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Improved ion-pair high-performance liquid chromatographic method for the quantification of a wide variety of nucleotides and sugar–nucleotides in animal cells

THOMAS RYLL and ROLAND WAGNER*

Arbeitsgruppe Zellkulturtechnik, Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-3300 Braunschweig (Germany)

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ABSTRACT

An improved method including extraction procedures is presented for the analysis of nucleotides in suspension-cultivated animal cells. Quantification was performed by ion-pair high-performance liquid chromatography after perchloric acid extraction. It was found that the amount of perchloric acid taken for extraction influenced the yield and that cell washing procedures caused deterioration of the analysis results for triphosphates. More than thirty nucleotides and sugar–nucleotides were separated within 25 min using a Supelcosil reversed-phase column (3 μm) with tetrabutylammonium hydrogensulphate as pairing agent and methanol–pH gradient elution. Cultivated hybridoma cells showed variations in intracellular nucleotide concentrations as well as relative amounts during different growth phases, which could reflect the physiological state of a cell culture.

INTRODUCTION

The field of animal cell technology has become widespread during the last ten years. After a period of reactor development, a multitude of special types of fermenters have become available for mass cultivation of animal cells, biomass production, antibodies or special recombinant proteins. In contrast to individual microorganisms, animal cells, which are part of a macroorganism, have to be considered together with their environment as a physiological unit. Therefore the development and optimization of production processes require the control of many physiological parameters, such as dissolved oxygen, pH, carbohydrates, lactate, amino acids, ammonium, phosphate, etc. [1,2]. In spite of new analytical methods and improved process control, little information is known about the interaction of these environmental parameters and the intracellular conditions during the growth cycle of animal cell cultures. McKeehan *et al.* [3] calculated theoretically that the antibody production with hybridoma cells would be increased by a factor of 720 if truly optimal conditions for a cell line could be guaranteed during a high-density cultivation. Hence, the optimization of the

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physiological unit, cell and environment, has increased in significance for industrial-scale production. This is also particularly important for product quality, which is negatively influenced by proteolytic attack of proteases released by the cells in serum-free culture supernatants [4]. However, improvement of the physiological conditions requires more information about the basic biology of cultured cells. Beside the detection of environmental parameters, the control of intracellular metabolism has to be more carefully monitored. Nucleotides have been shown to be one of the most important substances for cell metabolism. They are involved in a number of cellular processes and have widespread regulatory potential [5]. Hence, nucleotides were chosen as a group of intracellular substances considered to have trigger or even regulatory functions in the control of elementary physiological processes [6,7] which could give information about intracellular conditions and the cell status during growth.

The analysis of nucleotides using chromatographic methods is based on principles introduced by Cohn [8], who published the first method for separation of nucleotides by ion-exchange chromatography in 1949. Since then, the separation of nucleotides has become a standard method and has been varied and improved (for review, see refs. 9 and 10). At present essentially three different analytical methods, based on high-performance liquid chromatography (HPLC) of nucleotides are available. The method of ion-exchange chromatography gives good resolution for highly charged mono-, di- and triphosphates, but requires a long time for analyses and is less useful for nucleosides and nucleobases. Additionally, ion-exchange columns are not stable over long periods [11–16]. In general, more reproducible results and shorter analysis times for the separation of nucleotides can be achieved by using phosphate-buffered methanol or acetonitrile gradients on reversed-phase columns. However, the separation of highly charged nucleotides and activated sugars is difficult [17–23]. Often both systems have been used in parallel in order to analyse the whole spectrum of nucleotides from biological samples [24–26]. Hoffmann and Liao [27] and three years later Juengling and Kammermeier [28] presented a third method, which used added solvophobic ions such as tetrabutylammonium hydrogensulphate or tetrabutylammonium phosphate. With the help of such ions it is possible to separate nucleobases, nucleosides and nucleotides on reversed-phase columns within one run. Thus, this ion-pair HPLC method has displaced ion-exchange and reversed-phase HPLC of nucleotides over the last few years [29–37].

This report presents an improved method, which allows the separation of more than thirty substances within 25 min. Its utility is demonstrated by its application as a standard off-line procedure for the observation of animal cells *in vitro*, an important tool which could become as prerequisite for the development of bioprocesses.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of two HPLC pumps (Model 64, Knauer, Berlin, Germany), a mixing chamber (Knauer), an injector valve (Model 7125, Rheodyne, Cotati, CA, USA), and a two-channel UV detector (Model D 430, Kontron Instruments, Hamburg, Germany). Process control and evaluation were carried out by the MT 450 data system, running on an IBM compatible computer (both from Kontron Instruments). Column temperature control was realized with a column oven (Model K-1, Techlab, Evessen, Germany). A Supelcosil LC-18T column (150 mm × 4.6 mm I.D., 3 μm particle size) was used combined with a guard column cartridge (5 μm particle size, both from Supelco, Bad Homburg, Germany). Both columns contained octadecyldimethylsilyl phases.

Chemicals

Analytical-grade perchloric acid (PCA), potassium hydroxide, phosphoric acid, dipotassium hydrogenphosphate and potassium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany). Tetrabutylammonium hydrogensulphate was obtained from Fluka (Buchs, Switzerland). HPLC-grade methanol was supplied by Baker (Deventer, Netherlands). Highly purified water was prepared with a Millipore water system (Super Q, Millipore, Eschborn, Germany) and was used to prepare all solutions. ATP, ADP, AMP, cyclic AMP (c-AMP), CTP, CDP, CMP, GTP, GDP, GMP, NAD, NADH, NADP, NADPH, UTP, UDP, UMP, UDP-glucuronate (UDP-Gluc), UDP-galactose (UDP-Gal) and UDP-glucose (UDP-Glc) were obtained from Boehringer Mannheim (Mannheim, Germany). All other standard nucleotides [adenine, adenosine, ADP-ribose (ADP-Rib), cytosine, cytidine, FAD, guanine, guanosine, inosine, uracil, uridine, UDP-N-acetylgalactosamine (UDP-GalNac), UDP-N-acetylglucosamine (UDP-GlcNac) and xanthine] were purchased from Sigma (Deisenhofen, Germany).

Chromatographic conditions

The basic solution for mobile phases consisted of 100 mmol/l potassium dihydrogenphosphate–dipotassium hydrogenphosphate buffer (pH 6.0). This basic solution supplemented with 8 mmol/l tetrabutylammonium hydrogensulphate (pH 5.3) was used as buffer A; 70% buffer A plus 30% methanol (pH 5.9) was buffer B. The adjustment of pH was carried out by adding concentrated potassium hydroxide or phosphoric acid. The basic solution could be stored for several weeks at 4°C, whereas the buffers were freshly prepared every three to five days by mixing and filtration through a 0.2-μm filter (RC 58, Schleicher & Schuell, Dassel, Germany) under vacuum. Both buffers were stored under helium during the period of use. The gradient was: 100% buffer A for 2.5 min, 0–40% buffer B for 14 min, 40–100% buffer B for 1 min, 100% buffer B for 6 min, 100–0% buffer B

for 1 min, followed by an equilibration phase of 100% buffer A for 8 min. The flow-rate was 1.5 ml/min. Temperature was set to 26–34°C depending on the particular separation problem.

Peak identification and quantification

For identification and quantification, stock solutions (1 mmol/l) of all standard nucleotides were prepared and stored at −30°C or, in the case of NADH and NADPH, at −70°C. Peaks were identified by comparison of retention times, spiked samples, calcualtion of the ratio between absorbance at 254 and 280 nm, and on-line scanning of UV spectra. Quantification was carried out by integration of peak areas. Standard curves were achieved with all standard nucleotides in the range of 100–10 000 pmol, for which the peak area was linearly correlated with the amount present and was taken for quantification.

Extraction of cells

The PCE extraction method was chosen as it resulted in the best yield of triphosphates *versus* extraction with trichloroacetic acid (TCA), potassium hydroxide or methanol. For the advantages or disadvantages of different extraction methods see ref. 38, in which this extraction procedure is also recommended as the most efficient one.

After taking a sample from the reactor, 0.5–5 ml of cell suspension, corresponding to $2–5 \cdot 10^6$ cells, were centrifuged immediately at 0°C and 190 *g* for 3 min. The supernatant was discarded and the cell pellet was resuspended in 500 μl of ice-cooled 0.5 *M* PCA, and stored on ice for 2 min. The insoluble macromolecules were subsequently sedimented at 0°C and 2000 *g* for 3 min. The supernatant was put on ice and the pellet was extracted a second time by homogenizing in 500 μl of cooled 0.5 *M* PCA. After a second sedimentation step as described above, the supernatants were collected and neutralized to pH 6.5 by adding cooled 2.5 *M* potassium hydroxide in 1.5 *M* dipotassium hydrogenphosphate, and stored on ice for 2 min. The potassium perchlorate precipitate was finally removed by sedimentation at 0°C and 2000 *g* for 1 min. The clear supernatant was filtered through a 0.45-μm filter (SJHVLO4NS, Millipore) and stored in liquid nitrogen until analysis. The total procedure took about 20 min from the fermenter sample to liquid nitrogen storage.

RESULTS AND DISCUSSION

Separation of standard nucleotides

Fig. 1 shows a typical chromatogram of a mixture of 31 standard nucleotides. Run-to-run precision for this mixture is shown in Table I. The column dead time amounted to 1.18 min, resulting in a buffer velocity of 12.7 cm/min. The capacity factor k', the theoretical number of plates N and the resolution of two peaks R are calculated as:

$$k' = \frac{t_R - t_0}{t_0} \quad (1)$$

$$N = 5.54 \left(\frac{t_R}{w_{0.5}} \right)^2 \quad (2)$$

$$R = \frac{2(t_{R2} - t_{R1})}{w_1 + w_2} \quad (3)$$

where t_R = retention time, t_0 = dead time of the column, w = baseline width of a peak, $w_{0.5}$ = peak width at half-peak height, t_{R1} = retention time of substance 1 and t_{R2} = retention time of substance 2.

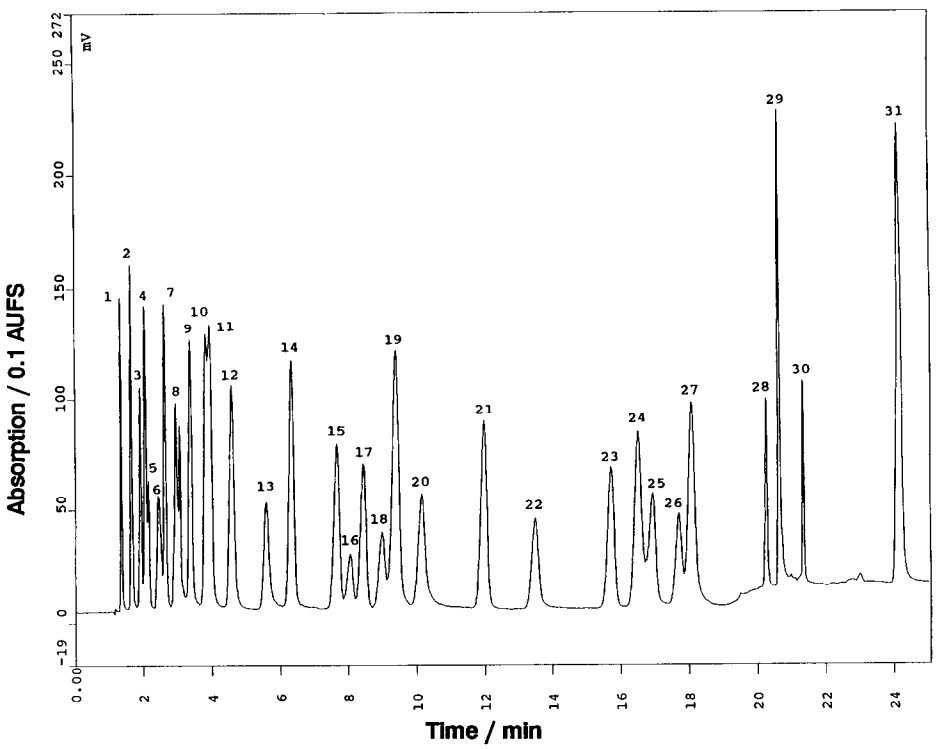

Fig. 1. Ion-pair chromatographic separation of standard nucleotides on a 3-μm reversed-phase column using methanol gradient elution. The temperature was set to 34°C. For details see chromatographic conditions. A 20-μl aliquot of a standard mixture was injected. The figures in brackets are the amount of each substance in pmol: 1 = cytosine (450); 2 = uracil (360); 3 = cytidine (330); 4 = guanine (320); 5 = xanthine (265); 6 = CMP (330); 7 = uridine (320); 8 = adenine (320); 9 = inosine (340); 10 = UMP (490); 11 = guanosine (310); 12 = GMP (330); 13 = CDP (490); 14 = NAD (330); 15 = adenosine (300); 16 = UDP-galactose (165); 17 = UDP-glucose (490); 18 = UDP (330); 19 = AMP (490); 20 = GDP (330); 21 = NADP (330); 22 = CTP (490); 23 = ADP-ribose (330); 24 = ADP (490); 25 = UDP-glucuronic acid (330); 26 = UTP (330); 27 = GTP (490); 28 = NADH (165); 29 = ATP (330); 30 = NADPH (165); 31 = FAD (330).

TABLE I

RUN-TO-RUN PRECISION FOR A MIXTURE OF 31 STANDARD NUCLEOTIDES

Values are expressed as mean values and standard deviations (n = 13). The resolution (R) related to the following peak. Temperature was 34°C. For details see chromatographic conditions. k' = capacity factor; N = theoretical plates; R = resolution of two peaks; t_R = retention time.

Substance	t_R (min)	k'	N	R
Cytosine	1.36 ± 0	0.153	4950 ± 490	3.29 ± 0.63
Uracil	1.67 ± 0.01	0.415	3940 ± 820	2.17 ± 0.25
Cytidine	1.94 ± 0.01	0.644	3720 ± 920	1.16 ± 0.27
Guanine	2.10 ± 0.01	0.780	3840 ± 670	0.74 ± 0.11
Xanthine	2.19 ± 0.01	0.856	6780 ± 220	1.48 ± 0.20
CMP	2.48 ± 0.01	1.10	1675 ± 260	0.94 ± 0.05
Uridine	2.68 ± 0.02	1.27	5360 ± 1400	1.40 ± 0.20
Adenine	3.00 ± 0.03	1.54	3780 ± 270	1.99 ± 0.65
Inosine	3.49 ± 0.04	1.96	4275 ± 420	1.58 ± 0.07
UMP	3.90 ± 0.02	2.31	4055 ± 325	0.73 ± 0.02
Guanosine	4.09 ± 0.05	2.47	3740 ± 310	1.96 ± 0.07
GMP	4.69 ± 0.04	2.97	4330 ± 430	2.89 ± 0.16
CDP	5.64 ± 0.02	3.78	4700 ± 360	2.51 ± 0.11
NAD	6.49 ± 0.05	4.50	7530 ± 580	3.87 ± 0.14
Adenosine	7.90 ± 0.08	5.69	6960 ± 2065	1.11 ± 0.38
UDP-Gal	8.16 ± 0.04	5.92	7715 ± 670	1.02 ± 0.04
UDP-Glc	8.56 ± 0.03	6.25	9760 ± 930	1.35 ± 0.04
UDP	9.10 ± 0.03	6.71	9040 ⊥ 920	1.10 ⊥ 0.06
AMP	9.56 ± 0.06	7.10	10000 ± 750	1.61 ± 0.08
GDP	10.26 ± 0.04	7.69	10360 ± 920	4.33 ± 0.12
NADP	12.10 ± 0.04	9.25	16590 ± 1370	3.32 ± 0.14
CTP	13.54 ± 0.02	10.47	16530 ± 1400	5.19 ± 0.09
ADP-Rib	15.85 ± 0.04	12.43	24890 ± 2140	1.70 ± 0.06
ADP	16.64 ± 0.04	13.10	23620 ± 2030	0.83 ± 0.02
UDP-Gluc	17.00 ± 0.02	13.41	22840 ± 540	1.66 ± 0.02
UTP	17.77 ± 0.02	14.09	34450 ± 4240	0.90 ± 0.05
GTP	18.15 ± 0.03	14.38	34000 ± 2990	7.50 ± 0.26
NADH	20.31 ± 0.01	16.21	412250 ± 28500	2.80 ± 0.07
ATP	20.67 ± 0.01	16.52	514820 ± 36860	6.18 ± 0.16
NADPH	21.38 ± 0.01	17.12	753750 + 61630	13.70 ± 0.35
FAD	24.25 ± 0.04	19.55	110770 ± 10000	–

The capacity factor can be considered as a standardized retention time which indicates the characteristic interaction of substance and separation material. The standard deviation for retention times as shown in Table I was about 1% or less for all substances and indicated the good run-to-run precision of this method. The column used supplied very high theoretical plates of 10 000, 20 000 and 500 000 for AMP, ADP and ATP, respectively, which can be compared with results gained from ion-exchange chromatography (NH_2), where values of only 3000 were reached for these compounds [39], demonstrating the high resolution under the conditions described.

Cell extraction

One method for extraction of cells from a suspension is performed by directly adding concentrated PCA to the suspension, in order to stop all enzymatic reactions. But this approach yields extracted substances which are diluted by the whole suspension volume. Consequently, processes such as evaporation are needed to concentrate nucleotides prior to injection. It is also possible to concentrate the cells by centrifugation before extraction. The influence of such a concentrating centrifugation step was studied by comparison of extracts from centrifuged cells (see Experimental section) with directly extracted samples from the same suspension without centrifugation. As seen in Table II, the yields of triphosphates remained constant within the standard deviation and the adenylate energy charge (AEC) was not affected by centrifugation.

In order to reduce possible interference from compounds derived from the culture supernatant, an intermediate purification step may be necessary. Hence, the influence of such a procedure with a cell wash with cooled PBS was tested by comparing the extraction yield of triphosphates and the AEC of a cell pellet without washing and identical cell pellets after one, two and three washings with 1 ml of ice-cold PBS (see Table III). In contrast to centrifugation, the cell wash with cooled PBS had a remarkable influence on the AEC and also on the yield of triphosphates, indicating that best results are obtained without the use of such procedures.

Another parameter which could also affect the extraction yield is the total volume of PCA used to extract a defined cell number. If cell pellets consisting of $2 \cdot 10^5$–$160 \cdot 10^5$ hybridoma cells were extracted with 1 ml, the extraction yield for the sum of ATP + GTP + UTP + CTP + NAD was constant for $2 \cdot 10^5$, $4 \cdot 10^5$, $8 \cdot 10^5$, $10 \cdot 10^5$, $20 \cdot 10^5$ and $40 \cdot 10^5$ cells at 8.76 ± 0.4 fmol per cell, and decreased to $7.7 \cdot 10^5$ and $7.1 \cdot 10^5$ for $80 \cdot 10^5$ and $160 \cdot 10^5$ cells, respectively. Hence, it was therefore important to take a minimum of about 0.2 ml of PCA per 10^6 cells for extraction in order to obtain comparable results. The influence of re-extraction of the macromolecular pellet was also checked. As shown in Fig. 2,

TABLE II

YIELDS OF TRIPHOSPHATES AFTER CENTRIFUGATION

Yields of triphosphates after centrifugation of 1 and 5 ml of a suspension of hybridoma cells ($7 \cdot 10^5$ cells per ml). The yield of triphosphates without centrifugation is set to 100%. The values are average values and standard deviations from eight experiments.

Centrifuged volume (ml)	Yield (%)				
	ATP	GTP	UTP	CTP	AEC
1	100 ± 05	95 ± 6	97 ± 6	97 ± 13	0.964 ± 0.01
5	90 ± 6	93 ± 4	100 ± 4	97 ± 6	0.956 ± 0.01

TABLE III

RECOVERIES OF NUCLEOTIDE TRIPHOSPHATES AFTER WASH PROCEDURES

Influence on a cell pellet of $2 \cdot 10^6$ hybridoma cells of the number of wash steps with ice-cold PBS before extraction with perchloric acid. The values are given as percentages based on an extraction without washing.

Washing with 1 ml of ice-cold PBS	Recovery (%)				
	ATP	GTP	UTP	CTP	AEC
Without	100	100	100	100	0.96
1 ×	96	100	94	100	0.94
2 ×	85	87	83	85	0.91
3 ×	74	73	68	70	0.89

it was possible to improve the extraction yield by about 30% by using 500 μl of PCA twice in succession rather than 1000 μl of PCA once. However, a third or fourth extraction step with 500 μl of PCA did not improve the yield.

Finally, the best results for extraction of suspended grown cells were obtained using conditions described in the Experimental section. The recoveries for this extraction method were proven by using both single standard substances and spiked samples, as shown in Table IV. The average recovery for all substances was 90 ± 6% for standard solutions. The loss of 10% of the nucleotides may be caused by co-precipitation with potassium perchlorate. For spiked samples the average value was slightly lower at 85 ± 5%. The additional loss could be due to adsorption of nucleotides on precipitated macromolecules.

Separation of cell extracts

In Figs. 3 and 4 typical chromatograms of extracted hybridoma cells are shown. Using the extraction conditions described above, ATP, ADP, AMP,

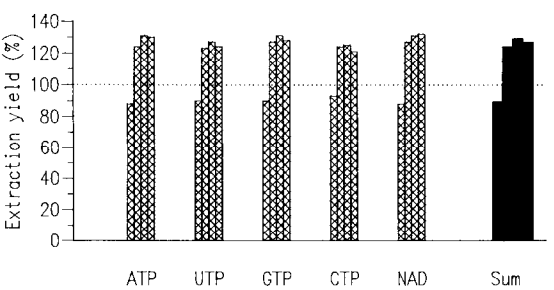

Fig. 2. Extraction yield of ATP, GTP, UTP, CTP and NAD from a cell pellet consisting of $2.4 \cdot 10^6$ hybridoma cells. The value 100% corresponds to the yield of one extraction with 1000 μl of PCA, whereas the bars represent the extraction yields using one, two, three and four extractions with 500 μl of PCA in succession with identical pellets.

TABLE IV

NUCLEOTIDE RECOVERIES, USING THE PCA EXTRACTION METHOD

Recoveries for standard substances were determined by adding 100 μl of 5 *M* PCA to 1000 μl of standard solution, followed by neutralization to pH 6.5 and sedimentation of formed potassium perchlorate. For spiked samples a pellet of $2 \cdot 10^6$ hybridoma cells was spiked with 5 nmol of each nucleotide and extracted as described in the Experimental section. The values are average values and standard deviations from eight experiments.

Substance	Recovery (%)	
	Standard solution	Spiked sample
ATP	90 ± 1	86 ± 1
GTP	89 ± 10	78 ± 2
UTP	80 ± 7	91 ± 3
CTP	95 ± 6	87 ± 7
ADP	85 ± 4	77 ± 5
GDP	80 ± 2	79 ± 1
UDP	74 ± 6	79 ± 4
CDP	86 ± 3	84 ± 7
AMP	93 ± 2	88 ± 2
GMP	96 ± 0.4	91 ± 1
UMP	93 ± 1	89 ± 3
CMP	94 ± 2	89 ± 2
NAD	93 ± 3	86 ± 4
NADP	93 ± 3	89 ± 3
FMN	96 ± 2	86 ± 2
FAD	94 ± 2	89 ± 2
c-AMP	97 ± 1	93 ± 6
ADP-Ribose	92 ± 3	91 ± 2
UDP-GalNac	91 ± 3	79 ± 11
UDP-GlcNac	91 ± 3	86 ± 9
UDP-Gal	95 ± 2	82 ± 5
UDP-Glc	91 ± 1	78 ± 2
UDP-Gluc	92 ± 1	92 ± 5

GTP, GDP, CTP, UTP, UDP-Glc, UDP-GalNac, UDP-GlcNac, NAD and NADP were detected. The cell-specific and the relative amounts of all detected nucleotides of the cells sampled from the logarithmic (see Fig. 3) and the stationary growth phases (see Fig. 4) of a perfused batch culture of hybridoma cells are listed in Table V. Whereas the relative amounts of the purine triphosphates were stable, the amounts of the pyrimidine triphosphates, UTP and CTP, showed a distinct variation and decreased to about 40% during cultivation, such that the ratio of purine to pyrimidine triphosphates changed during different growth phases [40]. In contrast, the amounts of the N-acetylated UDP-activated sugars (UDP-GNac) increased in the stationary growth phase [41].

In conclusion, with this method it is possible to detect variations in the physio-

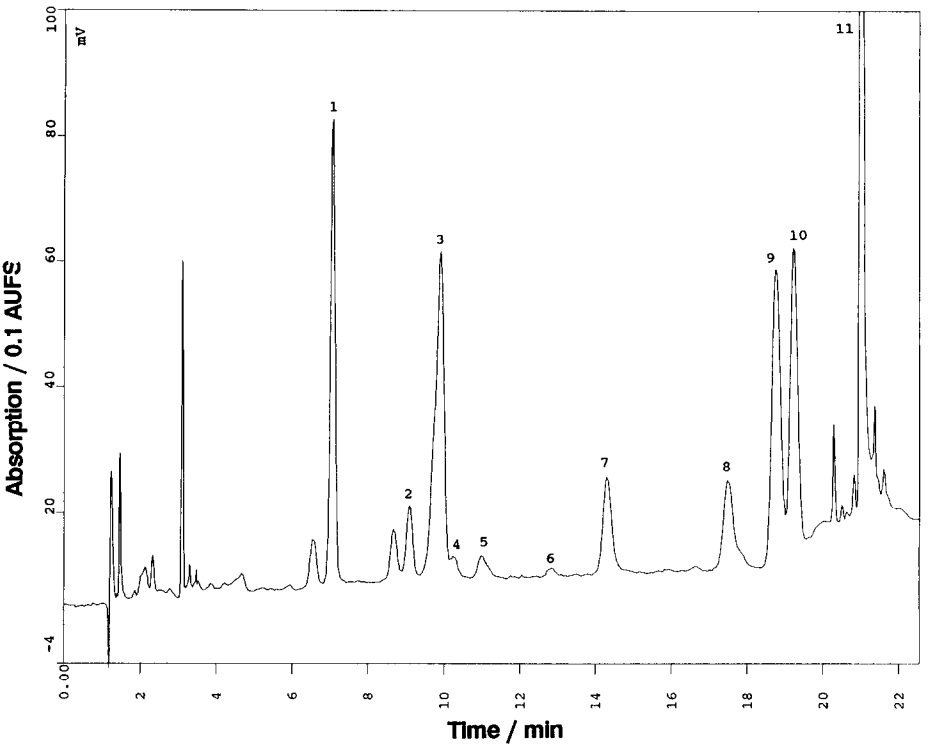

Fig. 3. Chromatogram of a cell extract corresponding to $3.5 \cdot 10^5$ hybridoma cells from the logarithmic growth phase of a culture. The injection volume was 100 μl. Temperature was set to 28°C. For elution conditions see the Experimental section. Peaks: 1 = NAD; 2 = UDP-Glc; 3 = UDP-GNac; 4 = AMP; 5 = GDP; 6 = NADP; 7 = CTP; 8 = ADP; 9 = UTP; 10 = GTP; 11 = ATP. All other peaks are unknown substances.

TABLE V

CELL-SPECIFIC AMOUNTS AND PERCENTAGES OF NUCLEOTIDES

Cell-specific amounts and percentages of nucleotides from the logarithmic and the stationary growth phases of a hybridoma culture. The chromatograms are shown in Figs. 3 and 4. UDP-GNac describes the sum of UDP-GalNac and UDP-GlcNac.

Nucleotide	Logarithmic phase		Statinary phase	
	fmol per cell	Percentage	fmol per cell	Percentage
ATP	5.51	40.3	2.42	43.6
ADP	0.42	3.1	0.13	2.3
AMP	0.05	0.4	0.03	0.6
GTP	1.24	9.1	0.55	9.9
GDP	0.08	0.6	0.04	0.7
CTP	0.92	6.7	0.22	3.9
UTP	2.31	16.9	0.54	9.8
UDP-Glc	0.36	2.6	0.06	1.1
UDP-GNac	1.87	13.7	0.98	17.6
NAD	0.89	6.5	0.57	10.3
NADP	0.01	0.1	0.01	0.2

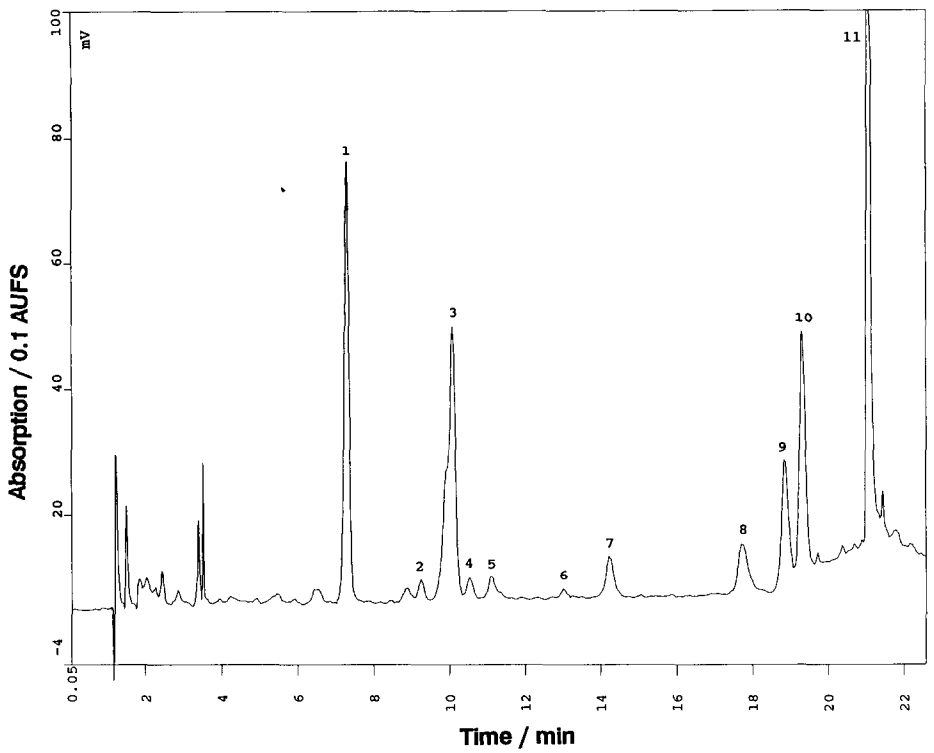

Fig. 4. Chromatogram of a cell extract corresponding to $3.3 \cdot 10^5$ hybridoma cells form the stationary growth phase of a culture. For details see Fig. 3.

logical state of growth of a cell culture within 1 h after cell sampling. Such investigations give additional information about the fundamental biology of cell cultures and therefore afford a new method for following optimization of production processes with animal cells.

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