 545. Solvent system J.

paper chromatography. This procedure is summarized schematically in Figure 8. The details of the procedure follow.

HYDROLYSIS AND PRIMARY FRACTIONATION. Yeast soluble-RNA (22 g), prepared by General Biochemicals according to the method of Holley *et al.* (1961), was degraded to its constituent nucleosides by means of whole snake venom and bacterial alkaline phosphatase as described previously (Hall, 1964a). The lyophilized digest weighed 21.5 g of which 11.5 g consisted of the free nucleosides as determined spectrophotometrically. The remainder of the material consisted of inorganic salts, moisture, and residual protein. The total digest was divided into four equal portions and each portion was fractionated by means of column partition chromatography (Hall, 1962a). The column consisted of precision-bore glass pipe (5.08 × 105 cm), fabricated by the Fischer-Porter Co. The column was charged with 690 g of Celite 545–Microcel E (9:1) which had been thoroughly mixed with 308 cc of the lower phase of solvent F (ethyl acetate–2-ethoxyethanol–water, 4:1:2). The Celite mixture was dry packed in 25-g increments with a close-fitting plunger machined from a Kel-F rod. The sample was dissolved in 35 cc of the lower phase of solvent F and, if necessary, the *p*H was adjusted to 7.0. The solution was filtered to remove insolubles

TABLE I: [a] Compounds Isolated from the Subfractions of Major Fractions by Paper Chromatography.

Major Fraction	Sub-fraction	Solvent System	Time (hours)	Rechroma-tography (if necessary)[b]	Compound Isolated	Amount (mg)
1	1	E	8		Adenosine	2.2
					Deoxyadenosine	3.8
					2′-*O*-Methyladenosine	3.4
					N^6-Methyladenosine	46.6
	2	A	24		Thymidine	3.6
					N^6-Methyladenosine	1.5
	3	A	24		2′-*O*-Methyluridine	3.4
3	1	A	40		Uridine[c]	15.5
					Uracil	0.28
	2	D	24		Uridine	400
4	1	E	8		N^6-Methyladenosine[d]	0.36
					3-Methyluridine	1.1
	2	D	24		Guanosine	0.90
					N^6-Methyladenosine	0.23
	3	E	24		2′-*O*-Methylguanosine	6.2
					Cytidine	1.7
					Uridine	1.7
					2′-*O*-Methylpseudo-uridine[e]	0.1
	4	E	24	A, 56	N^2-Methylguanosine	1.9
					N^2,N^2-Dimethyl-guanosine	3.3
					2′-*O*-Methylguanosine	Trace
	5	E	42		Guanosine	4.5
					Deoxyguanosine	
				A, 56	N^2,N^2-Dimethyl-guanosine	29.6
					1-Methylguanosine	12.0
				D, 20	1-Methylinosine	2.5
					2′-*O*-Methylcytidine	8.3
5	1	E	20		Adenosine	0.3
	2	E	36	A, 24	Adenosine-R	2.4
					5-Methylcytidine	5.2
					Deoxycytidine	0.2
				D, 20	1-Methylinosine	2.6
					2′-*O*-Methylcytidine	4.7
	3	D	30		2′-*O*-Methylguanosine	34
					Cytidine	47
	4	D	30		Guanosine	100
					Inosine	26
	5				Pseudouridine[f]	398

TABLE I (*Continued*)

Major Fraction	Subfraction	Solvent System	Time (hours)	Rechromatography (if necessary)[b]	Compound Isolated	Amount (mg)
6	1	D	36		Guanosine	64
					Cytidine	
					5-Methylcytidine	4.4
					3-Methylcytidine	2.0
	2	D	24		Guanosine	0.5
					Pseudouridine	63
					Cytidine	
	3	D	24		Guanosine	8
					Pseudouridine	25.7
					Cytidine	
					N^6-(Aminoacyl)-adenosine	5.7 (av mw 380)

[a] The compounds detected in each subfraction are listed in order of increasing distance from the starting line of the chromatogram. [b] In five instances, pairs of compounds did not separate under the conditions used. The material in the band was resolved by rechromatography in a second solvent system, indicated by the letter, for the number of hours indicated by the numeral. [c] The small fraction corresponding to peak 1 (Figure 3) and containing uridine appears to be either an artifact of the column, or perhaps the uridine in this peak originally contained a labile constituent which split off during subsequent isolation and remained undetected. [d] The appearance of N^6-methyladenosine in this major fraction as well as major fraction 1 can be attributed to a column artifact, or possibly the N^6-methyladenosine in this peak is the degradation product of a more complicated molecule. [e] This compound appears to be the same as that isolated from an RNA digest by another technique (Hall, 1964b) (which is considered to be 2′-*O*-methylpseudouridine). There is no direct proof that this is the correct structure. This structure was suggested by the fact that the compound has spectra similar to those of pseudouridine, is not affected by borate ion during electrophoresis, and moves somewhat faster than pseudouridine when chromatographed on paper. [f] The elution pattern in Figure 5 shows that pseudouridine is eluted rather slowly. Since it is the sole nucleoside in this fraction, its removal may be accelerated by discontinuing the flow of solvent at the end of fraction 4 and washing the column with 600 cc of water. In the example shown here only a portion of the pseudouridine was actually eluted by the solvent and a water wash was finally required to remove the bulk of the nucleoside. The amount of 398 mg represents the total amount isolated.

which included a portion of the guanosine, and the clear solution was mixed with 80 g of the Celite-Microcel mixture. This mixture was tamped on top of the column. The column was developed with the upper phase of solvent F at a flow rate of 600 cc/hour. The optical density of the effluent was monitored continuously at 270 mμ using a flow cell with a path length of 1 mm. The temperature of the laboratory was maintained at 25° ± 2°. The elution pattern which was identical for all four batches is shown in Figure 1. When the uridine fraction had been completely eluted (point A, Figure 1), the eluent was changed from the upper phase of solvent F to the upper phase of solvent G (ethyl acetate–1-butanol–ligroin–water, 1:2:1:1). This change in solvents occasionally caused the separation of a small amount of water from the next few hundred cc of the effluent. This condition appears to be owing to a combination of factors such as the mixing of the two solvent systems, as well as the fact that solutes with low solubility (methylated guanosines and guanosine) start to appear. No impairment in resolving power resulted. A total of about 30 hours was required to complete the separation, and, for convenience in laboratory scheduling, the column was operated on three consecutive days, a procedure which did not diminish resolving power. Each of the six fractions corresponding to the six peaks shown in Figure 1 were evaporated *in vacuo* to near dryness in a rotating flash evaporator (bath temperature 30°). Water (250 cc) was added and the mixture was reevaporated. This procedure was repeated three times in order to remove the organic solvent. Finally the residue was redissolved in water and the solution was filtered. At this point the corresponding fractions from each of the four column runs were combined and lyophilized, and the resulting six samples served as the starting mixtures for subsequent isolation of the minor nucleosides.

SUBFRACTIONATION AND FINAL ISOLATION BY PAPER

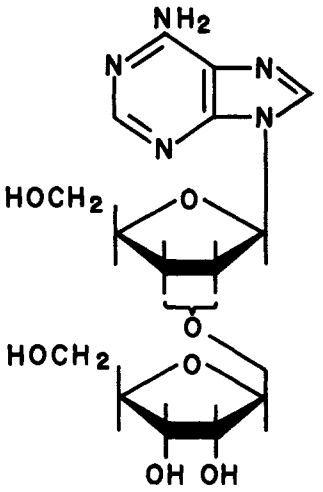

FIGURE 7: Structure of eighteenth nucleoside, 9[2′(3′)-*O*-ribosyl-β-D-ribofuranosyl]adenine.

CHROMATOGRAPHY. Each of the six fractions was fractionated (Figures 2–6)[4] on a smaller partition column (2.54 × 86 cm) constructed of heavy-wall precision-bore glass tubing (Fischer and Porter Co.). Each column was packed in the manner described with 150 g of Celite 545 mixed with a specified amount of the lower phase of the solvent system used. The lyophilized samples were dissolved in the specified quantity of the lower phase and the mixture was filtered and mixed with Celite 545. This charge was tamped on top of the column. The columns were developed with the upper phase of the solvent systems at a flow rate of 150 cc/hour and the optical density of the effluent was continuously monitored at 270 mμ. After the amount of solvent specified in the description had passed through the column, the column was washed with 500 cc of water to remove any material that might have been retained.

Final isolation of the minor nucleosides was accomplished by means of paper chromatography as follows (Table I): The effluent from each column was divided according to the subfractions shown in the elution profiles. These divisions in many cases were arbitrary, with the result that certain of the minor components appeared in more than one fraction. The subfractions were concentrated *in vacuo* so that the concentration of nucleosides in the final solution was approximately 5 mg/cc. The entire concentrate (for the larger fractions an aliquot was taken) was streaked on Whatman No. 3MM paper and developed in the indicated solvent system for a specified period of time.

The developed chromatogram was examined under ultraviolet light provided by a General Electric germicidal bulb. The light was filtered through a Corning filter, No. 7-54, which so reduced the background absorption of the paper that very faint ultraviolet absorbing bands could be detected. The ultraviolet absorbing bands were eluted and, if the material did not appear to be homogeneous on the basis of paper chromatography in several solvent systems, it was rechromatographed in a suitable solvent system. The amount of nucleoside isolated was calculated from the molar extinction value, and if this value was not known the value for the parent ribonucleoside was used. The eighteen minor nucleosides isolated are listed in Table II and the entire isolation scheme is summarized in Figure 8.

Major fraction 2 (Figure 1) was subfractionated on a 150-g column of Celite using solvent system E. A single bell-shaped peak, eluted between the 260 cc mark and 415 cc mark, was obtained. Paper chromatography of an aliquot of the material in this peak in solvent C showed two ultraviolet absorbing spots, the major corresponding to adenosine and the minor corresponding to 5-methyluridine. The calculated amount of the latter nucleoside in fraction 2 is 78 mg. There was no value to this subfractionation; it only demonstrated that under these conditions no other minor nucleosides were detected.

Identification of Minor Components. Each of the minor nucleosides was identified on the basis of three criteria: (1) comparison with standards by paper chromatography in five solvent systems (see Table III); (2) electrophoresis at *p*H 9.2 in the presence and absence of borate ion; (3) ultraviolet absorption spectra measured at *p*H 1.5, 7.0, and 11.5. Several of the authentic samples used for comparison with the isolated compounds were synthesized using literature methods: 1-methyladenosine (Jones and Robins, 1963), 3-methylcytidine (Brookes and Lawley, 1962), 1-methylinosine (Jones and Robins, 1963), 3-methyluridine (Miles, 1956), 5-methyluridine (Fox *et al.*, 1956). An synthetic sample of 1-methylguanosine was the gift of Dr. R. K. Robins, and a sample of pseudouridine was the gift of Dr. W. E. Cohn. 5-Methylcytidine, N^6-methyladenosine, and inosine were commercial preparations.

Synthetic samples of N^2-methylguanosine and N^2,N^2-dimethylguanosine were not available. The corresponding isolated nucleosides therefore were identified on the basis of the similarity of their ultraviolet spectra with those published by Smith and Dunn (1959a). More direct evidence for their identity was obtained by chromatographic comparison of the corresponding bases, released by hydrolysis in 1 N hydrochloric acid at 100° for 15 minutes, with synthetic samples obtained from Dr. G. E. Elion.

The group of N^6-(aminoacyl)adenosines was identified as previously described (Hall, 1964a). The 2-*O*-methylribosyl derivatives of the four common bases were identified as previously described (Hall, 1964b). Evidence for the identity of the fifth compound of this series, 2′-*O*-methylpseudouridine, remains at present only circumstantial (Hall, 1964b).

Adenosine-R. 9[2′(3′)-*O*-Ribosyl-β-D-ribofuranosyl]-adenine (Figure 7) was obtained from the fraction corresponding to peak 2 of the subfractionation of the main guanosine fraction (Figure 5). This subfraction was rechromatographed in solvent E (see Table I) and

[4] Major fraction 2 (adenosine) is not included in this series of figures since resolution of its components was not achieved (*vide infra*).

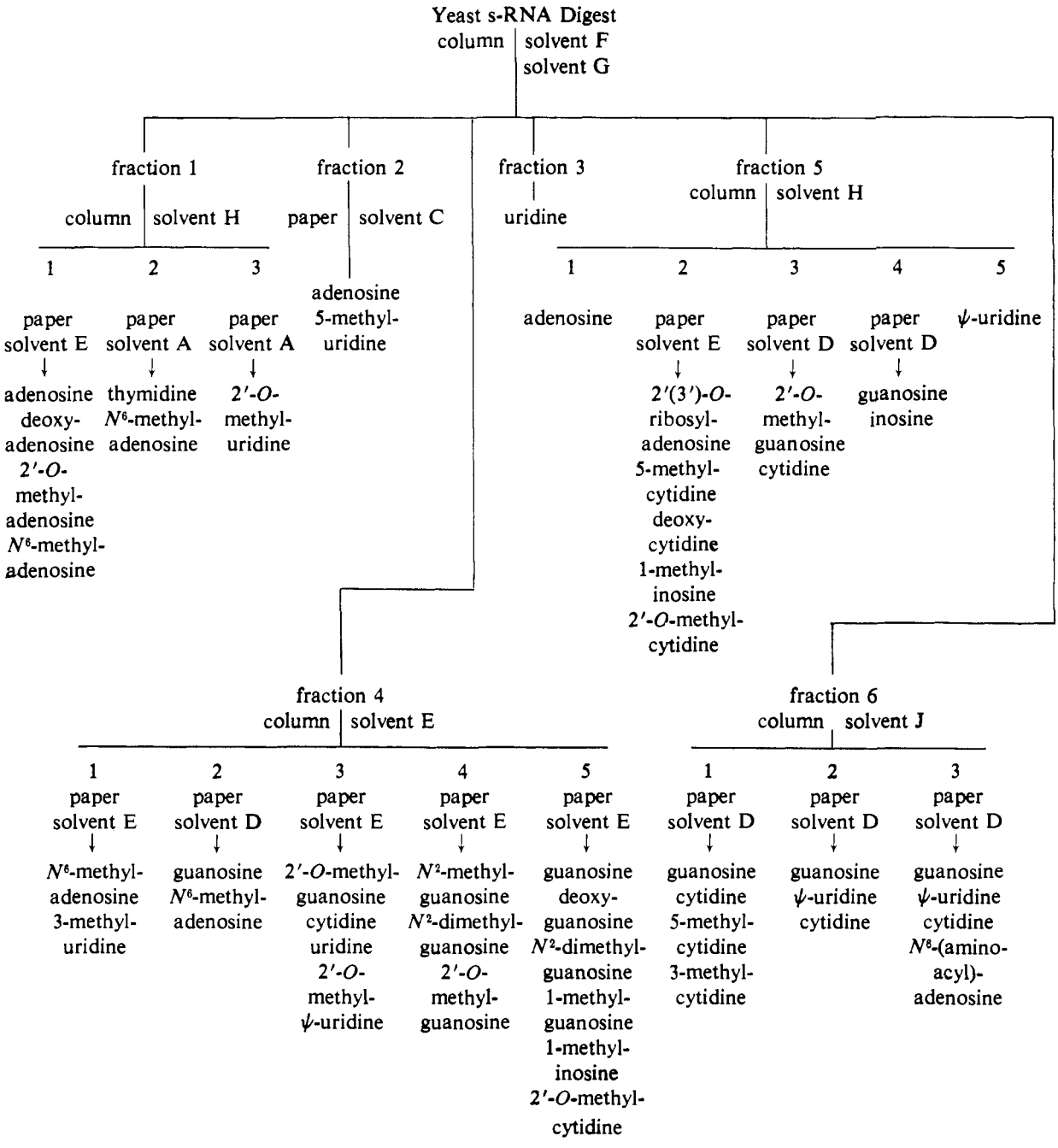

FIGURE 8: Isolation of the minor nucleosides from a digest of yeast s-RNA by partition chromatography on columns and on paper.

the material in the slowest-moving band was separated into two nucleosides by rechromatographing in solvent A for 24 hours. The faster-moving nucleoside was identified as 5-methylcytidine. The slower-moving component was homogeneous on the basis of paper chromatography in four solvent systems (Table III), and, since it was not recognizable as a known nucleoside, for purposes of discussion it was labeled *adenosine-R*. It has been assigned the structure 2′(3′)-*O*-ribosyladenosine, on the basis of the following criteria:

ULTRAVIOLET ABSORPTION SPECTRA. The spectra of adenosine-R measured at *p*H 1.5, 7.0, and 11.5 are identical with those of adenosine.

ACID HYDROLYSIS. Adenosine-R was heated at 100° in 0.1 N hydrochloric acid for 2 hours. Chromatography of the reaction mixture in five solvent systems showed a

TABLE II: Minor Nucleosides Isolated from Yeast s-RNA.[a]

Nucleoside	Total Amount Obtained from 11.5 g of Mixed Nucleoside in the Digest of s-RNA (mg)	Moles/1000 Moles of Total Nucleosides in the Digest	Previous Reference
N^6-Methyladenosine	48.7	3.9	Littlefield and Dunn (1958)
2′(3′)-*O*-Ribosyladenosine (adenosine-R)	2.4	0.13	
N^6-(Aminoacyl)adenosines	5.7	0.34	Hall (1964a)
1-Methylguanosine	12.0	0.90	Smith and Dunn (1959a)
N^2-Methylguanosine	1.9	0.14	Smith and Dunn (1959a)
N^2,N^2-Dimethylguanosine	32.9	2.36	Smith and Dunn (1959a)
3-Methylcytidine	2.0	0.18	Hall (1963b)
5-Methylcytidine	9.6	0.84	Dunn (1960)
3-Methyluridine	1.1	0.1	Hall (1963b)
5-Methyluridine	78	6.9	Littlefield and Dunn (1958)
Pseudouridine	488	45.0	Cohn (1960)
Inosine	26.0	2.2	Hall (1963d)
1-Methylinosine	5.1	0.4	Hall (1963d)
2′-*O*-Methyladenosine	3.4	0.28	Hall (1964b) Smith and Dunn (1959b)
2′-*O*-Methyluridine	3.4	0.29	Hall (1964b)
2′-*O*-Methylguanosine	40.2	3.1	Hall (1964b)
2′-*O*-Methylcytidine	13.0	1.1	Hall (1964b)
2′-*O*-Methylpseudouridine	0.1	0.009	Hall (1964b)

[a] Five additional compounds not detected in the present work have been isolated from the hydrolysates of RNA of yeast and/or other tissues: N^6,N^6-dimethyladenine and 2-methyladenine (Littlefield and Dunn, 1958), 1,5-diribosyluracil (Lis and Lis, 1962), 7-methylguanine (Dunn, 1963), and N^2-ribosylguanine (Hemmens, 1964; Shapiro and Gordon, 1964).

single spot corresponding to adenine. The ultraviolet absorption spectra of this product corresponded to those of adenine.

PARTIAL ACID HYDROLYSIS. Adenosine-R was heated at 100° in 0.1 N hydrochloric acid for 10 minutes. Chromatographic examination of this reaction mixture in solvent E revealed three ultraviolet absorbing spots corresponding to (a) starting material, (b) adenosine, and (c) adenine.

QUALITATIVE SUGAR ANALYSIS. The chromatograms of the acid-hydrolyzed compound (see Acid Hydrolysis) were sprayed with aniline hydrogen phthalate (Partridge, 1949). In each case a single spot corresponding to ribose appeared. Solvent E is particularly useful for the definitive separation of sugars (Hall, 1962b).

QUANTITATIVE SUGAR ANALYSIS. An aqueous solution containing 0.044 μmole of adenosine-R per cc (estimated spectrophotometrically, $\epsilon = 14.6$) was analyzed by the orcinol method (Dische, 1955). It contained 0.092 μmole of pentose per cc; that is, the ratio of ribose to base is 2:1.

ELECTROPHORESIS. At 1000 v (22 v/cm) for 5 hours, the distance moved from the origin was as follows: in 0.05 M glycine, *p*H 9.2, −4.4 cm for both adenosine and adenosine-R; in 0.05 M glycine–0.05 M borate, *p*H 9.2, +13.5 and +11.7 cm for adenosine and adenosine-R, respectively.

PERIODATE UPTAKE. The periodate uptake of adenosine-R and that of adenosine were measured under identical conditions spectrophotometrically (Dixon and Lipkin, 1954). An aqueous solution of adenosine and one of adenosine-R were prepared, each with an OD at 260 mμ of 0.61 which corresponds to 0.0418 μmole/cc, $\epsilon = 14.6$. The oxidation procedure was carried out as follows: One cc of nucleoside solution was placed in a 10-mm cuvet in a Cary Model 14 spectrophotometer at 25° and at time zero 1 cc of water containing 0.24 μmole of sodium periodate was added. The decrease in OD at 225 mμ was followed for 1 hour. The rate of decrease for each nucleoside was similar and the reaction had stopped essentially after 45 minutes.

The optical densities at 225 mμ for adenosine and adenosine-R, respectively, were as follows: at start, 0.50, 0.63; after 15 minutes, 0.33, 0.46; after 1 hour, 0.30, 0.39; net change, 0.20, 0.24. *Note:* The higher

TABLE III: R_F Values of Nucleosides Isolated from Yeast RNA.

Compound	R_F (solvents) A	B	C	D	E
Adenosine	0.27	0.58	0.34	0.54	0.35
2′-*O*-Methyladenosine	0.49	0.67	0.53	0.71	0.57
N^6-Methyladenosine	0.47	0.73	0.51	0.72	0.57
N^6-(Aminoacyl)adenosine	0.19	0.64	0.34	0.61	0.11
Cytidine	0.15	0.49	0.47	0.44	0.10
2′-*O*-Methylcytidine	0.30	0.67	0.64	0.62	0.19
3-Methylcytidine	0.34	0.62	0.54	0.62	0.04
5-Methylcytidine	0.20	0.51	0.48	0.44	0.07
Guanosine	0.05	0.47	0.30	0.26	0.13
2′-*O*-Methylguanosine	0.21	0.64	0.42	0.43	0.30
1-Methylguanosine	0.17	0.54	0.34	0.47	0.18
N^2-Methylguanosine			0.45	0.39	0.21
N^2,N^2-Dimethylguanosine	0.29	0.59	0.42	0.45	0.19
Inosine	0.05	0.53	0.33	0.37	0.14
1-Methylinosine	0.21	0.61	0.48	0.56	0.22
Uridine	0.10	0.62	0.64	0.38	0.27
2′-*O*-Methyluridine	0.30	0.73	0.86	0.52	0.58
3-Methyluridine	0.45	0.75	0.88	0.70	0.59
5-Methyluridine	0.27	0.68	0.74	0.54	0.44
Pseudouridine	0.04	0.50	0.52	0.29	0.11
2′-*O*-Methylpseudouridine			0.56	0.75	0.18
2′(3′)-*O*-Ribosyladenosine	0.18		0.42	0.45	0.08

starting OD for adenosine-R was owing to the presence of nonspecific absorption of this solution in the region of 225 mμ.

The periodate uptake was also measured at *p*H 5.5 volumetrically by the classical arsenite-iodine method. In this case 0.42 μmole of adenosine-R consumed 0.48 μmole of periodate. Thus adenosine-R under these conditions appears to consume slightly more than 1 mole of periodate per mole than adenosine, which suggests that one pair of vicinal hydroxyl groups in the molecule is blocked.

Discussion

The task of hydrolyzing the RNA molecule presents a considerable challenge if the structural integrity of all its constituent nucleosides is to be preserved. The enzymic procedure used in the present work is very mild compared with chemical degradation; nevertheless it possesses certain inherent disadvantages. It is possible that some minor nucleosides may be retained in difficultly hydrolyzable oligonucleotide fragments and thus would not be detected in the chromatographic procedures. Chemical changes in the structure of certain nucleosides may occur during the period of hydrolysis. It is known, for example, that under slightly alkaline conditions 1-methyladenosine can rearrange to N^6-methyladenosine (Elion, 1962) and 7-methylguanosine can be converted to 2-amino-4-hydroxy-5-methylformamido-6-ribosylaminopyrimidine (Haines *et al.*, 1962; Jones and Robins, 1963). Further, the N^6-(aminoacyl)adenosines appear to be mostly hydrolyzed to adenosine and amino acid on exposure for a long period to aqueous solution at *p*H 8.6 (Hall, 1964a).

The column elution patterns obtained are reproducible for a given sample. There is the possibility that individual partition coefficients may vary slightly depending on the composition of the starting mixture. Thus subtle differences in elution patterns of the minor components might occur with another type of RNA. For this reason, as well as consideration of the fact that RNA contains many unidentified components, it is essential to characterize rigorously every compound isolated. The sample of s-RNA used in this study contained a trace amount of DNA which was degraded to its constituent deoxyribonucleosides under the conditions of hydrolysis. These four compounds were readily separated from the other components of the digest as is seen in Table I and Figure 8.

Since the isolation procedure involves several steps, the absolute amount of the minor nucleosides recovered and recorded in Table II was not quantitative. It is difficult to calculate accurately the percentage recovery, although for some of the minor nucleosides it is possible to make a comparison with the amounts recovered in other types of procedures. Bell *et al.* (1964) report the isolation by an ion-exchange procedure of approximately 4 mole % of methylated base nucleotides from an alkaline hydrolysate of yeast s-RNA. In the present study approximately 1.6 mole % of the methylated

base nucleosides was obtained. Amounts of the 2′-*O*-methylribonucleosides isolated varies with individual members of the group when compared with the amounts obtained by a more direct procedure (Hall, 1964b). The amounts obtained of 2′-*O*-methyladenosine and 2′-*O*-methylguanosine are equivalent to those of the previous study whereas the amounts obtained of 2′-*O*-methyluridine and 2′-*O*-methylcytidine are less than half of that of the other procedure.

Isolation of the previously unknown nucleoside, adenosine-R, raises the question whether it is a genuine constituent of s-RNA or whether it arises from a fraction isolated fortuitously with the s-RNA. The latter possibility cannot be excluded and other lines of evidence will be needed to settle this point. The possibility that the snake venom is somehow responsible has been considered. On the basis of two control experiments, this compound does not appear either to originate from the *Crotalus adamanteus* venom or to be formed by its action. A solution of 2 g of *Crotalus adamanteus* venom was incubated at 37° for 18 hours and then lyophilized. The residue was examined by the partition column procedure outlined in Figure 1 and no adenosine-R was detected. In the second experiment 1 g of adenosine, 1 g of D-ribose, 100 mg of phosphoric acid, 1 g of *C. adamanteus* venom, and 20 mg of alkaline bacterial phosphatase were incubated for 18 hours at 37° in aqueous solution at *p*H 8.6. Examination of this mixture by the chromatographic procedure of Figure 1, which should have enabled detection of 100 μg of adenosine-R, revealed nothing unusual.

The structure of adenosine-R suggested by the available evidence is that shown in Figure 7. The similarity of the ultraviolet absorption spectra with those of adenosine makes it doubtful that the second ribose residue is attached at any point on the adenine residue since such substitution would presumably cause a noticeable change in the ultraviolet spectra. This particular point is raised since one of the intermediates in the biosynthesis of histidine is 1-ribosyladenosine 5′-triphosphate (Ames *et al.*, 1961). This compound possesses spectra which are sufficiently distinct from those of adenosine-R to eliminate it from consideration. Alternative structures for adenosine-R cannot be excluded. It has been assumed that this compound consists solely of adenine and ribose; however, it could contain additional undetected residues. If true, this fact would necessitate reinterpretation of the data.

In conclusion, the procedure for isolation of minor nucleosides presented in this paper is a flexible tool. It has proved its value for the detection and isolation of several minor nucleosides. Its application to the examination of hydrolysates of RNA from different sources will very probably reveal existence of more as yet unknown nucleosides. This possibility continues to receive our attention.

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