Journal of Chromatography, 599 (1992) 137–140
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2548

Monitoring the accumulation of nucleoside triphosphates by high-performance liquid chromatography

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ABSTRACT

An anion-exchange high-performance liquid chromatographic method was developed to examine the effect of uracil starvation on purine and pyrimidine metabolism. The isolation, separation and determination of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) from a mutant strain of *Escherichia coli* in a single run is described. The separation was performed using a Waters Radial-PAK Partisil SAX cartridge. The experimental procedure included the preparation of a neutralized Freon–amine extract and injection of 100 μl of it into the column. The results showed the accumulation of ATP and GTP under uracil starvation.

INTRODUCTION

Purine and pyrimidine nucleotides are important compounds for the syntheses of all macromolecules in living cells [1,2]. Dynamic changes and adjustments in intracellular nucleotides evoke metabolic reorientation of essential metabolites, alter the genetic expression of the genome and induce changes in macromolecular syntheses.

The pyrimidine biosynthetic pathway is universal and is obligately required for the balanced synthesis of nucleotide precursors of RNA and DNA [2]. The occurrence of bacterial mutants with an absolute requirement for pyrimidines shows that an alternative to the *de novo* pathway for the generation of pyrimidine nucleotides must exist. The fact that uracil, cytosine, uridine, cytidine, deoxyuridine and deoxycytidine satisfy the pyrimidine requirement of such mutants [3–5] further indicates that considerable interconversions may occur among the compounds. Accordingly, isotope studies show that any of the above mentioned pyrimidine compounds are equally effective as precursors for all the pyrimidine moieties of RNA and DNA [6–8].

Measurement of endogenous nucleotides has been revolutionized by the development of three different methods, based on high-performance liquid chromatography (HPLC): anion-exchange [9,10], reversed-phase [11] and ion-pair reversed-phase [12–14] chromatography. In our laboratory [10,14], the analysis of ribonucleotides was accomplished in the gradient anion-exchange HPLC mode in a single run. This method affords the detection of changes in the levels of low concentrations of nucleoside triphosphates in bacteria.

In this work we re-examined the effect of uracil starvation on purine and pyrimidine metabolism and developed a scheme for the efficient production of adenosine and guanosine nucleotides and their analogues using specially constructed mutant strains of bacteria.

EXPERIMENTAL

Chemicals and reagents

Nucleotides, trichloroacetic acid (TCA) and tri-*n*-octylamine were obtained from Sigma (St. Louis, MO, USA), 1,1,2-trichloro-1,2,2-trifluoroethane

0021-9673/92/$05.00

(Freon) and monobasic ammonium phosphate from Mallinckrodt (Paris, KY, USA) and potassium chloride from Eastman Kodak (Rochester, NY, USA). All other chemicals were of analytical-reagent grade and were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Bacterial strains

A mutant strain of *Escherichia coli* K12 having a genotype of pyrBI argG argI (designated here as TB2) was used.

Growth conditions

All cultures were grown at 37°C in a Lab Room Controlled Environmental Room (Lab-Line, Melrose Park, IL, USA) in M9 medium containing 0.2% glucose + 20% casamino acids + uracil (50 μg/ml). Liquid cultures were incubated in Klett erlenmeyer flasks (Kontes, Vineland, NJ, USA) in a G10 gyratory shaker (New Brunswick, Edison, NJ, USA) set at 120 rpm. The turbidity was measured every 20 min with a photoelectric Klett Summerson colorimeter using a No. 54 green filter and recorded as Klett units (KU), where 1 KU = $1 \cdot 10^7$ cells/ml. Volumes of 50 ml of bacterial cultures at different stages of the exponential phase were harvested, the actual KU recorded and then spun at 12 000 *g* at 4°C for 2 min. The supernatant was decanted and the cell pellet was used for nucleotide extraction.

The culture was not allowed to grow once the cells had reached a density of 85 KU. A 200-ml sample of this culture was then centrifuged and washed and the pellet was resuspended in 200 ml of the same medium without uracil. The cells at this stage showed a density of 25 KU. The cells were shaken for 1 h before harvesting in the same manner as before.

Extraction of ribonucleotides

A 1-ml volume of ice-cold 6% (w/v) TCA was added to the cell pellet and thoroughly mixed for 2 min in a vortex mixer. The mixture was allowed to stand at 4°C for 30 min before further centrifugation at 12 000 *g* for 10 min. The clear supernatant was then neutralized with an equal volume of ice-cold Freon–amine [15] solution (0.7 *M* tri-*n*-octylamine in Freon 113, 1.06 ml of amine per 5 ml of Freon). The Freon-amine mixture was agitated on a vortex mixer for 2 min and then allowed to separate while standing at 4°C for 10 min. The top aqueous layer, which contained the ribonucleotide pool extract, was filtered through a 0.45-μm ACRO LC3A filter (Gelman, Ann Arbor, MI, USA) and kept frozen at −20°C until used.

Chromatographic apparatus and conditions

The HPLC equipment (Waters, Milford, MA, USA) consisted of two Model 510 pumps, a Model 680 automated gradient controller, a U6K injector and a Model 481 LC spectrophotometer. Ribonucleotides were detected by monitoring the column effluent at 254 nm with the sensitivity fixed at 0.05 V aufs. Separations were performed on an anion-exchange Waters Radial-PAK Partisil SAX cartridge (10 cm × 0.8 cm I.D.) inside a Waters Radial Compression Z-module system.

Nucleotide samples (100 μl) obtained from bacteria were injected into the column. A linear gradient [10] of eluent A, 7 m*M* $NH_4H_2PO_4$ (pH 3.8), to eluent B, 250 m*M* $NH_4H_2PO_4$ (pH 4.5) containing 500 m*M* potassium chloride, was applied for 20 min followed by an isocratic period of 10 min with eluent B. The flow-rate was maintained at 4.5 ml/min and all analyses were performed at ambient temperature. Peaks were integrated either manually on a Cole-Palmer (Chicago, IL, USA) strip-chart recorder or a Waters 740 Data Module.

Individual components of the ribonucleotide pool mixture were identified on the basis of their retention times in comparison with standards and by injecting known internal standards. The recoveries of ribonucleotides from solutions of ribonucleotide standards were determined by measuring peak heights before and after the extraction procedures. The recoveries of added NTP (mean of three experiments ± standard error of the mean) were UTP 97 ± 5.1, CTP 88 ± 1.5, ATP 98 ± 4.1 and GTP 96 ± 2.3%.

Calculation

The concentration of the sample was calculated by comparing its peak height with the standard, for which the concentration was known (1 m*M*). Values in micromoles per gram dry weight for all nucleotides were calculated as described previously [10].

RESULTS AND DISCUSSION

The ATP and GTP levels of the culture were monitored during initial growth in M9 + uracil + casamino acid medium by HPLC. Fig. 1a shows a typical profile of the nucleotides from *E. coli* strain TB2. The range of buffer pH in this study was 3.8–4.5. It was observed that the resolution of the nucleoside triphosphates was very sensitive to the pH of the high-phosphate buffer B. The levels of ATP and GTP continued to accumulate, reaching a maximum when the cells reached a density of 75 KU after 265 min. Cells were grown to various densities from 60 to 100 KU. The precipitous increase in ATP concentration occurred to the greatest extent when uracil was removed from strain TB2 at 75 KU. At this stage, the cells were harvested, centrifuged, washed and resuspended in a medium without uracil.

The bacterial suspension at this stage contained $2.5 \cdot 10^8$ cells/ml. The cells were allowed to grow for 1 h, at which time a density of $3.0 \cdot 10^8$ cells/ml was attained. When the ATP and GTP pools were measured for this uracil-less culture, a six-fold increase was noted in comparison with cells having the same density during initial growth in the presence of uracil (Fig. 1b and Table I). The ATP and GTP levels started to decrease after reaching their peak as growth continued in the absence of uracil in the medium.

The massive elevation and accumulation of ATP and GTP (at molar concentrations) under uracil starvation are noteworthy. Nucleoside triphosphates increase specifically whenever cells are subjected to stress. The older literature [16] leaves no doubt that purines regulate pyrimidine synthesis but that pyrimidines do not regulate the synthesis of purines. The only logical interpretation can be offered in terms of the old concept wherein the ATP concentration is taken as the key mediator in metabolic control [17]. As can be seen in Pyr^- mytant, uracil is an absolute requirement for the ultimate production of UTP and CTP to be used for the synthesis of RNA. When uracil is removed, RNA synthesis stops, RNA is degraded and nucleotides accumulate. Thus, the cell starts to produce and accumulate massive amounts of ATP and GTP. If this were a wild-type cell (*i.e.*, Pyr^+), all four nucleoside triphosphates would accumulate. However, as strain TB2 is a uracil-requiring mutant, then UTP and CTP do not accumulate. Further, as RNA is not produced, vital ATP- and GTP-requiring steps

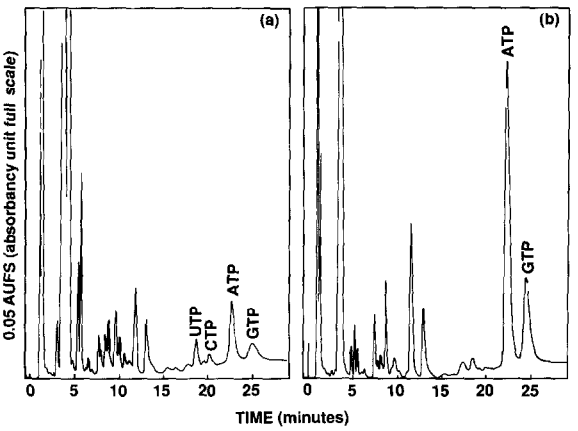

Fig. 1. Elution profiles of ribonucleoside mono-, di- and triphosphates from *E. coli* strain TB2. (a) Grown in M9 medium containing 0.2% (w/v) glucose + 0.4% (w/v) casamino acids and uracil (50 μg/ml). (b) Cells were grown in uracil as in (a) to 75 KU/ml, and were then harvested, washed and resuspended at 25 KU/ml in the same medium without uracil. They were starved of uracil for 1 h.

TABLE I

ACCUMULATION OF ATP AND GTP LEVELS UNDER VARIOUS GROWTH CONDITIONS

Growth conditions	Actual KU	ATP (μmol/g dry weight)	GTP (μmol/g dry weight)
M9 + uracil + casamino acids	30	1.92	0.74
M9 + casamino acids[a]	30	11.61	4.49
M9 + uracil + casamino acids	75	5.79	2.03

[a] Cells were starved of uracil at this stage.

in protein biosynthesis no longer occur such that they do not require ATP and GTP. Hence cellular ATP and GTP might accumulate. Taken together, this massive accumulation of ATP and GTP is a logical extension of the uracil starvation.

To our knowledge, this study is the first to provide a new direction for the manufacture of adenosine and guanosine nucleotides industrially. Kawamoto *et al.* [18] were able to show that a uracil-requiring mutant of *Arthrobacter paraffineus* accumulated orotic acid and orotidine when grown on *n*-paraffins as a sole carbon source. A decoyinine-resistant mutant [19,20] of *Bacillus ammoniagenes* was used to show the direct production of 5'-guanine nucleotides from a carbohydrate by mixed cultivation of 5'-XMP-accumulating strain and 5'-XMP-converting mutant. However, no study so far has shown that an extensive accumulation of ATP and GTP can occur in a uracil-requiring strain. Although this technique of producing ATP and GTP certainly shows great promise for the future, similar work should be conducted on industrial strains such as those of *B. ammoniagenes* and *Corynebacterium glutamicum* using large-scale fermentation for industrial adaptation.

In conclusion, this study has shown that it is possible to develop an ion-exchange HPLC method for monitoring nucleoside triphosphate levels in bacteria. The rapid information that can be deduced from these high-resolution separations is invaluable in the characterization and quality control of the nucleoside triphosphate-derived products. Finally, this technique puts forward the concept of using mutant strains of bacteria in the field of biotechnology.

REFERENCES

1 A. G. Moat and H. Friedman, *Bacteriol. Rev.*, 24 (1960) 339.
2 G. A. O'Donovan and J. Neuhard, *Bacteriol. Rev.*, 34 (1970) 278.
3 J. C. Gerhard, *Curr. Top. Cell. Regul.*, 2 (1970) 275.
4 S. S. Cohen, *Cold Spring Harbor Symp. Quant. Biol.*, 18 (1953) 221.
5 A. M. Moore and J. B. Boylen, *Arch. Biochem. Biophys.*, 54 (1955) 312.
6 E. T. Bolton, *Proc. Natl. Acad. Sci. U.S.A.*, 40 (1954) 764.
7 E. T. Bolton and A. M. Reynard *Biochim. Biophys. Acta*, 13 (1954) 381.
8 L. Siminovitch and A. F. Graham, *Can. J. Microbiol.*, 1 (1955) 721.
9 A. L. Poglotti and D. V. Santi, *Anal. Biochem.*, 126 (1982) 335.
10 P. K. Dutta and G. A. O'Donovan, *J. Chromatogr.*, 385 (1987) 119.
11 R. A. Cunha, A. M. Sebastiao and J. A. Ribiero, *Chromatographia*, 28 (1989) 610.
12 V. Stocchi, L. Cucchiarini, F. Canestari, M. P. Piacentini and G. Fornaini, *Anal. Biochem.*, 167 (1987) 181.
13 T. Ryll and R. Wagner, *J. Chromatogr.*, 570 (1991) 77.
14 G. A. O'Donovan, S. Herlick, D. E. Beck and P. K. Dutta, *Arch. Microbiol.*, 153 (1989) 19.
15 J. X. Khym, *Clin. Chem.*, 21 (1975) 1245.
16 *Purine and Pyrimidine Metabolism (Ciba Foundation Symposium*, No. 48), Elsevier, New York, 1977.
17 D. E. Atkinson, *Cellular Energy Metabolism and its Regulation*, Academic Press, New York, 1977.
18 I. Kawamoto, T. Nara, M. Misawa and S. Kinoshita, *Agric. Biol. Chem.*, 34 (1970) 1142.
19 A. Furuya, S. Abe and S. Kinoshita, *Biotechnol. Bioeng.*, 13 (1971) 229.
20 A. Furuya, R. Okachi, K. Takayami and S. Abe, *Biotechnol. Bioeng.*, 15 (1973) 795.