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Note

Determination of nucleotides in adenosine-treated cultured lymphoid cells by high-performance anion-exchange column chromatography

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Excessive phosphorylation of adenosine kinase results in a lethal interruption of pyrimidine synthesis at a late stage of the biosynthetic pathway in cultured mammalian cells [1, 2]. Added adenosine is readily phosphorylated and raises the levels of adenine nucleotides, while inhibiting the synthesis of pyrimidine nucleotides. The inhibition of orotidine 5′-phosphate decarboxylase by AMP may also explain the inhibition of cell growth as a consequence of the addition of adenosine to cell culture [3]. Human glutamine phosphoribosyl—pyrophosphate amidotransferase is most effectively inhibited by AMP and GMP, but the corresponding diphosphates are only about one half as effective as inhibitors, and triphosphates are about one quarter as effective as the monophosphates [4]. However, Brenton et al. [5] reported that ATP concentrations in cultured human lymphoid cells are 6—10 times the ADP concentrations and 20—50 times the combined AMP/IMP concentrations. They indicated that, in view of the relatively elevated concentrations of the diphosphates and triphosphates, a regulating role specifically for AMP and GMP within the cell is less certain.

In the present study, the simultaneous determination of the ribonucleoside monophosphate, diphosphate and triphosphate by high-performance liquid chromatography (HPLC) shows the relatively high concentration of AMP. It seems that the depletion of UMP in adenosine-treated lymphoid cells results in the increase of the AMP level.

EXPERIMENTAL

Chemicals

Analytical-grade ammonium acetate, acetic acid, adenosine, adenine, cyto-

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sine, guanosine, thymine, uracil and uridine were purchased from Wako (Doshumachi, Higashi-ku, Osaka, Japan). The purine and pyrimidine nucleotides, such as CMP, AMP, UMP, GMP, IMP, TMP, ADP, UDP, GDP, IDP, CTP, ATP, UTP, GTP and ITP, were purchased from P.-L. Biochemicals (Milwaukee, WI, U.S.A.). cAMP and CDP were purchased from Sigma (St. Louis, MO, U.S.A.).

High-performance anion-exchange column chromatography

A Shimadzu Model LC-4A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) was used for the nucleotide analysis. A jacketed, stainless-steel column, 250 × 4.6 mm I.D. was used, and prepacked with MCI CDR-10 anion-exchange resin (Mitsubishi Kasei, Tokyo, Japan) [6] with a mean particle size of 7 μm (Umetani packed column). The column temperature was maintained at 60°C with a Shimadzu CTO-2AS constant-temperature circulator. A JASCO UVIDEC-100 UV spectrophotometer (Japan Spectroscopic, Tokyo, Japan) and Chromatopac C-RIA (Shimadzu) were used for monitoring and recording of the UV signal and for the determination of peak areas.

A 40-μl cell extract sample was eluted using a linear gradient from 100% of the low-strength eluent to 100% of the high-strength eluent at a flow-rate of 0.8 ml/min. The low-strength eluent was water and the high-strength eluent was 6.0 *M* ammonium acetate buffer (pH 4.4). With the increase of buffer concentration, the inlet pressure was raised from 70 to 135 kg/cm^2. The eluent was monitored at 254 nm at 0.16 a.u.f.s. After analysis, the column was washed with distilled water for 30 min.

Cell culture

Mouse leukaemic lymphoid cells were used [7]. The cells were grown in Dulbecco-Vogt's modification of Eagle MEM (Nissui) supplemented with streptomycin (75 mg/l), penicillin (50 mg/l), glucose (1.5 g/l), sodium bicarbonate (1.3 g/l) and 10% horse serum.

Preparation of cell extracts

The extraction of nucleotides was done by the method of Khym [8]. Acid-soluble nucleotides were extracted from cells with 0.5 *M* perchloric acid. Equal volumes of cell extract sample and amine—Freon solution (ca. 0.5 mol/l of Alamine 336) were mixed gently for 3—4 min. After neutralizing the acid solutions, nucleotides, nucleosides and purine and pyrimidine bases remained in the aqueous (top) phase, which had a pH of 4—5. The aqueous solution was withdrawn with a capillary, concentrated to dryness in a rotary evaporator and then dissolved in 0.5 ml of water.

RESULTS

A chromatogram of a standard mixture of purine and pyrimidine nucleotides is shown in Fig. 1. The nucleosides and its bases are eluted within 10 min. All the ribonucleoside monophosphates, diphosphates and triphosphates, except for UMP, TMP, ADP and UDP, are readily separated. Fig. 2 shows the pattern given by adenosine-treated cells. When cultured cells were incubated in a

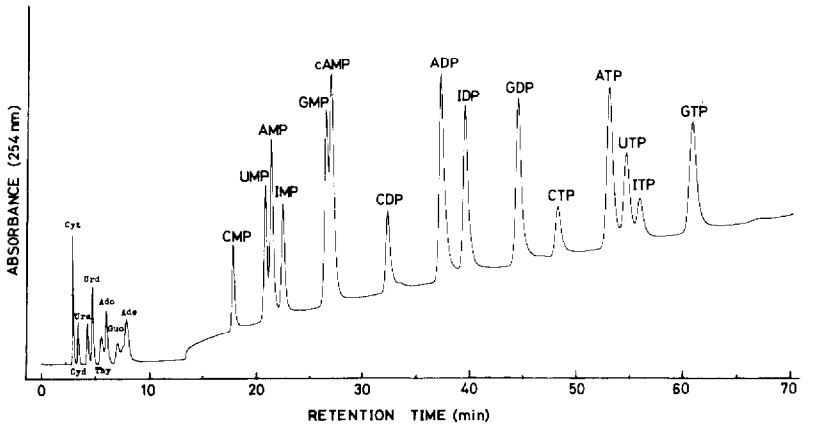

Fig. 1. Chromatogram of a standard mixture of ribonucleosides, ribonucleotides and bases, each at a concentration of 0.3 μg (Cyt = cytosine), 0.2 μg (Cyd = cytidine; Ura = uracil; Urd = uridine; Thy = thymine; Ado = adenosine; Guo = guanosine; Ade = adenine), 1 μg (AMP; IMP), 2 μg (CMP; UMP; GMP; cAMP), 4 μg (CDP; ADP; IDP; GDP; CTP; ATP; UTP; ITP; GTP) per 10 μl. Column: MCI GEL CDR-10, 7 μm average particle size (250 × 4.6 mm I.D.); eluent: acetate buffer (pH 4.4), 0—6.0 *M* linear gradient; flow-rate: 0.8 ml/min; temperature: 60° C; detection: UV, 254 nm.

medium containing adenosine at a concentration of 0.1 or 0.3 m*M*, the AMP peak was obviously higher than in the control (Fig. 2A) whereas the UMP peak was notably reduced (Fig. 2B and C).

Table I shows the amounts of the nucleoside monophosphates, diphosphates and triphosphate obtained by measurement of the areas under the peaks of Fig. 2. The presence of 0.1 m*M* adenosine in the medium led to 57, 32 and 18% increases in the pools of AMP, ADP and ATP, respectively, compared with the control. Conversely, UMP was reduced by ca. 21%, while UTP remained practically constant. A similar tendency in the nucleotide contents was observed in the experiment with 0.3 m*M* adenosine-treated cells. CDP and GTP were also increased, although no change in the GMP or GDP level was observed.

DISCUSSION

The toxicity of adenosine depends on its direct conversion to AMP by adenosine kinase [1]. Increased levels of dATP were believed to cause cytotoxicity by inhibiting ribonucleotide reductase and depleting cells of other deoxynucleoside triphosphates required for DNA synthesis [9—11]. However, since the concentrations of adenine deoxynucleotides in cells are generally two orders of magnitude lower than those of adenine ribonucleotides, and there is a feedback inhibition of ribonucleotide reductase, one cannot expect large quantities of dATP to be synthesized [12, 13].

Adenine and adenosine concentrations of 0.1 m*M* in the medium produced modest increases in the intracellular adenine nucleotides of cultured human lymphoid cells, which were grown in MEM supplemented with adenine or adenosine, while causing decreases in the uridine nucleotides and no decrease in CTP [5]. In the present study, the levels of UTP, CMP and CDP were not

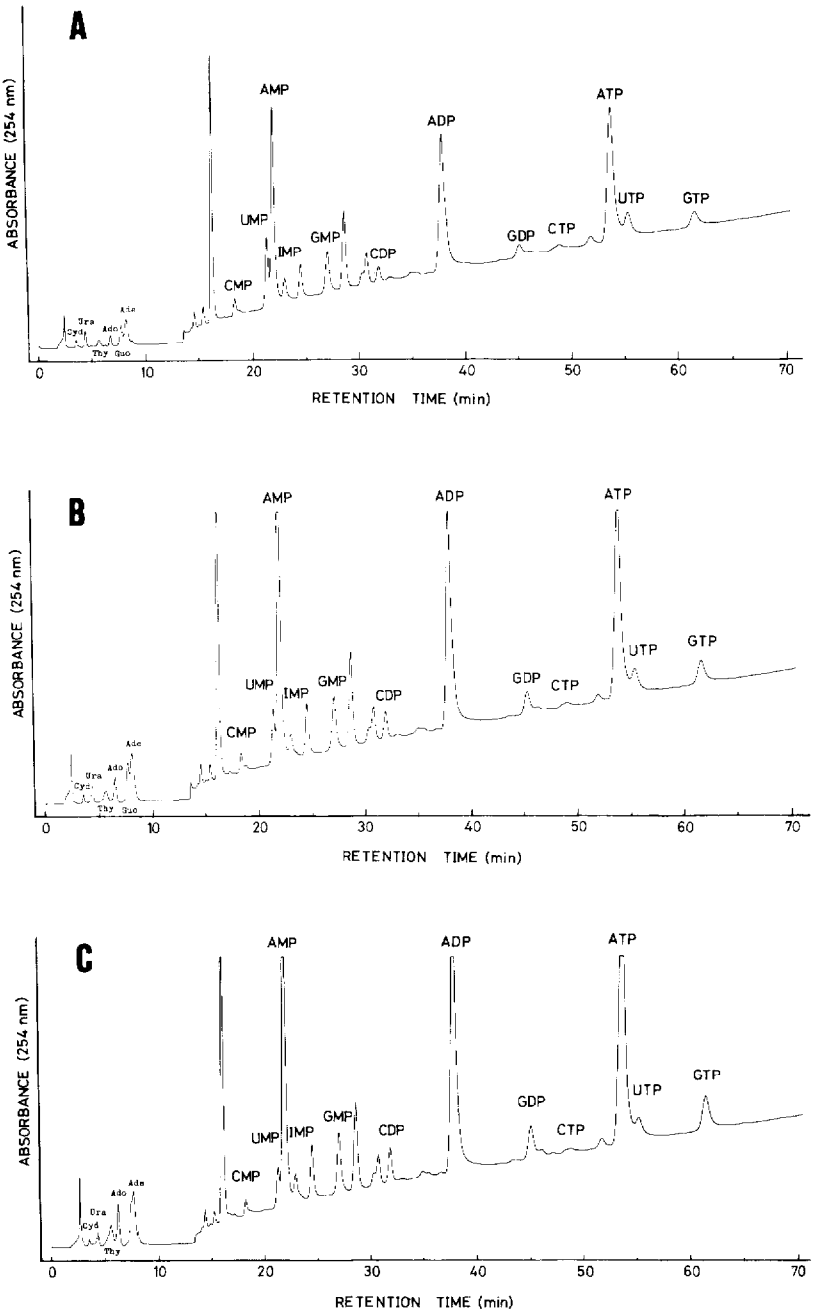

Fig. 2. Chromatograms of SC-1 cell extract analysed using the anion-exchange resin column. The conditions are the same as in Fig. 1. (A) Control; (B, C) cell extracts from SC-1 cells treated with 0.1 and 0.3 m*M* adenosine for 6 h, respectively.

decreased in the presence of extracellular adenosine for 6 h, but there was a noticeable decrease in the UMP level.

In spite of the high 5'-nucleotidase activity in lymphoid cells [14, 15], AMP concentration was ca. 30% of the ATP concentration (Table I). Since

TABLE I

NUCLEOTIDE POOL IN SC-1 CELLS TREATED WITH ADENOSINE

Nucleotide	Amount (mean ± R.S.D., n = 4) (nmol per 10^8 cells)		
	Control	0.1 m*M* Adenosine	0.3 m*M* Adenosine
AMP	22.5 ± 1.4	35.4 ± 2.1	37.9 ± 2.3
ADP	40.5 ± 2.4	53.5 ± 3.2	66.9 ± 4.0
ATP	76.3 ± 4.6	90.2 ± 5.4	126.2 ± 7.6
IMP	4.1 ± 0.3	4.1 ± 0.2	4.8 ± 0.3
GMP	7.2 ± 0.4	7.9 ± 0.5	8.4 ± 0.5
GDP	6.1 ± 1.0	13.1 ± 0.8	17.2 ± 1.0
GTP	9.0 ± 0.5	11.9 ± 0.7	17.0 ± 1.0
UMP	20.1 ± 1.2	15.9 ± 1.0	11.6 ± 0.7
UDP	–	–	–
UTP	9.7 ± 0.6	9.7 ± 0.6	8.1 ± 0.5
CMP	9.8 ± 0.6	10.9 ± 0.7	10.3 ± 0.6
CDP	7.3 ± 0.4	9.1 ± 0.5	11.6 ± 0.7
CTP	–	–	–

added uridine could reverse the growth-inhibiting effect of adenosine in SC-1 cells [16], the toxicity of adenosine resulted in the interruption of pyrimidine synthesis.

Adenosine is clearly identified in the serum of patients deficient in adenosine deaminase [17], and it serves as a marker for this enzyme defect known to result in severe alterations of the immune system.

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