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Note

Simple ion-exchange column fractionation of ribonucleotides

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Hydrolysis of ribonucleic acids by alkali and by many enzymes yields the nucleoside-2′ (or -3′)-monophosphates while snake venom phosphodiesterase yields the 5′-isomers. Numerous methods for the separation of the individual ribonucleotides have been described, including several using ion-exchange column chromatography[1–7]. We have simplified the procedures of Katz and Comb[3] to provide a rapid and simple two-column chromatographic separation of the major nucleotides in ribonucleic acid (RNA) hydrolyzates, whether present as the 2′- (or 3′-) or 5′-isomers.

EXPERIMENTAL

The 2′- (or 3′-) and 5′-monophosphates of adenosine (AMP), guanosine (GMP), cytidine (CMP) and uridine (UMP) were obtained from Sigma, St. Louis, Mo., U.S.A. Ion-exchange resins AG 50W-X4 (200–400 mesh) and AG 1-X8 (200–400 mesh) were obtained from Bio-Rad Labs., Richmond, Calif., U.S.A. The resins were washed copiously with water prior to use.

Column preparation

Columns were prepared in glass tubes (1 cm I.D.). The bed volumes of the AG 50W and AG 1 columns were 5.3 and 1.8 ml respectively, corresponding to column heights of 6 and 2 cm. The AG 50W column was converted into the H^+ form by treatment with 3 *N* hydrochloric acid, was washed with water until neutral and then equilibrated with 0.5 *N* hydrochloric acid for 1 h. The AG 1 column was used in the formate form after treatment with 3 *N* formic acid followed by water until neutral.

Extraction and hydrolysis of RNA

RNA was extracted from freeze-thawed *Chlorella pyrenoidosa* cells with 10% cold perchloric acid in an ice bath (three 2 h extractions). The acid extracts were treated with potassium hydroxide to pH 8–8.5 at 0°. The resulting precipitate of potassium perchlorate was removed by centrifugation. RNA was precipitated from the supernatant with 2 volumes of cold ethanol, washed with 0.001 *M* phosphate buffer (pH 6.7), reprecipitated and dried by passage through the series ethanol, ethanol–diethyl ether and finally diethyl ether[8].

Elution procedure

The sample (30–60 μl) of mixed nucleotides was applied to the top of the AG 50W column and allowed to drain into the resin bed. Elution was commenced using 0.05 *N* HCl at a flow-rate of 60–65 ml/h. The eluate was monitored continuously at 254 nm by passing it through the flow-cell of a UV analyzer. Elution was continued until the first peak (UMP) was completely eluted, as indicated by the UV monitor. This required a maximum of 5–6 min, after which the eluent was changed to water with the same flow-rate as for the 0.05 *N* HCl. A second peak (GMP) was eluted within a further 6–7 min.

After complete removal of GMP, elution with water was continued at the same flow-rate, but the eluate, after passing through the UV analyzer, was fed directly on to the top of the AG 1 (HCO_2^-) column. A single broad peak consisting of CMP and AMP, eluted from the AG 50W resin, was thus transferred directly to the AG 1

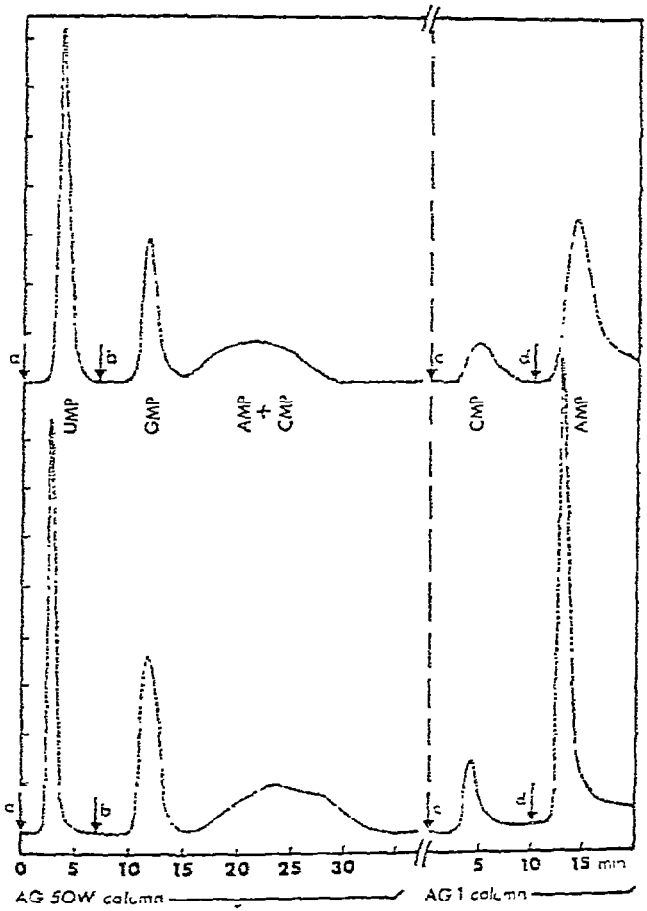

Fig. 1. Separation of ribonucleoside 2′- (or 3′-)monophosphates (upper trace) and 5′-isomers (lower trace) on AG 50W-X4 (H^+) and AG 1 (HCO_2^-) columns in sequence. The elution was monitored with an Isco UA-2 flow-cell and UV monitor. Ordinate, recorder units. The arrows indicate the start of elution with (a) 0.05 *N* HCl, (b) water, (c) 0.1 *N* formic acid and (d) 0.8 *N* formic acid; flow-rate 60–65 ml/h.

column. CMP was then eluted over a period of *ca.* 7 min with the aid of 0.1 *N* formic acid. After the complete elution of CMP, the eluent was changed to 0.8 *N* formic acid, and AMP was then eluted in a further 7 min.

After elution of the mixed CMP–AMP peak, the AG 50W resin was regenerated by treatment with three bed volumes of 0.1 *N* HCl and one bed volume of water, and finally washed with 0.05 *N* HCl for 30 min in order to allow it to equilibrate prior to re-use. The AG 1 column was regenerated by treatment with six bed volumes of 2 *N* formic acid, followed by washing with water to neutrality.

RESULTS AND DISCUSSION

The separation of mixtures of ribonucleotide standards by the procedures described is shown in Fig. 1. The traces of the UV monitor show the separation of the four 2'- (or 3'-)monophosphates (upper trace) and the comparable separation of the four 5'-isomers (lower trace). The separation of the 2'- (or 3'-)monophosphates resulting from the alkali hydrolysis of *Chlorella* RNA is shown in Fig. 2, and the results of a typical base analysis are given in Table I.

Eluates of the four peaks were subjected to co-chromatography on 3MM paper using the solvent system isopropyl alcohol–6 *N* hydrochloric acid (2:1) for 26 h at room temperature[8]. This confirmed the identity of the four nucleotides. Phosphorus analyses showed that the first eluate (UMP) also contained traces of orthophosphate derived from the buffer used in washing the RNA sample.

Both figures illustrate the clear separation of the four major ribonucleotides, permitting their quantitative estimation, either directly by integration of the areas of the peaks recorded by the UV analyzer or by conventional spectrophotometry. The

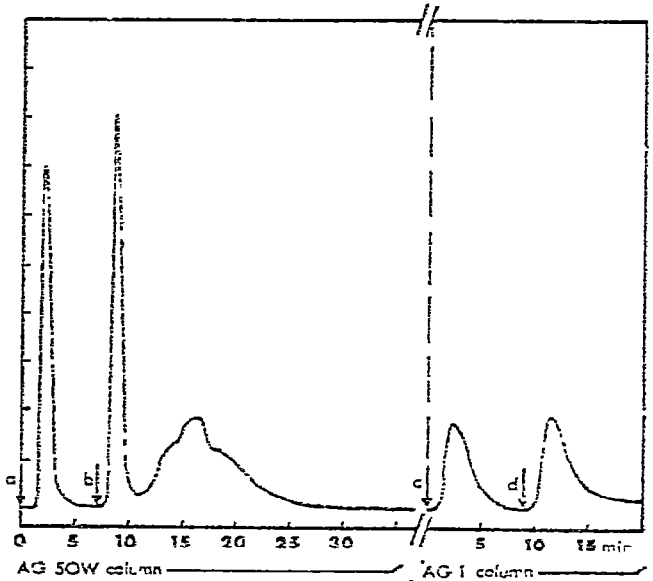

Fig. 2. Separation of nucleoside monophosphates in an alkali hydrolyzate of *Chlorella* RNA. The elution was monitored with a LKB Uvicord II instrument. Other conditions as in Fig. 1.

TABLE I

BASE COMPOSITION OF RNA EXTRACTED FROM *Chlorella pyrenoidosa* CELLS

The extracts were hydrolyzed with 0.3 *N* KOH for 18 h at 37°. The data are presented as the means of two determinations.

Nucleotide	*Molar percentage of RNA*
AMP	24.8
GMP	26.2
CMP	24.6
UMP	24.4

entire separation procedure can be completed within 60 min at room temperature and is a modification of that of Katz and Comb[3], with improvements in the resolution of the chromatographic separations, their extension to the fractionation of the 5'-nucleotides, and the utilization of direct quantitative absorptiometry in order to follow the elution process.

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