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DETERMINATION OF NUCLEOTIDES, NUCLEOSIDES AND NUCLEOBASES IN CELLS OF DIFFERENT COMPLEXITY BY REVERSED-PHASE AND ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Procedures are presented for the analysis of profiles of purine and pyridine compounds in human and rabbit red blood cells by reversed-phase high-performance liquid chromatography and in Ehrlich ascites tumour cells of mouse by ion-pair high-performance liquid chromatography. These compounds are present in rabbit erythrocytes in higher concentrations than in human blood cells, and in rabbit reticulocytes the concentration of purine compounds is still higher. During glucose-free incubation, human red cells accumulate adenosine and adenine in the presence of coformycin owing to the inhibition of adenosine and AMP deamination. Ehrlich ascites tumour cells lose major portions of purine mono-, di- and triphosphates between the seventh and eleventh day after inoculation into mouse peritoneal cavities.

INTRODUCTION

The development and optimization of high-performance liquid chromatography (HPLC) for the separation and determination of purine and pyrimidine bases, nucleosides, and nucleotides and pyridine dinucleotides is a field of intensive research. Various modes of this technique are available. Ion-exchange HPLC widely applied to nucleotide analysis is restricted by the need for highly concentrated elution buffers and long analysis times [1]. The separation of nucleosides

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and bases by the ion-exchange mode has been accomplished on ODS columns with phosphate-buffered methanol eluents [2–5]. Currently reversed-phase and ion-pair chromatography have superseded the ion-exchange procedures; the predominant reason seems to be the possibility of simultaneous determination of nucleotides, nucleosides and nucleobases [6–9].

To choose an appropriate HPLC procedure for the analysis of biological samples one has to consider the complexity of the nucleotide metabolism, e.g. the variety of enzymatic pathways, turnover rates, concentration profiles. This paper presents results of determinations of nucleotides, nucleosides and nucleobases in cells of different complexity by use of reversed-phase and ion-pair HPLC. Concentration profiles are examined of rabbit red blood cells of different maturity and of Ehrlich mouse ascites tumour cells at two stages of proliferation. Additionally the influence of coformycin on the purine nucleotide degradation of human erythrocytes in glucose-free media was studied.

EXPERIMENTAL

Chemicals

All reference standards (purine bases; purine and pyridine compounds) of the highest analytical grade available were purchased from Boehringer (Mannheim, F.R.G.), Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogenphosphate and disodium hydrogenphosphate were from Ferak (West Berlin), triethanolamine (TRA) from Boehringer, and ammonium dihydrogenphosphate was from Fisher (Fair Lawn, NJ, U.S.A.). Methanol and acetonitrile were obtained from Merck (Darmstadt, F.R.G.). Coformycin was from Calbiochem (La Jolla, CA, U.S.A.) and tetrabutylammonium phosphate (TBA, PIC reagent A) from Waters Assoc. (Milford, MA, U.S.A.). Water for HPLC analyses was glass-distilled twice.

HPLC

Two HPLC instruments were used: (A) Du Pont (Wilmington, DE, U.S.A.) and (B) Waters Assoc.

System A. The chromatographic system was assembled from Series 8800 gradient liquid chromatographic system, 850 column compartment, 8800 pump module, absorbance detector (254 nm) and variable-wavelength detector (280 nm) (all Du Pont) and an SP 4100 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.) for peak-area calculations. The following columns were used: Zorbax ODS (Du Pont), 7 μm, 250×4.6 mm I.D.; stainless-steel guard column, 50×4.6 mm I.D. The mobile phase consisted of 0.15 *M* potassium dihydrogenphosphate–disodium hydrogenphosphate buffer, pH 6.85 (buffer A), and the same phosphate buffer containing 20% methanol (v/v, buffer B). The gradient was: 7 min 100% buffer A, 5 min 0–20% buffer B, 5 min 20–50% buffer B, 20 min 50% buffer B. The flow-rate was 1 ml/min. At the end of gradient elution the system was flushed with buffer A for 15 min. The temperature was 18–22°C. The peaks were identified as follows: (i) some of the biological samples were divided, and to the aliquot was added one of each of the sixteen reference compounds before

the HPLC analysis was performed; (ii) the UV absorbance of biological samples as well as of a mixture containing all the reference compounds was measured at two wavelengths: 254 and 280 nm. For quantitation several diluted mixtures of constant proportions of all reference compounds as well as mixtures of various proportions of these compounds were injected onto the column. The mixture containing the reference compounds was treated with perchloric acid, neutralized and analysed in the same manner as the biological samples.

Buffer solutions were filtered through 0.45-μm HA membrane filters, and acetonitrile and methanol through 0.45-μm HF filters from Millipore Intertech (France). Before use the buffer solutions were degassed with helium.

System B. This consisted of an M510 and an M45 solvent-delivery system Model 441, a Lambda-Max Model 481 absorbance detector, a U6K Universal liquid chromatograph injector, a Model 660 solvent programmer and a Z-Module RCSS radial compression separation system. The absorbance was recorded at 254 and 280 nm, and the peaks were integrated by a 730 data module (Waters Assoc.). The column was a Nova Pak C_{18} plastic cartridge (100×8 mm I.D.) (Waters Assoc.) and the flow-rate was 2.50 ml/min. The eluent was a 0.01 *M* ammonium dihydrogenphosphate buffer containing 2 m*M* TBA and acetonitrile (15%, v/v). The separation was carried out in the isocratic mode.

Cell preparation

Venous blood was drawn from young human beings and heparinized, and plasma and buffy-coat were removed by centrifugation. Subsequently the cell pellet was washed twice with glucose-free, isotonic triethanolamine hydrochloride buffer (pH 7.4). The haematocrit was adjusted to ~45%, and this erythrocyte suspension was used for in vitro incubation studies. The incubations were performed at 37°C.

Rabbit blood was taken from the ear vein. Reticulocytosis was induced by daily bleeding of 50–60 ml. At days 6–8 the reticulocyte count was 25–45%. Reticulocyte counts up to 70% have been achieved by centrifugation for 20 min at 500 *g*. The supernatant was removed, the top layer of cells with a high percentage of reticulocytes was taken, and the cells were suspended in cold isotonic saline solution.

The Ehrlich ascites cells of mice were withdrawn on the seventh and eleventh day after inoculation by puncture of the peritoneal cavity.

Extraction procedure and sample preparation

The cell suspensions were deproteinized with chilled 6% (v/v) perchloric acid, centrifuged for 10 min at 1200 *g* and neutralized with triethanolamine–potassium carbonate. After filtration of the sample, 50 μl of the supernatant were analysed by HPLC.

RESULTS AND DISCUSSION

Reversed-phase HPLC of human erythrocyte extracts

The elution profile of a standard mixture separated by reversed-phase HPLC is shown in Fig. 1.The purine mono-, di- and triphosphates, as well as the nucleo-

sides and bases applied, are readily separated within 40 min. This also holds true for the reduced and oxidized pyridine nucleotides.

Perchloric acid extracts of human erythrocytes were analysed by reversed-phase HPLC, and the chromatogram is shown in Fig. 2. In order to detect nucleobases, nucleosides and reduced pyridine nucleotides, the absorbance sensitivity was increased four-fold during the run. Table I summarizes data from washed human erythrocytes. The results are in fair accordance with those obtained by various chromatographic techniques [10–13].

Purine nucleotide catabolism in human erythrocytes

In previous investigations in this laboratory [14] a rapid degradation of adenine nucleotides occurred in erythrocytes under glucose deprivation. The catabolism of adenine nucleotides yields AMP, which is degraded by deamination and dephosphorylation via AMP deaminase and phosphatase, or vice versa via phosphatase and adenosine deaminase. In both routes inosine is produced, which is further converted into hypoxanthine and ribose 1-phosphate via nucleoside phosphorylase. Little is known about the relative importance of the two routes.

The present studies dealt with the analysis of the breakdown products under glucose deprivation in the presence of the inhibitor coformycin. During glucose-deprived incubation at 37°C in absence of coformycin, hypoxanthine accumulated (data not shown), but adenosine and adenine were not observed (Figs. 3 and 4). At low concentrations (1 μM) coformycin inhibits adenosine deaminase, and at higher concentrations (above 10 μM) it inhibits adenylate deaminase [15]. At 1 μM coformycin, adenosine accumulated and hypoxanthine production was diminished. Adenosine accumulated because adenosine deamination was blocked (Fig. 3), but accumulation of adenine could not be detected (Fig. 4). A further accumulation of adenosine at 10 μM coformycin can be explained by inhibition of AMP deaminase and a consequent increase of dephosphorylation of AMP to adenosine (Fig. 3). Recently, comparable findings were reported [15] about the effect of adenosine deaminase inhibition by deoxycoformycin. The formation of adenine may be due to the phosphorylysis of adenosine by nucleoside phosphorylase (Fig. 4), but adenine seems to be detectable only when adenosine has accumulated to a certain extent. The recent observation in this laboratory that a crude preparation of nucleoside phosphorylase from human erythrocytes converts adenosine into adenine (unpublished data) supports this conclusion.

Reversed-phase HPLC of rabbit reticulocytes

The maturation of reticulocytes to erythrocytes is accompanied by loss of organelles and energy-requiring processes, as well as the switch from aerobic to anaerobic ATP production [16,17]. Little is known about the changes in the nucleotide metabolism during this period. Fig. 5 shows the chromatogram of a perchloric acid extract taken from a suspension of rabbit red blood cells, containing 66.7% reticulocytes. Estimated blood values of different reticulocyte counts were extrapolated to values for pure erythrocytes or reticulocytes. In some experiments reticulocytes were further enriched by centrifugation. Table II gives a synopsis of these data.

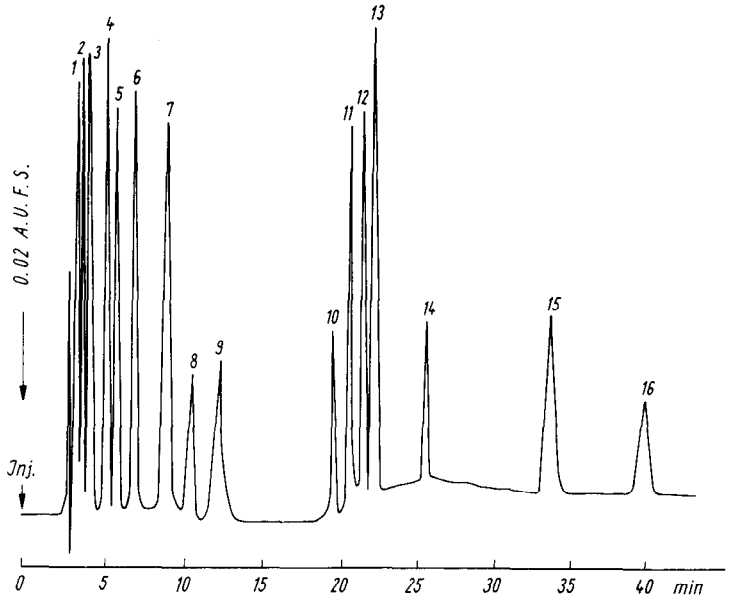


Fig. 1. Separation of a standard mixture containing 800 pmol of each substance. Separation conditions are described in Experimental (see *HPLC system A*). Peaks: 1 = GTP; 2 = GDP; 3 = IMP; 4 = ATP; 5 = ADP; 6 = AMP; 7 = $NADP^+$; 8 = xanthine; 9 = hypoxanthine; 10 = NADPH; 11 = inosine; 12 = guanosine; 13 = NAD^+; 14 = NADH; 15 = adenosine; 16 = adenine.

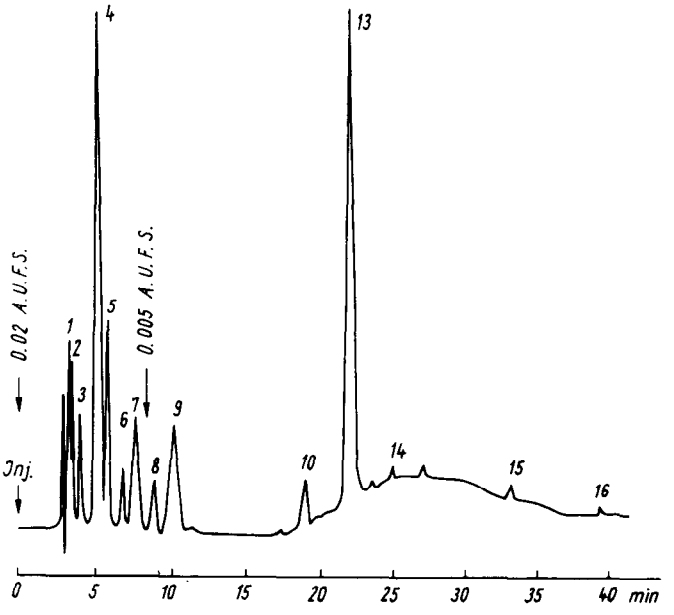
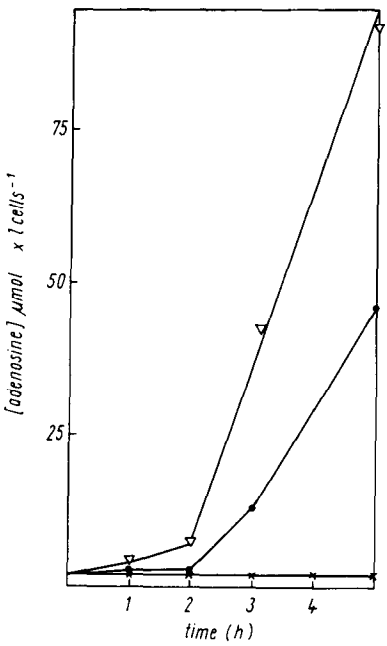


Fig. 2. Separation of nucleotides from an extract of human red blood cells. In this chromatogram inosine and guanosine were below the detection limit. Peaks as in Fig. 1.

TABLE I

CONCENTRATIONS OF PURINE AND PYRIDINE NUCLEOTIDES IN WASHED HUMAN RED BLOOD CELLS

Metabolite	Concentration (mean ± S.D., $n=10$) (μmol/l of cells)
ATP	1140 ± 70
ADP	134 ± 10
AMP	12 ± 2.8
Ado	0.6 ± 0.2
Ade	0.3 ± 0.15
GTP	60 ± 5.5
GDP	10 ± 2.0
IMP	70 ± 6.1
Hyp	8.2 ± 1.3
Xan	2.5 ± 0.6
$NADP^+$	39 ± 4.9
NAD^+	43 ± 6.5



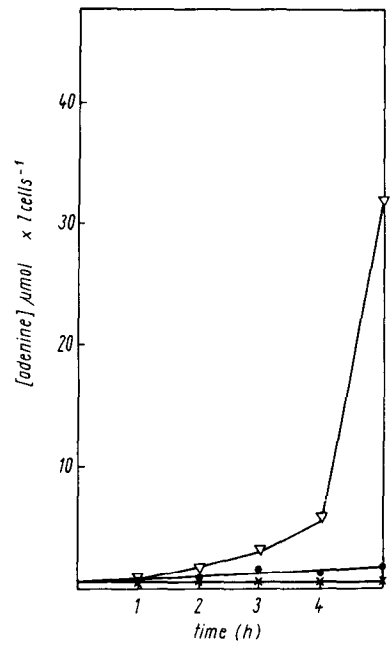


Fig. 3. Incubation of washed human red blood cells in glucose-free TRA buffer. Conditions: pH 7.4 for 5 h at 37 °C; coformycin concentration, 10^{-7} M (●) or 10^{-5} M (▽). Values are the average of three experiments. Adenosine production was measured by reversed-phase separation of cell extracts. The data points marked with crosses are from control experiments.

Fig. 4. Incubation of washed human red blood cells. For details see Fig. 3. Adenine production was measured by reversed-phase separation of cell extracts.

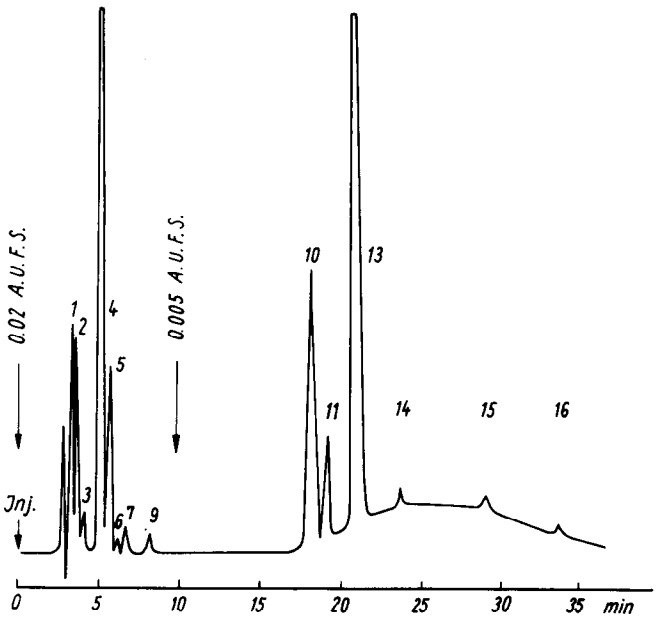


Fig. 5. Chromatogram of an extract of a reticulocyte-rich suspension. Separation conditions are described in Experimental (see *HPLC system A*). Peaks as in Fig. 1.

The concentrations of purine mono- and triphosphates are two- to four-fold greater in reticulocytes than in erythrocytes. Still greater differences occur for nucleosides and nucleobases. Reticulocytes show also higher ratios between purine tri- and diphosphates. Little or no difference is seen for the oxidized pyridine dinucleotides. These observations indicate the higher metabolic activity and diversity of reticulocytes. Comparable data for the concentrations of adenine nucleotides were found in rabbit red cell suspensions containing 90% reticulocytes [18].

Differences between the nucleotide profiles of animal species may be derived by comparison of Table I with Table II. Generally the concentrations are higher in rabbit erythrocytes, whereas the ratios of ATP to ADP and of GTP to GDP are smaller.

Ion-pair chromatography of Ehrlich ascites tumour cells

Fig. 6 shows the chromatogram of a standard mixture containing nucleotides, ca. 600 pmol of each. The chromatogram of an acidic extract of ascites tumour cells is shown in Fig. 7. The retention characteristics of purine and pyrimidine compounds are changed with respect to the reversed-phase mode. Monophosphates are eluted first, followed by di- and triphosphates. The complete analysis is performed within 20 min and it is therefore appreciably faster than that in the reversed-phase system. Table III shows that there are profound differences when cells are analysed on the seventh or eleventh day after inoculation: these represent a period of proliferation and a plateau, respectively. Comparable data for adenine nucleotides at days 7–9 after inoculation have been published [19].

TABLE II

CONCENTRATIONS OF METABOLITES IN MATURE AND IMMATURE RABBIT RED BLOOD CELLS

Metabolite	Concentration (μmol/l of cells)	
	Erythrocytes	Reticulocytes
ATP	1395	3580
ADP	310	395
AMP	40	161
Ado	0.6	3.6
Ade	0.5	3.3
GTP	216	680
GDP	63	95
IMP	50	110
Ino	2.5	41
Hyp	12	68
$NADP^+$	48	51
NAD^+	82	110

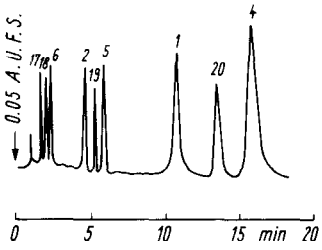


Fig. 6. Chromatogram of a standard mixture containing 600 pmol of each substance. Separation conditions are described in Experimental (see *HPLC system B*). Peaks: 17 = GMP; 18 = UMP; 6 = AMP; 2 = GDP; 19 = UDP; 5 = ADP; 1 = GTP; 20 = UTP; 4 = ATP.

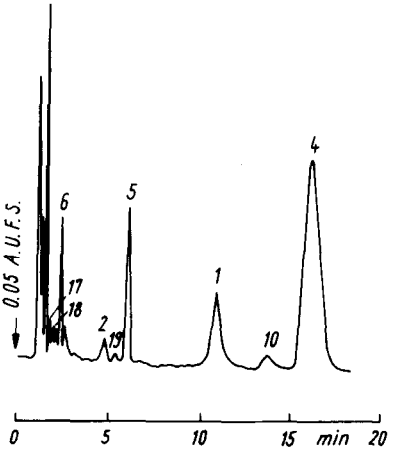


Fig. 7. Chromatographic peak pattern of an extract of Ehrlich ascites cells. Separation conditions are described in Experimental (see *HPLC system B*). Peaks: 17 = GMP; 18 = UMP; 6 = AMP; 2 = GDP; 19 = UDP; 5 = ADP; 1 = GTP; 20 = UTP; 4 = ATP.

TABLE III

NUCLEOTIDE LEVELS IN EHRLICH ASCITES TUMOUR CELLS

Metabolite	Concentration (mean ± S.D., $n=4$) (μmol/l of cells)	
	Day 7	Day 11
ATP	2512 ± 373	270 ± 42
ADP	881 ± 297	214 ± 48
AMP	166 ± 52	112 ± 62
GTP	639 ± 225	124 ± 31
GDP	201 ± 16	57 ± 13
GMP	118 ± 22	92 ± 14
UTP	99 ± 60	10.8 ± 0.2
UDP	32 ± 17	1 ± 0.1
UMP	0	0
Σ AdN	3559 ± 480	596 ± 89
Σ GN	953 ± 227	273 ± 36
Σ UN	131 ± 62	12 ± 0.2

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