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Changes of nucleotide patterns in liver, muscle and blood during the growth of Ehrlich ascites cells: application of the reversed-phase and ion-pair reversed-phase high-performance liquid chromatography with radial compression column[a]

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ABSTRACT

The pool of purine compounds was analysed in liver, skeletal muscle and blood of mice during the growth of Ehrlich ascites tumour cells. Three fast isocratic high-performance liquid chromatographic methods were used. (1) Determination of nucleotides by an isocratic ion-pair reversed-phase chromatography with a 10 m*M* ammonium phosphate buffer containing acetonitrile and tetrabutylammonium phosphate. (2) Separation of nucleosides and nucleobases in cell extracts by a reversed-phase system with methanol and 50 m*M* potassium phosphate buffer as eluent. (3) Nucleosides and nucleobases in body fluids were analysed by a reversed-phase system with 10 m*M* potassium phosphate containing methanol. These methods allow the rapid determination of purine compounds in small biological samples from various cell types and body fluids, with high accuracy and sensitivity. The pool of cellular nucleotides increased during the exponential phase of tumour growth. Adenosine accumulated significantly in all tissues in the stationary phase of tumour growth.

INTRODUCTION

Cells of the Ehrlich ascites tumour show tremendous changes after inoculation into a host animal (mouse). This is accompanied by variation of a multitude of cellular structures and functions, *e.g.* substrate utilization, energy production and synthesis of specific proteins. The growth in the host proceeds in two phases, called the exponential and stationary phases [1]. The transition from the exponen-

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tial to the stationary phase is accompanied by a decline of the incorporation rate of adenine, hypoxanthine and inosine, as well as a loss of purine compounds [2,3]. The mechanisms of these changes are still unknown. The metabolism of tumour cells is intimately related to the metabolism of the host tissues, one indication of which is the disturbance of the pattern of enzyme activities in various tissues during tumour growth [4,5]. Owing to the importance of the purine nucleotides and their precursors in tumour growth, the interrelations between the pools of purine compounds of tumour cells and host should be of paramount significance. Our aim is to analyse the changes of purine pools in liver, skeletal muscle and blood of mouse during the various phases of ascites tumour growth.

For the determination of nucleotides by high-performance liquid chromatography (HPLC) various techniques have been applied: anion-exchange [6], reversed-phase [7] and ion-pair reversed-phase [8,9]. Nucleosides and nucleobases may be measured by reversed-phase [10] and ion-pair reversed-phase HPLC [8,9]. In our laboratory the analysis of nucleobases, nucleosides and nucleotides was accomplished in the gradient ion-pair reversed-phase mode in a single run [11,12]. In order to fulfill the specific requirements of the particular aim of this study a modified version had to be developed.

EXPERIMENTAL

Materials

All reference standards were purchased from Boehringer (Mannheim, F.R.G.). $NH_4H_2PO_4$ and KH_2PO_4 were obtained from Fisher (Fairlawn, NJ, U.S.A.). Acetonitrile and methanol (HPLC grade) were from Merck (Darmstadt, F.R.G.). Tetrabutylammonium phosphate (PIC reagent A) was from Waters (Milford, MA, U.S.A.).

Sample preparation and extraction procedure

Female ICR mice, weighing 15–20 g, were used. The Ehrlich ascites tumour cells obtained from mice seven to eight days after inoculation of the tumour were suspended in saline solution to a concentration of $5 \cdot 10^7$ cells per ml; 0.5 ml of this suspension was inoculated into the recipient animal.

For the experiments the animals were grouped in the following way: group I, the control mice; group II, mice on the fifth day after tumour inoculation (the exponential phase of tumour growth); group III, mice on the twelfth day after tumour inoculation (stationary phase of tumour growth). The mice were anaesthetized with diethyl ether. The ascites fluid was aspirated. Then the liver and femoral muscle were excised and frozen immediately in liquid nitrogen. Blood was drawn from the heart with a syringe. Blood and ascites fluid were centrifuged (900 *g*, 2 min). Plasma and the supernatant of ascites fluid were extracted. Red blood cells were resuspended in saline solution and extracted. The hematocrit of blood and erythrocyte suspensions was measured. The frozen pieces of tissues

were weighed. Samples were extracted with ice-cold 6% perchloric acid. The extracts were centrifuged for 10 min at 1200 *g*, neutralized with thriethanolamine–K_2CO_3 and centrifuged. The aliquots were stored at −20°C. After thawing, 10 or 20 μl were analysed by HPLC.

HPLC instrument

A system from Waters, consisting of an M510 pump, an U6K universal liquid chromatograph injector, a variable-wavelength detector (adjusted to 280 nm), a 254-nm wavelength detector, a Model 730 data module, a 740 data module and a Model 640 system controller, was used. The 5-μm Nova Pak C_{18} cartridge (100 mm × 8 mm I.D.) with a Z-module compression system was used.

HPLC analysis

Nucleotides were determined in cell extracts by isocratic ion-pair reversed-phase HPLC. The buffer contained 10 m*M* $NH_4H_2PO_4$, 2 m*M* PIC reagent A and 20% acetonitrile. The flow-rate was 2 ml/min.

Nucleosides and nucleobases were assayed in cell extracts, by an isocratic reversed-phase system. The eluent was a buffer containing 7% methanol and 50 m*M* KH_2PO_4 (pH 4.1).

Nucleosides and nucleobases in body fluids were analysed in the isocratic reversed-phase mode using a buffer containing 8% methanol and 10 m*M* KH_2PO_4 (pH 5.9).

The separation time, particularly that of adenosine, was reduced by use of a flow gradient. The flow-rate was 1.5 ml/min for 0–6 min. During the next 60 s the rate was increased from 1.5 to 3 ml/min, then maintained at the value for 6 min. Thereafter the rate was reduced again to 1.5 ml/min. Peak identification was performed by coelution of biological samples with standard mixtures.

The Students-*t*-test was used for statistical evaluation.

RESULTS AND DISCUSSION

The methods presented here allow the determination of a wide range of purine compounds. As shown in Fig. 1, the mono-, di- and tri-phosphorylated nucleotides of adenine and guanine were well resolved by isocratic ion-pair reversed-phase HPLC. IMP and GMP were eluted together (Fig. 1A), which is in accordance with most previous results. Furthermore, the deoxynucleotides were *a priori* excluded from the analysis. In Table I the retention times, recoveries, response factors and absorbance ratios 254 nm/280 nm of the reference compounds are presented. Fig. 1B–D demonstrates the capability of this assay to analyse those compounds in perchloric acid extracts of liver, erythrocytes and muscle.

Both reversed-phase techniques allow the separation of hypoxanthine, xanthine, uric acid, inosine, guanosine, adenine and adenosine (Figs. 2 and 3). Chromatogram of standard mixtures are shown in Figs. 2A and 3A. Hypoxanthine,

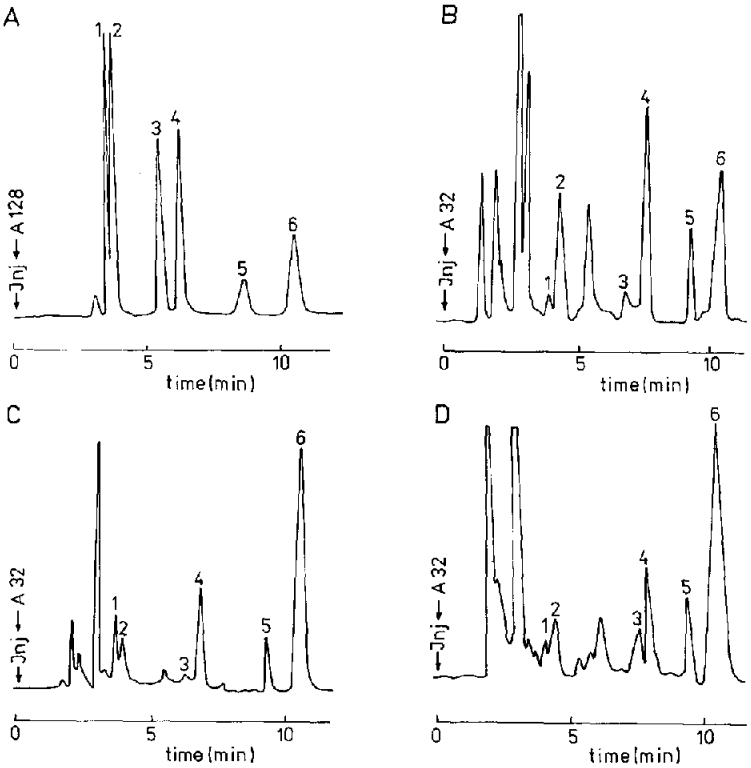

Fig. 1. Analysis of nucleotide patterns in several tissues of tumour-bearing mice. (A) Standard mixture; (B) liver; (C) erythrocytes; (D) skeletal muscle. The detection wavelength was 254 nm. Peaks: 1 = GMP + IMP; 2 = AMP; 3 = GDP; 4 = ADP; 5 = GTP; 6 = ATP.

TABLE I

PARAMETERS OF THE ISOCRATIC ION-PAIR REVERSED-PHASE ANALYSIS OF NUCLEOTIDES

Values are mean ± S.D.; $n = 3$.

Nucleotide	Retention time (min)	Recovery (%)	Response factor ($\cdot 10^{-4}$)	A_{254}/A_{280}
ATP	10.57	95 ± 4	0.4146	4.1
ADP	6.29	91 ± 4	0.3645	4.1
AMP	3.90	91 ± 7	0.1165	4.1
GTP	8.71	90 ± 3	0.4379	1.8
GDP	5.43	91 ± 6	0.1523	1.8
GMP	3.53	85 ± 7	0.1725	1.8

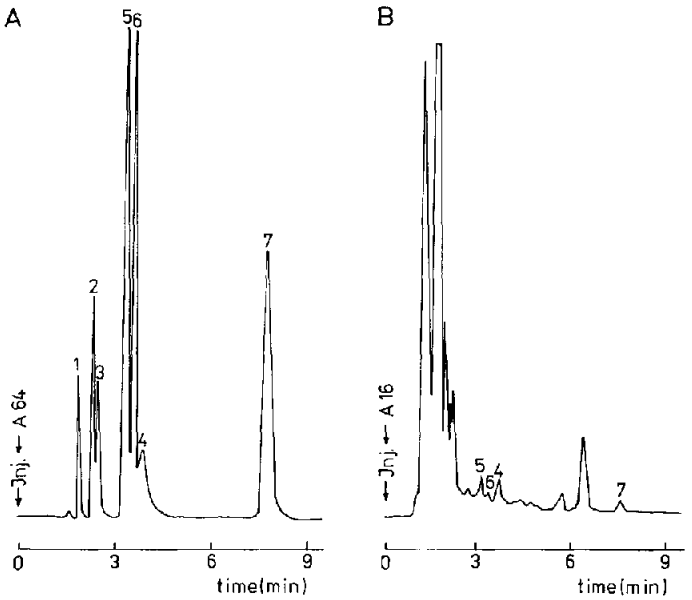

Fig. 2. Analysis of nucleosides and nucleobases in plasma. (A) Standard mixture; (B) plasma. Peaks: 1 = uric acid; 2 = Hyp; 3 = Xan; 4 = Ade; 5 = Ino; 6 = Guo; 7 = Ado.

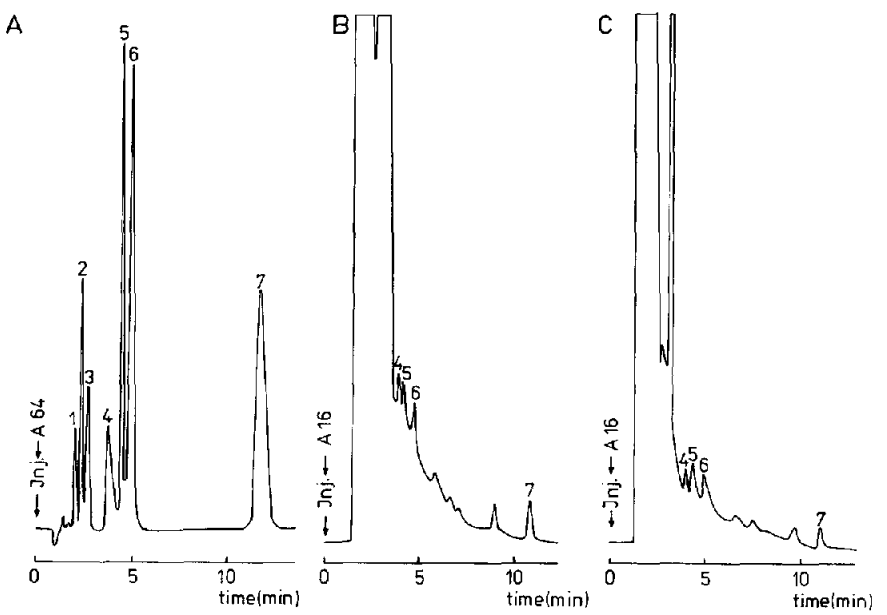

Fig. 3. Nucleoside and nucleobase analysis in cells. (A) Standard mixture; (B) skeletal muscle; (C) erythrocytes. For peak numbers see Fig. 2.

TABLE II

DEPENDENCE ON pH OF RETENTION TIMES OF NUCLEOSIDES AND NUCLEOBASES IN A REVERSED-PHASE SYSTEM

Eluent, 10 m*M* ammonium phosphate and 8% methanol; $n = 3$.

Purine	Retention time (min)				
	pH 4.0	pH 4.5	pH 5.0	pH 5.5	pH 6.0
Uric acid	2.5	2.3	1.9	1.8	1.8
Hyp	2.8	2.8	2.5	2.3	2.2
Xan	3.1	3.0	2.8	2.5	2.5
Ade	4.2	4.3	4.3	4.2	4.0
Ino	4.8	4.3	3.8	3.6	3.2
Guo	5.3	4.7	4.2	4.0	3.5
Ado	12.1	11.2	10.1	9.0	7.7

xanthine and uric acid coelute with unidentified compounds of the extracts. Therefore, these oxypurines were not evaluated here. Figs. 2B and C and 3B and C show that these methods are suitable for the estimation of adenine and various nucleosides.

TABLE III

PARAMETERS OF THE REVERSED-PHASE METHODS OF NUCLEOSIDE AND NUCLEOBASE ANALYSIS

Values are mean ± S.D.; $n = 3$.

Purine	Retention time (min)	Recovery (%)	Response factor ($\cdot 10^{-4}$)	A_{254}/A_{280}
50 mM $NH_4H_2PO_4$, 7% methanol, pH 4.1				
Uric acid	2.11	92 ± 2	0.9276	1.9
Hyp	2.40	89 ± 3	0.3882	4.8
Xan	2.69	84 ± 8	0.5174	1.5
Ade	3.75	88 ± 4	0.2804	4.1
Ino	4.42	90 ± 5	0.2928	5.2
Guo	5.01	92 ± 5	0.2625	1.8
Ado	11.35	93 ± 3	0.2303	4.1
10 mM $NH_4H_2PO_4$, 8% methanol, pH 5.9				
Uric acid	1.84	91 ± 3	0.9276	1.9
Hyp	2.21	88 ± 5	0.3882	4.8
Xan	2.53	82 ± 7	0.5174	1.5
Ade	3.97	91 ± 4	0.2804	4.1
Ino	3.23	87 ± 5	0.2928	5.2
Guo	3.54	92 ± 4	0.2625	1.8
Ado	7.70	94 ± 6	0.2303	4.1

The separation of the nucleosides and nucleobases is strongly dependent on the pH of the eluent (see Table II). The retention times, recoveries, response factors and absorbance ratios 254 nm/280 nm for both methods are shown in Table III.

TABLE IV

CONCENTRATIONS OF NUCLEOTIDES IN VARIOUS ORGANS OF MICE

Animals were controls (without tumour transplants) or on the fifth and twelfth day of Ehrlich ascites tumour growth.

Nucleotide	Sample	Concentration (mean ± S.D., $n = 5$) (nmol/g wet weight)		
		Liver	Skeletal muscle	Erythrocytes
ATP	Control	1512 ± 172	2401 ± 224	1702 ± 443
	Day 5	1904 ± 122[a,d]	2839 ± 367	2017 ± 163
	Day 12	1546 ± 185	1508 ± 308[b,f]	2020 ± 110
ADP	Control	1059 ± 38	375 ± 23	174 ± 25
	Day 5	1301 ± 190	467 ± 53	198 ± 54
	Day 12	1052 ± 123	519 ± 151	513 ± 106[b,e,f]
AMP	Control	269 ± 13	61 ± 19	37 ± 17
	Day 5	319 ± 50	54 ± 11	78 ± 18
	Day 12	262 ± 48	116 ± 12[b,e,f]	242 ± 81[b,e,f]
GTP	Control	497 ± 154	143 ± 9	159 ± 33
	Day 5	399 ± 35	187 ± 16[b,d]	170 ± 26
	Day 12	312 ± 35	140 ± 33[c,f]	485 ± 85[b,e,f]
GDP	Control	93 ± 23	31 ± 3	23 ± 5
	Day 5	85 ± 11	24 ± 8	34 ± 6
	Day 12	78 ± 10	51 ± 7[b,e]	45 ± 5[a,e]
GMP	Control	86 ± 30	64 ± 26	14 ± 2
	Day 5	71 ± 14	62 ± 22	30 ± 9
	Day 12	57 ± 13	44 ± 10[a,e,f]	79 ± 18[a,e,f]
AdN	Control	2840	2837	1913
	Day 5	3524	3360	2293
	Day 12	2860	2143	2775
ATP/	Control	1.43	6.40	9.78
ADP	Day 5	1.46	6.08	10.18
	Day 12	1.47	2.90	3.94
GdN	Control	676	238	196
	Day 5	555	273	234
	Day 12	447	235	609
GTP/	Control	5.34	4.61	6.91
GDP	Day 5	4.69	7.79	5.00
	Day 12	4.00	2.74	10.78

[a] $p < 0.01$.
[b] $p < 0.05$.
[c] $p < 0.001$.
[d] Day 5 *vs.* control.
[e] Day 12 *vs.* control.
[f] Day 12 *vs.* day 5.

In previous papers from this laboratory, gradient procedures for ion-pair reversed-phase HPLC were modified to perform the analysis of nucleobases, nucleosides as well as nucleotides [11,12]. The advantage of those procedures is the simultaneous determination of all three groups of purine compounds in a single run. However, there are two shortcomings. (i) Purine bases and nucleosides are eluted in the region where the baseline is rising. Thus the sensitivity and accuracy of the determination is limited. (ii) The time required for a single run approaches 1 h.

The aim of this study was to characterize the pattern of purine nucleotides and their precursors in various tissues of mice during tumour growth. Therefore, the methods must be tailored to meet the following two requirements: first, low concentrations should be estimated accurately, and in this way small variations during tumour growth should be detectable; second, the method should be appropriate for samples of various tissues.

Such an aim cannot be realized with a single run. The combination of isocratic ion-pair reversed-phase HPLC for the separation of nucleotides with isocratic

TABLE V

CONCENTRATIONS OF ADENINE AND NUCLEOSIDES IN VARIOUS ORGANS AND BODY FLUIDS OF MICE

Animals were controls (without tumour transplants) or on the fifth or twelfth day of Ehrlich ascites tumour growth.

Purine	Sample	Liver	Concentration (mean ± S.D., $n = 5$) (nmol/g wet weight)			
			Skeletal muscle	Erythrocytes	Ascites fluid	Plasma
Ado	Control	1.9 ± 0.1	2.6 ± 0.7	1.0 ± 0.1		1.2 ± 0.3
	Day 5	1.7 ± 0.3	2.5 ± 0.7	0.9 ± 0.2	0.01 ± 0.01	1.3 ± 0.2
	Day 12	5.1 ± 1.1[a,f]	5.3 ± 1.7	6.2 ± 1.9[a,e,f]	0.03 ± 0.01[a,f]	2.9 ± 0.6[a,e,f]
Ade	Control	0.9 ± 0.6	1.3 ± 0.1	1.6 ± 0.1		3.2 ± 0.9
	Day 5	1.5 ± 0.3	1.6 ± 0.7	0.8 ± 0.1[a,d]	1.2 ± 0.3	2.7 ± 0.2
	Day 12	0.7 ± 0.1[a,f]	3.3 ± 1.8	2.6 ± 0.3[c,e,f]	1.3 ± 0.2	2.8 ± 0.5
Ino	Control	4.7 ± 0.6	2.4 ± 1.0	2.6 ± 0.4		2.1 ± 0.4
	Day 5	7.2 ± 1.1	2.5 ± 1.1	0.7 ± 0.2[b,d]	1.2 ± 0.1	1.7 ± 0.4
	Day 12	5.4 ± 1.0	4.0 ± 1.8	2.6 ± 1.4[b,f]	1.3 ± 0.1	3.3 ± 0.6[a,f]
Guo	Control	6.9 ± 1.1	4.5 ± 1.1	1.7 ± 0.4		0.8 ± 0.2
	Day 5	5.6 ± 0.9	5.2 ± 2.2	2.0 ± 0.2	0.8 ± 0.1	0.7 ± 0.1
	Day 12	3.6 ± 0.6[a,e]	2.0 ± 0.6	2.3 ± 0.4	0.5 ± 0.1[a,f]	0.9 ± 0.2

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.
[d] Day 5 *vs.* control.
[e] Day 12 *vs.* control.
[f] Day 12 *vs.* day 5.

reversed-phase HPLC for nucleobases and nucleosides fulfills the requirements. For both procedures the accuracy was enhanced by using the isocratic mode. The time necessary for one separation cycle was considerably shortened by an increased flow-rate using a radial compression column. Switching from one isocratic mode to the other after a series of runs gives no complications. The time required for the complete analysis of the purine compounds is shorter than for the gradient procedure.

The concentrations of purines are shown in Tables IV and V. In general, an increase of cellular adenine nucleotide concentrations (liver, muscle, erythrocytes) was found during the exponential phase of tumour growth. In the stationary phase the sum of purine nucleotides in liver and muscle is scaled down. Simultaneously the levels of nucleosides and bases changed in blood plasma, ascites fluid and host tissues. There was a drastic increase of the adenosine concentration at day 12 after transplantation of the tumour.

Summing up these observations, one may conclude that the profile of purine compounds in extracts of various tissues can be estimated by the method presented here with high accuracy and sensitivity in a short time.

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