Journal of Chromatography, 563 (1991) 172–177
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5601

Note

Application of ion-pair high-performance liquid chromatography with radioisotope detection to *in vitro* studies of nucleoside metabolism in mitochondria[a]

MATHIAS ZIEGLER, WOLFGANG DUBIEL, WOLFGANG HENKE[b] and GERHARD GERBER

Institute of Biochemistry, Humboldt University Berlin, Hessische Strasse 3-4, 1040 Berlin (G.D.R.)

and

ALEXANDER M. PIMENOV*, YURII V. TIKHONOV and RUSLAN T. TOGUZOV

Department of Biochemistry, Central Research Laboratory, N.I. Pirogov 2nd Moscow Medical Institute, Ostrovityanova 1, Moscow 117869 (U.S.S.R.)

(First received April 25th, 1990; revised manuscript received August 8th, 1990)

A number of high-performance liquid chromatographic (HPLC) techniques have been employed for the determination of nucleotides and their derivatives in various biological samples. The development of reversed-phase columns with ion-pairing reagents in the mobile phase has permitted the simultaneous determination of a whole range of charged and hydrophobic nucleic acid constituents (*i.e.*, nucleotides, nucleosides and nucleobases) [1,2]. It was also shown that reversed-phase ion-pair HPLC in the isocratic mode is a useful tool if only some of these compounds (*e.g.*, nucleosides and their monophosphates, or nucleotides only) are of interest [3,4]. In this instance, isocratic elution has all the advantages of HPLC with much shorter separation times.

In most studies, metabolic changes caused by particular experimental conditions, *e.g.*, at hypoxia, are investigated using UV detection. However, for the analysis of metabolic pathways or flux rates, the application of radiolabelled compounds and the determination of their metabolites is essential.

In this study, the principle routes of purine nucleoside metabolism of mitochondria were examined. As it is still an open question whether these organelles are capable of taking up and metabolizing these compounds, ^{14}C-labelled nucleosides were used in order to approach this problem. Intact and fractured (and thus

[a] Presented at the *11th International Symposium on Biomedical Applications of Chromatography and Electrophoresis, Tallinn, April 24–28th, 1990.*

[b] Division of Experimental Organ Transplantation.

0378-4347/91/$03.50

deprived of the membrane barrier) mitochondria were incubated with such nucleosides and the redistribution of the radiolabel was studied.

A previously developed isocratic ion-pair HPLC procedure [5] was modified in order to achieve an appropriate separation of purine bases, nucleosides and nucleoside monophosphates, yielding satisfactory resolution in the case of radioisotope detection.

EXPERIMENTAL

Chemicals

Nucleotides, nucleosides and nucleobases were obtained from Sigma (St. Louis, MO, U.S.A.), ^{14}C-labelled compounds from Amersham (Aylesbury, U.K.), potassium dihydrogenphosphate from Prolabo (Paris, France), tetrabutylammonium phosphate (TBA, PIC reagent A) from Beckman (San Ramon, CA, U.S.A.) and HPLC-grade acetonitrile (CromAR) from Promochem (Wesel, F.R.G.).

Sample preparation

Mitochondria were isolated from the livers of male Wistar rats as described previously [6]. Intact or fractured (by sonication) mitochondria (15–20 mg of protein) were incubated in the presence of 1 m*M* ATP, 1 m*M* $MgCl_2$, 5 m*M* KH_2PO_4 and 100 μM ^{14}C-labelled nucleoside (adenosine or guanosine, 0.2 μCi/ml). After several time intervals, acid-soluble fractions (ASF) were obtained by precipitation of 200-μl aliquots of the mitochondrial suspension with 1 volume of ice-cold perchloric acid (0.6 *M*). After centrifugation the supernatant was neutralized with 0.25 volume of K_2CO_3 (1.3 *M*). Aliquots (50 μl) of the neutralized supernatant were analysed by HPLC.

Instrumentation

HPLC analysis was carried out using the GOLD method development system, consisting of Model 126 programmable solvent module, Model 167, scanning detector module, Model 171 radioisotope detector (solid version, 125-μl cell volume), and Altex 210A injection valve (all from Beckman, Fullerton, CA, U.S.A.). A 3-μm Ultrasphere XL-ODS cartridge column (75 mm × 4.6 mm I.D.) was used for the separations. Version 3.1 GOLD Software for data collection and controller functions and ChromatoGraphic Software for radioactive data acquisition were applied.

RESULTS AND DISCUSSION

Optimization of the chromatographic procedure

In analogy with the cytosolic nucleotide metabolism, the initial steps of mitochondrial purine nucleoside utilization might be (i) degradation via nucleoside

phosphorylase or via deamination (adenosine), or (ii) phosphorylation to the corresponding monophosphates. The aim of this study was to investigate these possible routes. The interconversion of purine nucleoside mono-, di- and triphosphates has already been shown to take place within mitochondria [7]. Therefore, an isocratic elution mode separating nucleobases, nucleosides and mononucleotides was applicable.

A rapid method for this purpose was recently developed and applied to erythrocytes [5]. Using radioisotope detection, however, it was necessary to increase the resolution because of the relatively large volume of the solid scintillation cell (125 μl). In particular, the retention of GMP and IMP had to be increased, as high activities of initially added [^{14}C]guanosine produced very large peaks, possibly overlapping with those of the nucleoside monophosphates.

Application of the ODS-XL cartridge column with a 3-μm particle size permitted a satisfactory separation to be achieved with a mobile phase composed of 40 m*M* KH_2PO_4–1 m*M* TBA–2.5% acetonitrile (pH 5.1) at a flow-rate of 0.5 ml/min. The use of medium ionic strength and a low content of organic modifier under non-saturation conditions of the ion-pair reagent on a C_{18} stationary phase allowed the retention of charged nucleoside monophosphates to be increased, while the pattern of the separation of bases and nucleosides remained unchanged (Fig. 1).

Analysis of purine nucleoside metabolism in mitochondria

There is increasing evidence that mitochondria possess an autonomous metabolism of purine compounds [6–11] which might be of importance for processes such as turnover of nucleic acids, regulation of energetic functions and others proceeding within these organelles. In recent studies it was shown that endogenous purine nucleotides can be degraded to their nucleosides [6,11]. In this investigation, nucleoside utilization by isolated rat liver mitochondria was assessed.

As shown in Fig. 2 (for fractured mitochondria), adenosine was primarily deaminated to inosine, which was subsequently converted to hypoxanthine. Guanosine was predominantly degraded to guanine (Fig. 3).

In Fig. 4, the time courses of adenosine and guanosine degradation are presented. Using fractured mitochondria the rates of degradation are higher, providing an indication of the intramitochondrial localization of this process, because with intact organelles the nucleoside must first be taken up. Guanosine transport into mitochondria has been described [10], whereas adenosine uptake by liver mitochondria has not. For heart mitochondria only slow [12] or even no [13,14] adenosine incorporation was observed. Possibly, under the conditions chosen in our experiments (*i.e.*, in the presence of 1 m*M* ATP, 5 m*M* P_i and 1 m*M* $MgCl_2$), this nucleoside was able to penetrate into the organelles.

No radioactivity could be detected in the nucleoside monophosphate regions (Figs. 2 and 3). Therefore, phosphorylation of the nucleosides did not occur or was negligible in comparison with their degradation. It is noteworthy that deoxy-

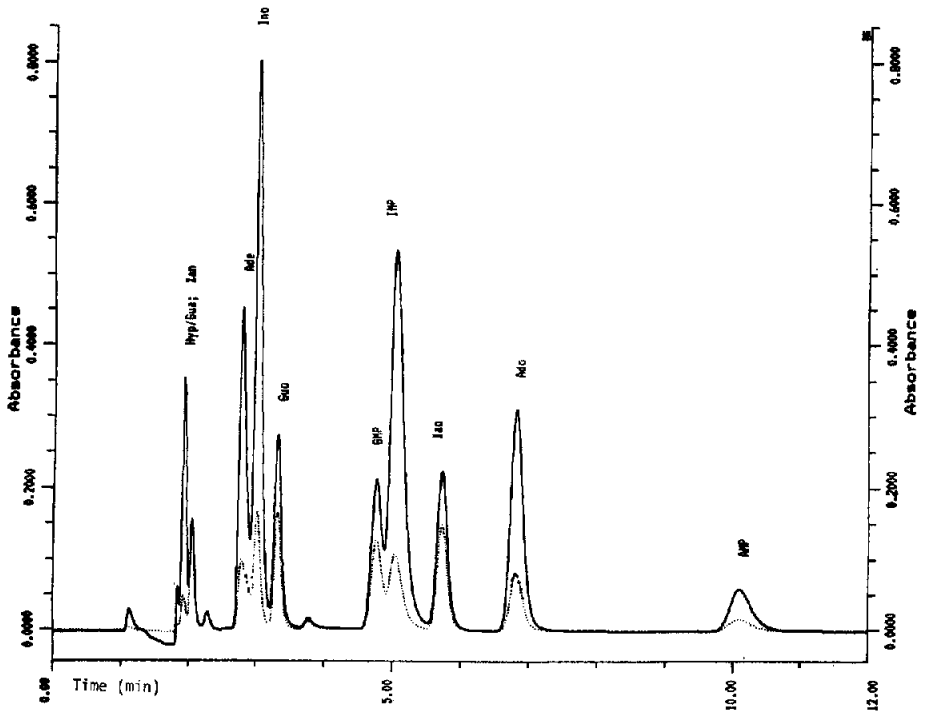

Fig. 1. Isocratic separation of standard purine nucleobases, nucleosides and nucleoside monophosphates. Conditions: 3-μm Ultrasphere XL-ODS cartridge column (75 mm × 4.6 mm I.D.); mobile phase, 40 m*M* KH_2PO_4–1 m*M* TBA–2.5% acetonitrile (pH 5.1); flow-rate, 0.5 ml/min. Detection at (full line) 254 nm and (dotted line) 280 nm.

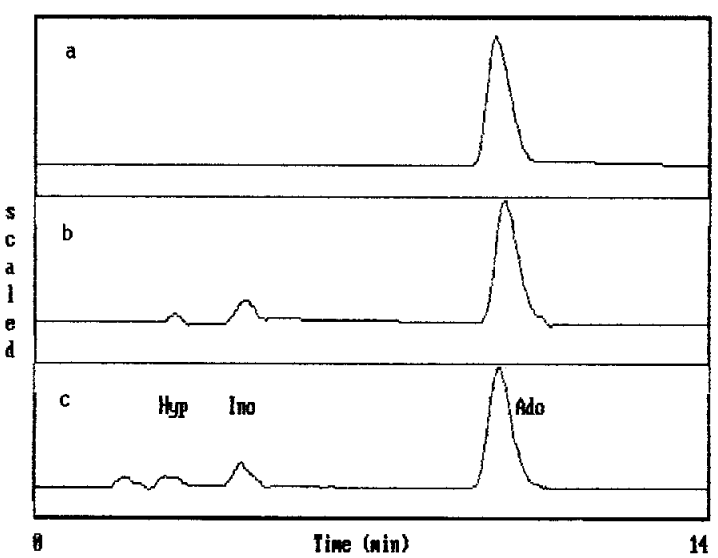

Fig. 2. Chromatograms of ASF of sonicated rat liver mitochondria obtained after (a) 0, (b) 5 and (c) 15 min of incubation in the presence of 100 μM [U-^{14}C]adenosine. For conditions, see Experimental. Detection of ^{14}C radioactivity.

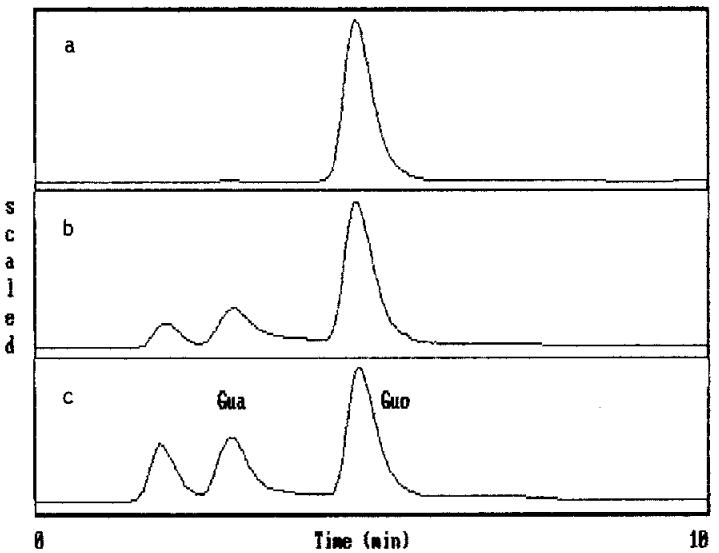

Fig. 3. Chromatograms of ASF of sonicated rat liver mitochondria obtained after (a) 0, (b) 5 and (c) 15 min of incubation in the presence of 100 μM [U-^{14}C]guanosine. For conditions, see Experimental. Detection of ^{14}C radioactivity.

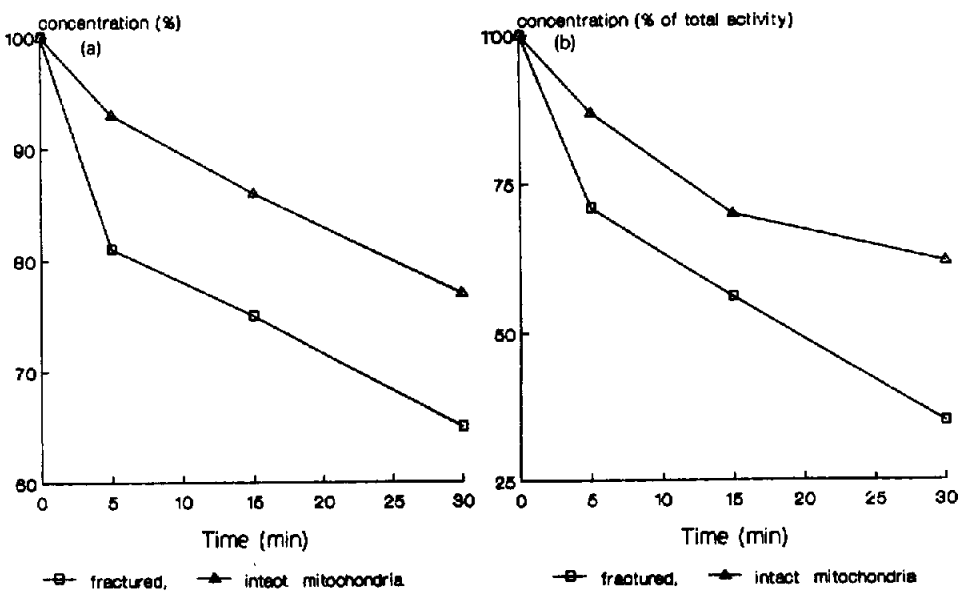

Fig. 4. Time courses of (a) adenosine and (b) guanosine degradation in intact and fractured mitochondria. △, Intact mitochondria; □, fractured mitochondria.

guanosine is taken up and phosphorylated in rat liver mitochondria under the same conditions as used in our experiments [15].

In conclusion, the chromatographic procedure including detection of radioactivity allowed us to examine the mitochondrial pathways of purine nucleoside degradation. The data presented here demonstrate that mitochondria possess enzyme activities that catalyse purine nucleoside degradation, possibly adenosine deaminase, as suggested recently [6], and purine nucleoside phosphorylase. In addition, the lower rates of nucleoside utilization in intact mitochondria, as compared with fractured organelles, indicate intramitochondrial localization of these enzymes. This, in turn, implies that mitochondria are capable of taking up adenosine and guanosine under the conditions used.

REFERENCES

1 A. A. Darwish and R. K. Prichard, *J. Liq. Chromatogr.*, 4 (1981) 1511.
2 A. M. Pimenov, Yu. V. Tikhonov, I. S. Meisner and R. T. Toguzov, *J. Chromatogr.*, 365 (1986) 221.
3 P. A. Perrone and P. R. Brown, *J. Chromatogr.*, 307 (1984) 53.
4 J. P. Caronia, J. B. Crowther and R. A. Hartwick, *J. Liq. Chromatogr.*, 6 (1983) 1673.
5 R. T. Toguzov, Yu. V. Tikhonov, A. M. Pimenov, V. Yu. Prokudin, W. Dubiel, M. Ziegler and G. Gerber, *J. Chromatogr.*, 434 (1988) 447.
6 M. Ziegler, W. Dubiel, A. M. Pimenov, Yu. V. Tikhonov, R. T. Toguzov, W. Henke and G. Gerber, *Mol. Cell. Biochem.*, 93 (1990) 7.
7 H.-D. Söling, in H. Sies (Editor), *Metabolic Compartmentation*, Academic Press, New York, 1982, pp. 123–146.
8 J. Greger and K. Fabianowska, *Enzyme*, 24 (1979) 54.
9 A. S. Tsiftsoglou and J. G. Georgatsos, *Biochim. Biophys. Acta*, 262 (1972) 239.
10 M. Ziegler, W. Dubiel, A. M. Pimenov, Yu. V. Tikhonov, R. T. Toguzov, W. Henke and G. Gerber, *FEBS Lett.*, 248 (1989) 182.
11 W. Henke, M. Ziegler, W. Dubiel and K. Jung, *FEBS Lett.*, 254 (1989) 5.
12 P. S. Fitt, N. Sharma, J. Attia and B. Korecky, *Mol. Cell. Biochem.*, 78 (1987) 37.
13 R. D. Bukoshi, H. V. Sparks and L. M. Mela-Riker, *Biochim. Biophys. Acta*, 884 (1986) 5.
14 G. K. Asimakis, D. E. Wilson and V. R. Conti, *Life Sci.*, 37 (1985) 2373.
15 L. F. Watkins and R. A. Lewis, *Mol. Cell. Biochem.*, 77 (1987) 71.